Strain-specific probiotic (Lactobacillus helveticus) inhibition of Campylobacter jejuni invasion of human intestinal epithelial cells

Eytan Wine, Mélanie G. Gareau, Kathene Johnson-Henry & Philip M. Sherman

Correspondence: Philip M. Sherman, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5G 1X8. Tel.: +416 813 7734; fax: +416 813 6531; e-mail: philip.sherman@sickkids.ca

Present address: Eytan Wine, Pediatric Gastroenterology, University of Alberta, Aberhart Center 1, Room 9219, 11402 University Avenue, Edmonton, AB, Canada T6G 1Z3.

Received 22 May 2009; accepted 24 August 2009.
Final version published online 18 September 2009.
DOI:10.1111/j.1574-6968.2009.01781.x

Editor: Rustam Aminov

Abstract

Campylobacter jejuni is the most common bacterial cause of enterocolitis in humans, leading to diarrhoea and chronic extraintestinal diseases. Although probiotics are effective in preventing other enteric infections, beneficial microorganisms have not been extensively studied with C. jejuni. The aim of this study was to delineate the ability of selected probiotic Lactobacillus strains to reduce epithelial cell invasion by C. jejuni. Human colon T84 and embryonic intestine 407 epithelial cells were pretreated with Lactobacillus strains and then infected with two prototypic C. jejuni pathogens. Lactobacillus helveticus, strain R0052 reduced C. jejuni invasion into T84 cells by 35–41%, whereas Lactobacillus rhamnosus R0011 did not reduce pathogen invasion. Lactobacillus helveticus R0052 also decreased invasion of one C. jejuni isolate (strain 11168) into intestine 407 cells by 55%. Lactobacillus helveticus R0052 adhered to both epithelial cell types, which suggest that competitive exclusion could contribute to protection by probiotics. Taken together, these findings indicate that the ability of selected probiotics to prevent C. jejuni-mediated disease pathogenesis depends on the pathogen strain, probiotic strain and the epithelial cell type selected. The data support the concept of probiotic strain selectivity, which is dependent on the setting in which it is being evaluated and tested.

Introduction

Campylobacter jejuni is identified as a leading cause of bacterial-induced enterocolitis in humans in most countries across the globe, being isolated, for instance, in 56% of cases of acute diarrhoea in a large Swedish study (Ternhag et al., 2008). Typically, acute diarrhoea develops, ranging from watery stools to dysentery that is characterized by haematochezia with pus and mucus (Young et al., 2007). Some of the long-term systemic complications of acute C. jejuni infection include reactive arthritis and Guillain–Barré syndrome (Crushell et al., 2004), as well as irritable bowel syndrome and chronic inflammatory bowel diseases (Garcia Rodriguez et al., 2006; Marshall et al., 2006). The ability of some C. jejuni strains to invade the cytosol of human cells has been demonstrated and appears to correlate with both pathogen virulence and disease severity (Byrne et al., 2007; Kalischuk et al., 2007).

Probiotics are defined as live microorganisms, which provide beneficial effects when administered in adequate quantities (Ventura et al., 2009). Some of the best-characterized and validated beneficial effects attributed to probiotics are in the setting of infectious viral-induced diarrhoea. Probiotic strains shorten the duration of acute rotavirus-induced intestinal illness in children (Szaewska et al., 2007) and are effective in preventing Clostridium difficile-associated diarrhoea (Parkes et al., 2009). The proposed mechanisms of action of probiotics include competitive exclusion, alteration of the intestinal microbial communities, enhancement of host barrier defences and modification of host signalling (Sherman et al., 2009).

The aim of this study was to determine the potential for selected Lactobacillus strains to reduce epithelial cell invasion by C. jejuni. The findings support the usefulness of Lactobacillus helveticus, strain R0052 in inhibiting the ability of C. jejuni to invade human intestinal epithelial cells, but also highlight the strain specificity of this effect, which limits the capacity to generalize findings in probiotics research from one setting to another.
Materials and methods

Epithelial cells in tissue culture

T84 human colon cancer epithelial cells [American Type Culture Collection (ATCC) CCL-248, Manassas, VA] were cultured in Dulbecco’s minimal essential medium/F-12, 10% (v/v) heat-inactivated foetal bovine serum (FBS), 2% penicillin–streptomycin, 2% sodium bicarbonate and 0.6% L-glutamine. Human embryonic intestine 407 cells (ATCC CCL-6) were cultivated in MEM, 10% FBS and 2% penicillin–streptomycin (all from Gibco, Grand Island, NY). Cells were maintained in 25-cm² flasks (Corning Glass Works, Corning, NY) and then seeded into six-well plates (5 × 10⁵ cells per well) and grown until semi-confluent (37 °C; 5% CO₂; Corning Glass Works).

