Probiotics prevent enterohaemorrhagic *Escherichia coli* O157:H7-mediated inhibition of interferon- γ -induced tyrosine phosphorylation of STAT-1

Narveen Jandu, Zoë Jingjing Zeng, Kathene C. Johnson-Henry and Philip M. Sherman

Correspondence

Philip M. Sherman
philip.sherman@sickkids.ca

Research Institute, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 inhibits interferon (IFN)- γ -stimulated tyrosine phosphorylation of signal transducer and activator of transcription (STAT)-1 in epithelial cells. We determined the effects of probiotics on EHEC-mediated disruption of IFN- γ -stimulated STAT-1 activation in epithelial cell lines. Confluent Intestine 407, HEp-2 and Caco-2 epithelial cells were pre-treated (3 h) with either probiotics or surface-layer proteins derived from *Lactobacillus helveticus* R0052 prior to infection with EHEC O157:H7 strain CL56 (m.o.i. 100:1, 6 h, 37 °C in 5% CO₂). Subsequently, cells were washed and stimulated with human recombinant IFN- γ (50 ng ml⁻¹, 0.5 h, 37 °C) followed by whole-cell protein extraction and immunoblotting for tyrosine-phosphorylated STAT-1. Relative to uninfected cells, STAT-1-activation was reduced after EHEC O157:H7 infection. Pre-incubation with the probiotic *L. helveticus* R0052 followed by EHEC infection abrogated pathogen-mediated disruption of IFN- γ -STAT-1 signalling. As determined using Transwell inserts, probiotic-mediated protection was independent of epithelial cell contact. In contrast, pre-incubation with boiled *L. helveticus* R0052, an equal concentration of viable *Lactobacillus rhamnosus* R0011, or surface-layer proteins (0.14 mg ml⁻¹) did not restore STAT-1 signalling in EHEC-infected cells. The viable probiotic agent *L. helveticus* R0052 prevented EHEC O157:H7-mediated subversion of epithelial cell signal transduction responses.

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INTRODUCTION

Outbreaks of enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 infection occur frequently across the developed world (Serna & Boedeker, 2008). This foodborne enteric pathogen is acquired from contaminated foodstuffs and non-chlorinated drinking water (Karch *et al.*, 2005; Maki, 2006; Rangel *et al.*, 2005). In the severest cases of infection (~10–15% of cases), the haemolytic uraemic syndrome develops as a systemic complication, leading to patient hospitalization, kidney failure and death (Serna & Boedeker, 2008; Tarr *et al.*, 2005). Renal disease results from bacterial cytotoxin [Shiga-like toxin (Stx)-1 and -2] production in the gut, release into the systemic circulation and damage to vascular endothelial cells in the renal glomerulus (Blackall & Marques, 2004). EHEC pathogenesis is also attributed to intimate bacterial adhesion conferred by the locus of enterocyte effacement (LEE) pathogenicity island, the activity of effector proteins

secreted through a type three secretion system (T3SS), and pathogen modulation of host cell signal transduction cascades (Bhavsar *et al.*, 2007; Kaper *et al.*, 2004).

EHEC disease pathogenesis includes subversion of epithelial cell signal transduction cascades involved in host innate immune responses to infection (Bhavsar *et al.*, 2007). For instance, EHEC inhibits interferon (IFN)- γ -stimulated tyrosine phosphorylation of signal transducer and activator of transcription-1 (STAT-1) in multiple cell lines (Ceponis *et al.*, 2003), which is mediated by a bacterially encoded factor (Jandu *et al.*, 2006). EHEC-mediated disruption of STAT-1 activation is independent of EHEC-derived toxins (Stx-1 and Stx-2), the LEE pathogenicity island, the O157:H7 plasmid (pO157), and H7 flagellin (Ceponis *et al.*, 2003). Gene knockout mice provide evidence that the IFN- γ -Jak1,2-STAT-1 signal transduction cascade is an essential innate immune response required to combat microbial infections (Shtrichman & Samuel, 2001).