Bacterial strains

Two prototype C. jejuni strains were used to represent different potential virulence mechanisms: NCTC 11168, the first sequenced C. jejuni strain (Dorrell & Wren, 2007) and the invasive strain, ATCC 81-176 (Crushell et al., 2004). Both C. jejuni strains were incubated in Mueller–Hinton broth and regrown on Columbia blood agar plates under microaerophilic conditions (48 h; 37 °C) before use for epithelial cell infection.

Probiotic strains included Lactobacillus rhamnosus, strain R0011 and L. helveticus, strain R0052 (Sherman et al., 2005), which were kindly provided by Dr Thomas Tompkins (Institut Rosell-Lallemand Inc., Montreal, QC, Canada). Probiotics were grown overnight at 37 °C in de Man, Rogosa and Sharpe (MRS) broth (Becton Dickenson, Sparks, MD). Lactobacillus rhamnosus GG (LGG; ATCC 53103) was cultured in blood agar and grown overnight at 37 °C in MRS broth (Johnson-Henry et al., 2008).

Tissue culture infection and invasion assays

Tissue culture medium was replaced to antibiotic-free medium 1 day before bacterial infection. Live or heat-killed (HK; 100 °C; 45 min) Lactobacillus strains were added [multiplicity of infection (MOI) 200:1] 1 h before infection, or in a subset of experiments, together with C. jejuni. Campylobacter jejuni strains (81-176 or 11168; MOI 20:1) were then added to the medium (without washing off the probiotics strains) and left to incubate for an additional 4 h (37 °C; 5% CO₂). Wells were then washed three times with phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent bacteria; fresh antibiotic-free cell culture medium was then added (2 h; 37 °C) with gentamicin (Sandoz, Boucherville, QC, Canada). Gentamicin (100 µg mL⁻¹) effectively killed all extracellular bacteria (Watson & Galan, 2008). Cells were then washed three times with PBS and lysed with 0.1% Triton X-100 (15 min; 20 °C; Sigma). Aliquots (10 µL) were plated in serial 10-fold dilutions onto blood agar plates and grown under microaerophilic conditions (48 h; 37 °C). Bacteria recovered from wells with gentamicin represent internalized organisms. Invasion of C. jejuni strains with probiotic treatment is expressed relative to invasion rates in wells in which probiotic strains were not added. The ability of L. helveticus R0052 to directly inhibit C. jejuni growth was assessed by aliquoting a 20-µL L. helveticus suspension onto blood agar plates carpeted with C. jejuni and marking the area of growth inhibition (Johnson-Henry et al., 2005).

Electron microscopy

For transmission electron microscopy, intestine 407 cells were grown in 10-cm tissue culture dishes and infected with C. jejuni, strain 81-176 (MOI: 20:1; 4 h; 37 °C). After four washes, cells were fixed in formaldehyde (4%) and glutaraldehyde (1%) in phosphate buffer, and postfixed in osmium tetroxide (1%) for 2 h at 20 °C. Specimens were then dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined in a transmission electron microscope (JEM-1011, JEOL USA Corp., Peabody, MA) at an accelerating voltage of 75 kV. Digital micrographs were acquired directly using a CCD camera system (AMT Corp., Danvers, MA).

For scanning electron microscopy, T84 cells were prepared as described previously (Sherman et al., 2005). Briefly, semi-confluent epithelial cells were treated with L. helveticus R0052 for 3 h (37 °C; 5% CO₂). Cells were then collected and fixed overnight in parafomaldehyde and glutaraldehyde. Samples were critically point dried, and sputter coated with gold before images were acquired with a scanning electron microscope (model JSM 820; Joel Ltd, Boston, MA).

Statistics

Results are expressed as means ± SEM. N represents the number of individual experiments. ANOVA and unpaired Student’s t-test were conducted using INSTAT3 (GraphPad, San Diego, CA). When a normal distribution was ascertained, means were compared using ANOVA and Tukey’s post hoc test; when the distribution was not considered normal the nonparametric Kruskal–Wallis test was used.