Probiotics are live, non-pathogenic micro-organisms that confer health benefits to the host (Senok *et al.*, 2005). Probiotics are increasingly being employed in the management of a variety of human diseases (Servin, 2004). For

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; IFN, interferon; LEE, locus of enterocyte effacement; STAT-1, signal transducer and activator of transcription-1.

instance, pathogen adhesion and invasion, and changes in epithelial cell permeability induced by enteroinvasive *E. coli* (Resta-Lenert & Barrett, 2006), *Salmonella typhimurium* (Gill *et al.*, 2001; O'Hara *et al.*, 2006; Silva *et al.*, 2004), *Shigella flexneri* (Tien *et al.*, 2006) and *Yersinia enterocolitica* (Frick *et al.*, 2007), are prevented using probiotics administered either as a single strain or as combination therapy. Enteric pathogen-induced changes in the activation, expression and secretion of pro-inflammatory cytokines (tumour necrosis factor- α ; TNF α) and chemokines (interleukin 8) by epithelial cells are also prevented by probiotics (Frick *et al.*, 2007; O'Hara *et al.*, 2006; Tien *et al.*, 2006).

The aim of the current study was to characterize the effects of the probiotic *Lactobacillus helveticus* strain R0052 and *Lactobacillus rhamnosus* R0011 on EHEC O157:H7 inhibition of STAT-1 activation in response to IFN- γ . Herein, we show that probiotic-mediated protection against EHEC subversion of the innate immune response is species- and strain-specific, involves heat-labile probiotics, and is independent of direct probiotic contact with epithelial cells.

METHODS

Tissue culture. Intestine (Int) 407, HEp-2 and Caco-2 epithelial cell lines (American Type Culture Collection) were utilized as *in vitro* models of infected epithelia. Tissue-culture cells were grown as previously described (Ceponis *et al.*, 2003). Briefly, Int 407 cells were grown in tissue-culture medium composed of minimal essential medium containing 10% (v/v) fetal bovine serum and 2% (v/v) penicillin-streptomycin. HEp-2 cells were grown in minimal essential medium containing 15% (v/v) fetal bovine serum, 2% (v/v) sodium bicarbonate, 2.5% (v/v) penicillin-streptomycin, and 1% (v/v) fungizone (all from Invitrogen Canada). Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum, 2% (v/v) penicillin-streptomycin, 1.1% sodium pyruvate and 0.3% (v/v) human transferrin (all from Invitrogen Canada).

Cells were grown in T25 flasks (Corning) at 37 °C in 5% CO₂ until confluent (2×10^6 – 3×10^6 cells per flask). Once confluent, cells were trypsinized (0.05% trypsin) for 20 min at 37 °C in 5% CO₂ and centrifuged at 500 r.p.m. for 5 min in a Beckman Coulter centrifuge. Cell pellets were resuspended in tissue-culture medium, seeded into 6 cm Petri dishes (Becton Dickinson Labware), and grown at 37 °C in 5% CO₂ until confluent. Twenty-four hours prior to bacterial infection, cells were incubated in antibiotic-free and serum-free tissue-culture medium (Jandu *et al.*, 2006). To determine whether the benefits of probiotics required epithelial cell contact, cultured epithelia were grown in a 12-well tissue-culture plate fitted with 0.4 μ m pore-size Transwell filter membranes (Costar). Bacteria were physically separated from epithelial cells by growing the epithelia in the top chamber of the Transwell, while bacteria were added into the bottom chamber (Ceponis *et al.*, 2003).

Bacterial strains and growth conditions. Bacterial strains used in this study included EHEC strain CL56 (serotype O157:H7), enteropathogenic *E. coli* (EPEC) strain E2348/69 (serotype O127:H6) and the non-pathogenic laboratory *E. coli* strain HB101. EHEC O157:H7, strain CL56, which produces both Stx-1 and Stx-2, was originally isolated from a child with haemorrhagic colitis and

haemolytic uraemic syndrome at the Hospital for Sick Children (Toronto, ON, Canada) (Karmali *et al.*, 1985). The *eae*-positive EPEC strain E2348/69 and non-pathogenic *E. coli* strain HB101 were used for comparative purposes. Bacteria were grown as previously described (Ceponis *et al.*, 2003; Jandu *et al.*, 2006, 2007). Briefly, strains were grown on 5% sheep blood agar plates (Becton Dickinson) at 37 °C for 24 h. Prior to infecting epithelial cells, bacteria were inoculated into 10 ml Penassay broth (Becton Dickinson) and grown overnight at 37 °C.

Gram-positive probiotics *L. helveticus* R0052 (formerly *Lactobacillus acidophilus* R0052; Naser *et al.*, 2006) and *L. rhamnosus* R0011 were provided by Institut Rosell-Lallemand. As previously described (Johnson-Henry *et al.*, 2005), probiotics were grown in 10 ml de Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson) at 37 °C for 24 h prior to pre-treatment of epithelial cells. For pre-treatment studies, confluent epithelial cells were pre-treated with an overnight growth of probiotics (5×10^8 c.f.u. ml⁻¹) at 37 °C in 5% CO₂ for 3 h. The probiotic strain tested was then left on the epithelial cells (i.e. cells were not washed), and EHEC O157:H7 was applied directly onto the cells plus probiotics for a 6 h infection period. For co-treatment studies, probiotics were added to epithelial cells at the same time as EHEC; both the probiotic and the pathogen were then left with the epithelial cells for the entire 6 h infection period.