Results

Campylobacter jejuni adhesion and invasion of epithelial cells

Because the ability of C. jejuni strains to invade epithelial cells is cell-type dependent (Wine et al., 2008), we
determined the capacity of lactobacilli to inhibit \textit{C. jejuni} invasion in both human colon T84 cells and another human intestinal epithelial cell line commonly used to evaluate \textit{C. jejuni} invasion, intestine 407 cells (Larson et al., 2008). Absolute invasion rates of \textit{C. jejuni}, strains 81-176 and 11168 were 0.32 ± 0.14 and 0.06 ± 0.03 bacterial CFU per infected epithelial cell, respectively, in T84 cells and 0.42 ± 0.16 and 0.08 ± 0.03 in intestine 407 cells. Both adhesion and invasion of \textit{C. jejuni} 81-176 to intestine 407 cells are demonstrated by transmission electron microscopy (Fig. 1).

**Lactobacillus helveticus, strain R0052 reduces \textit{C. jejuni} invasion of T84 cells**

One hour preincubation of T84 cells with the probiotic strain \textit{L. helveticus} R0052 (MOI 200 : 1) impaired the ability of both \textit{C. jejuni} strains 81-176 and 11168 (MOI 20 : 1) to invade cells (41 ± 9% and 35 ± 5% reduction, respectively, relative to \textit{C. jejuni} invasion without probiotics; \textit{N} = 5–7; \textit{ANOVA}: \( P < 0.01; \) Fig. 2). The reduction in bacterial invasion was not due to changes in epithelial cell viability, as assessed using the exclusion of trypan blue (data not shown). HK \textit{L. helveticus} were still able to reduce T84 invasion by \textit{C. jejuni}, although to a lesser extent than live microorganisms (by 24 ± 8% and 27 ± 9% for \textit{C. jejuni} 81-176 and 11168, respectively; \textit{N} = 4–6; \( P < 0.05 \) relative to \textit{C. jejuni} infection without pretreatment and \( P > 0.05 \) relative to pretreatment with live organisms; Fig. 2). These findings suggest that the inhibitory effect of \textit{L. helveticus} R0052 does not completely depend on the presence of metabolically active bacteria. Coinfection of \textit{C. jejuni}, strain 81-176 with \textit{L. helveticus} R0052 resulted in a 15% reduction in \textit{C. jejuni} invasion (data not shown), suggesting that this approach is more suitable for prevention of invasion than as a therapeutic measure.

**Campylobacter jejuni invasion is not prevented by \textit{L. rhamnosus}, strain R0011**

In contrast to the protective effect shown for pretreatment with \textit{L. helveticus} R0052, incubation with \textit{L. rhamnosus}, strain R0011 did not impair the ability of \textit{C. jejuni}, strain 81-176 to invade T84 cells (10 ± 39% increase, relative to infection without pretreatment; \textit{N} = 5; \textit{ANOVA}: \( P > 0.05; \) Fig. 3). There was also no protective effect of \textit{L. rhamnosus}, strain R0011 on invasion of \textit{C. jejuni}, strain 11168 (13 ± 13% increase; \textit{N} = 3; \( P = \text{NS}; \) Fig. 3).

**Protection of cells from \textit{C. jejuni} invasion by lactobacilli depends on strain specificity of both the pathogen and the probiotic**

While invasion of \textit{C. jejuni}, strain 11168 into intestine 407 cells was reduced by 55 ± 9% with \textit{L. helveticus} R0052 pretreatment (\textit{N} = 5; \textit{t}-test: \( P < 0.0005 \)), invasion of \textit{C. jejuni}, strain 81-176 was not affected by the presence of probiotics (\( t \)-test: \( P > 0.05; \) Fig. 4). An opposite result was
**Lactobacillus helveticus inhibits C. jejuni invasion**

**L. helveticus in infection without strain R0052 reduces C. jejuni with L. helveticus intestine 407 cells**

Fig. 2. Pretreatment of T84 epithelial cells with Lactobacillus helveticus, strain R0052 reduces Campylobacter jejuni invasion. Relative to C. jejuni infection without L. helveticus pretreatment, 1 h incubation of T84 cells with L. helveticus R0052 before a 4 h C. jejuni infection reduced invasion of C. jejuni strains 81-176 (black bars) and 11168 (grey bars) by 41% and 35%, respectively. Pretreatment with HK, nonviable L. helveticus R0052 reduced invasion of C. jejuni strains by 24% and 27%, respectively. N = 4–6; **P < 0.05; ***P < 0.0005. In contrast, invasion of C. jejuni, strain 81-176 (black bars) was not affected by pretreatment with the same probiotic strain (N = 3). In contrast, LGG was unable to inhibit invasion of C. jejuni, strain 11168, but did show a trend for reduction of invasion by strain 81-176 of 37% (N=2). These findings indicate that the protective effect of probiotics was dependent on the properties of both the Lactobacillus strain used to interrupt the infectious process and the C. jejuni strain used as the infectious challenge.

**Lactobacillus rhamnosus adheres to T84 and intestine 407 cells**

Fig. 3. Lactobacillus rhamnosus R0011 does not prevent Campylobacter jejuni invasion of T84 cells. Incubation of T84 cells with L. rhamnosus R0011, before pathogen challenge did not impair the ability of C. jejuni, strains 81-176 (black bars) and 11168 (grey bars) to invade cells. N = 3–5; P > 0.05.

**Discussion**

The burden of disease inflicted by C. jejuni remains considerable (Crushell et al., 2004). Poultry are most effectively colonized and serve as an important environmental reservoir, typically without causing symptoms. Thus, infection in humans is frequently acquired by ingesting undercooked poultry (Stafford et al., 2008), as well as from drinking untreated contaminated water and unpasteurized milk and by direct contact with colonized domesticated animals (Kuusi et al., 2005). Although antibiotics are commonly used to treat patients infected with C. jejuni, emergence of antibiotic resistant strains highlights the necessity for developing novel alternative approaches (Alfredson & Korolik, 2007). Furthermore, prevention of disease in humans and a reduction of the pathogen reservoir in farm animals, without the need for antibiotics, are of both ecological and financial benefits to society.
strains (Chaveerach et al., 2004), a finding associated with downregulation of the flagellar factor, flaA (Ding et al., 2005).

Other studies document a protective effect for probiotic strains on animals infected with campylobacters. For example, administration of the butyrate-producing Butyribivio fibrisolvens, strain MDT-1 to mice both before and throughout the course of infection with C. coli reduces enterocolitis and weight loss (Ohkawara et al., 2006). Two studies have reported the efficacy of mixed probiotics preparations in inhibiting both pathogen shedding and colonization of C. jejuni in 1-day-old chicks (Morishita et al., 1997; Willis & Reid, 2008) and another study describes a reduction in C. jejuni colonization of immunodeficient and immunocompetent mice with combination probiotic therapy (Wagner et al., 2009).

By contrast, no previous studies have directly focused on the ability of potential probiotic strains to inhibit C. jejuni growth and invasion either in humans or in the setting of mixed cultures with intestinal cells in vitro. Epithelial cell invasion by C. jejuni is a key component of disease pathogenesis and is associated with other well-defined disease traits, including pathogen adhesion to gut epithelia (Byrne et al., 2007), induction of cell death (Kalischuk et al., 2007) and disruption of mucosal barrier function (Wine et al., 2008). As shown for other intestinal pathogens, interfering with the ability of bacteria to invade epithelial cells can prevent intestinal injury and improve clinical outcomes (Searle et al., 2009).

For these reasons, the present study focused on invasion of human epithelial cell as a target for probiotic therapies. Our findings show, for the first time, the capacity of L. helveticus R0052 to impair the ability of two well-characterized C. jejuni strains to invade human epithelial T84 cells under in vitro culture conditions. Although this reductionist model does not completely recapitulate the in vivo setting, it does provide a valuable opportunity to study the interaction between an enteric pathogen, potentially beneficial microorganisms, and host epithelial cells – each of which are key players in the intestinal ecosystem.

Because we found, in keeping with other studies (Sherman et al., 2005; Viefort et al., 2008), that lactobacilli adhere to epithelial cells, it appears that one of the mechanisms of inhibition of C. jejuni invasion occurs because of competitive exclusion. It is possible that differences in adhesion of the lactobacilli are responsible for the strain specificity of their protection. Alternatively, probiotic strains may have a bactericidal effect on campylobacters, as demonstrated for bacteria isolated from chickens (Chaveerach et al., 2004). Nevertheless, alterations in the signalling events involved in invasion are also likely to be affected by probiotics because HK metabolically inactive lactobacilli partially maintain the potential to inhibit C. jejuni invasion, possibly through a

Fig. 5. Lactobacillus helveticus adheres to epithelial cells. (a) Using scanning electron microscopy of T84 cells incubated with L. helveticus R0052 for 3 h, in the absence of Campylobacter jejuni, multiple adherent lactobacilli were observed (arrows). Approximate original magnification, × 4300. (b) Transmission electron microscopy of intestine 407 cells pretreated with L. helveticus R0052 for 1 h and infected with C. jejuni, strain 81-176 for 4 h shows adherent lactobacilli (arrows) adjacent to a loosely adherent C. jejuni (arrowheads). Scale bar = 1 µm.