To determine whether viable probiotics were required for protection, the effects of boiled lactobacilli (100 °C, 60 min), surface-layer proteins (Johnson-Henry *et al.*, 2007), conditioned medium, and culture supernatants derived from *L. helveticus* R0052 were examined. Surface-layer proteins (0.14 mg ml⁻¹) were reconstituted in antibiotic-free tissue-culture medium and then incubated with epithelial cells at 37 °C in 5% CO₂ for 3 h prior to EHEC infection. For conditioned medium, epithelial cells were incubated with *L. helveticus* R0052 at 37 °C in 5% CO₂ for 9 h. Medium was then collected, centrifuged at 3000 r.p.m. for 10 min, passed through a 0.45 μ m filter and stored at -20 °C until use. Culture supernatants were collected after an overnight growth of *L. helveticus* R0052 and centrifuged at 3000 r.p.m. for 10 min. Supernatants were then collected and filter-concentrated using an Amicon ultracentrifugal filter column (Millipore). Columns were centrifuged at 3000 r.p.m. for 30 min and samples were then stored at -20 °C. Epithelial cells were pre-incubated for 3 h at 37 °C in 5% CO₂ in either conditioned medium or culture supernatant followed by EHEC O157:H7 infection for 6 h.

Epithelial cell infection and whole-cell protein extraction.

Epithelial cells were either pre-treated with probiotics (*L. helveticus* R0052 or *L. rhamnosus* R0011) prior to infection, or co-incubated with probiotics and EHEC. EHEC infection was performed at an m.o.i. of 100:1 (Ceponis *et al.*, 2003). Briefly, an overnight bacterial culture was centrifuged at 3000 r.p.m. for 5 min, the supernatant decanted and the bacterial pellet resuspended in 0.1 ml tissue-culture medium without antibiotics or fetal bovine serum. The bacterial suspension was then used to infect (2×10^8 c.f.u. ml⁻¹) confluent epithelial cells grown in 6 cm Petri dishes for 6 h at 37 °C in 5% CO₂. At the end of the infection period, cell viability assays were performed for some experiments, while whole-cell protein extraction was performed in others.

Epithelial cell viability was determined by the exclusion of Trypan blue (Sigma). Briefly, after infection of cultured epithelial cells with EHEC O157:H7 alone, probiotics alone or probiotics and EHEC, cells were trypsinized and centrifuged. Cell pellets were resuspended in 10 ml tissue-culture medium, and an aliquot of the cell suspension was combined with 0.4% Trypan blue in a 1:3 (v/v) ratio, vortexed well and placed onto a haemocytometer slide. Using bright-field microscopy, viable and non-viable cells were then enumerated, with the number of viable cells calculated as a percentage of the total cell count.

At the end of the 6 h infection period, cells were washed once with sterile PBS (pH 7.4) to remove non-adherent bacteria and then stimulated with human recombinant IFN- γ (50 ng ml⁻¹; R&D Systems) for 30 min at 37 °C in 5 % CO₂ (Ceponis *et al.*, 2003; Jandu *et al.*, 2006, 2007). Subsequently, cells were washed three times with ice-cold PBS and collected, using a rubber scraper, into a final 1 ml volume of PBS.

Whole-cell protein extracts were prepared as described previously (Ceponis *et al.*, 2003; Jandu *et al.*, 2006, 2007). Briefly, cell extracts were centrifuged at 13 000 r.p.m. for 20 s at 20 °C, and the supernatants were decanted and pellets resuspended in 0.15 ml RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxyolate, 0.1 % SDS in PBS) supplemented with 150 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μ g PMSF ml⁻¹, 15 μ g aprotinin ml⁻¹, 2 μ g leupeptin ml⁻¹ and 2 μ g pepstatin A ml⁻¹ (all from Sigma Aldrich), vortexed well and left on ice for 20 min. Resuspended pellets were centrifuged again at 12 000 r.p.m. for 10 min at 4 °C, and supernatants were stored at -80 °C until further analysis by immunoblotting.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were conducted as previously described (Ceponis *et al.*, 2003; Jandu *et al.*, 2006, 2007). Briefly, cell extracts were prepared for SDS-PAGE by combining loading buffer with whole protein extracts in a 1:2 (v/v) ratio. Samples were heated at 100 °C for 3 min, loaded into precast 10 % polyacrylamide gels (Ready Gel; Bio-Rad Laboratories) and electrophoresed at 150 V for 1 h. Migrated protein was transferred onto nitrocellulose membranes (BioTrace NT; Pall Corporation) at 110 V for 1.5–2 h at 4 °C.