The use of probiotics to prevent and treat bacterial infections of the intestine is an exploding area of current biomedical research (Ventura et al., 2009). Previous studies describe inhibition of C. jejuni growth in the absence of mammalian cells. For instance, Fooks & Gibson (2003) report reduced C. jejuni growth in mixed culture by a symbiotic combination of Lactobacillus plantarum 0407 and Bifidobacterium bifidum Bb12 together with oligofructose and xylo-oligosaccharides, while individual human-derived lactobacilli also have a similar effect in vitro (Fernandez et al., 2003). Other lactobacilli and bifidobacteria, some of which were isolated from chicken intestine, also inhibit the growth of various C. jejuni and Campylobacter coli
secreted factor. Of note, absolute invasion rates in our study were lower than in some previous reports (Kalischuk et al., 2007; Zheng et al., 2008), possibly due to the fact that we did not centrifuge the infected plates or due to differences in culture conditions, strain characteristics and atmospheric growth conditions.

Other factors, such as the innate and adaptive immune systems, additional microorganisms in the commensal microbiota and the mucus layer derived from goblet cell mucins and trefoil factors are not accounted for in this reductionist immortalized epithelial cell model. Each could further alter the interaction between microbial pathogens and the intestinal epithelia. For example, various probiotic strains modulate host responses through activity on the adaptive immune system (Round & Mazmanian, 2009). By contrast, some Lactobacillus strains enhance mucosal protection by increasing mucin production and secretion (Mack et al., 2003). Increased mucins could potentially interfere with the ability of C. jejuni to invade epithelial cells (Byrne et al., 2007).

One of the important points highlighted by the present study is the strain specificity of the described effects. Our results demonstrate that L. helveticus R0052 is more effective than either L. rhamnosus R0011 or LGG in interfering with C. jejuni invasion into intestinal epithelial cells. Similar findings have been described in the case of interferon-γ-stimulated STAT-1 inhibition by Escherichia coli O157:H7, which is also inhibited by L. helveticus R0052, but not by L. rhamnosus R0011 (Jandu et al., 2009). Although not directly addressed in this study, it is possible that surface-layer proteins present on L. helveticus R0052, but not on L. rhamnosus R0011, which inhibits adhesion of E. coli O157:H7 to epithelial cells (Johnson-Henry et al., 2007), may explain the differential effect. Furthermore, in the case of intestine 407 cells, there was a difference in the ability of L. helveticus R0052 to inhibit invasion of the tested C. jejuni strains. This observation highlights the complexity of interactions between microorganisms and mammalian cells, as well as the need for caution in interpreting and generalizing studies describing the protective effects of probiotics.

Better characterization of the mechanisms of action of probiotic strains is required in order to further define and apply the potential uses in a variety of clinical settings. This study not only advances knowledge on the ability of selected probiotic strains for use in subverting the infectious process, but also offers further insight into potential mechanisms involved by recognizing the strain specificity of the effect. Inhibiting the ability of pathogens to invade epithelial cells is critical to maintaining the integrity of intestinal epithelial barrier structure and function. The pathogen and probiotic strain-specific effects shown herein provide an opportunity for future studies to focus on the identification of specific microbial factors present in one strain, but not in the other, that are responsible for the observed effect. Further research is also now required to elucidate the underlying mechanism of action of beneficial probiotic strains in this setting, and to establish the therapeutic potential of probiotics to interrupt the infectious process and thereby prevent human disease caused by C. jejuni infection.

Acknowledgements

The authors thank Yew Meng Heng, Department of Pediatric Laboratory Medicine, Hospital for Sick Children, for his expertise in microscopy techniques. This work was supported by an operating grant from the Canadian Institutes of Health Research (CIHR) and a Fay Shapiro Cutler Grant-in-Aid from the Crohn’s and Colitis Foundation of Canada (CCFC). E.W. was supported by a fellowship award from the Canadian Association of Gastroenterology/CIHR/Astra Zeneca partnership. P.M.S. is the recipient of a Canada Research Chair in Gastrointestinal Disease.

References