Nitrocellulose membranes were initially incubated in Odyssey blocking buffer (LI-COR Biosciences) for 1 h at 20 °C, followed by incubation with primary and secondary antibodies. Primary antibodies for STAT-1 bind to both the alpha and beta isoforms of this signalling molecule (Baran-Marszak *et al.*, 2004). Antibodies employed included an anti-latent-STAT-1 antibody (Santa Cruz Biotechnologies; 1:5000 dilution in Odyssey buffer) and an anti-phospho-STAT-1 antibody (Cell Signalling for HEp-2 cells; Zymed for Int 407 cells; both at 1:1000 dilution). An anti- β -actin primary antibody (1:5000 dilution; Sigma) was employed as a loading control. Membranes were incubated with primary antibodies at 4 °C overnight on a shaker and then washed four times with PBS + 0.1 % Tween (5 min per wash). Secondary antibodies, including IRDye 800 goat anti-rabbit IgG (1:20 000 dilution; Rockland Immunochemicals) and Alexa Fluor 680 goat anti-mouse IgG (1:20 000 dilution; Molecular Probes) were incubated for 1 h at 20 °C on a shaker. Subsequently, membranes were washed four times with PBS + 0.1 % Tween (5 min per wash); the final wash step included PBS without Tween.

The Odyssey Infrared Imaging System (LI-COR Biosciences) was used to scan nitrocellulose membranes and detect positively stained bands. Scanning was performed with both the 700 and 800 nm channels on, at a resolution of 169 μ m (Jandu *et al.*, 2007). The integrated intensity of all bands was calculated using automated software provided by the manufacturer. For immunoblots with dual anti-latent-STAT-1 and anti- β -actin or dual anti-phospho-STAT-1 and anti- β -actin staining, the integrated intensity values for anti-latent-STAT-1 and anti-phospho-STAT-1 bands were normalized using intensity values obtained for corresponding anti- β -actin bands (Jandu *et al.*, 2007). Values obtained from bands of uninfected IFN- γ -stimulated cells were then set to 100 %, with samples derived from microbial treatments calculated as a percentage relative to uninfected cells.

Statistics. Results are presented as means $\pm$ SEM. To determine statistical significances ($P < 0.05$) between multiple groups, ANOVA was performed. Where indicated, a two-tailed, paired Student's *t* test

was performed to determine statistical difference in levels of STAT-1 activation between two groups (Norman & Streiner, 2003).

RESULTS

EHEC O157:H7 inhibits tyrosine phosphorylation of STAT-1 in Int 407 epithelial cells

Immunoblotting and densitometry were employed to determine the effects of EHEC O157:H7 on STAT-1 signalling in Int 407 cells. As shown by immunoblotting in Fig. 1(a), IFN- γ -dependent STAT-1 tyrosine phosphoryla-

Fig. 1. EHEC O157:H7 disrupts IFN- γ -induced tyrosine phosphorylation of STAT-1 in Int 407 cells. Int 407 cells were infected with *E. coli* strains (m.o.i. 100:1 for 6 h at 37 °C in 5 % CO₂) and then incubated in the absence or presence of IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5 % CO₂. Whole-cell protein extracts were collected and immunoblots probed with anti-latent-STAT-1 (I-STAT-1), anti-phospho-STAT-1 (pY-STAT-1) or anti- β -actin primary antibodies, followed by the respective secondary antibodies. Positively stained bands were detected using an IR imaging system. β -Actin served as a protein loading control. (a) Lanes: 1 and 2, uninfected epithelial cells in the absence and presence of IFN- γ , respectively; 3 and 4, cells infected with EPEC strain E2348/69 (serotype O127:H6); 5 and 6, cells infected with a non-pathogenic laboratory *E. coli*, strain HB101; 7 and 8, cells infected with EHEC O157:H7 strain CL56. Only the EHEC strain (lane 8) blocked IFN- γ activation of STAT-1. (b) Densitometry of positively stained bands was quantified using software embedded in the IR scanner (three separate experiments; * $P < 0.05$, ANOVA).

tion was reduced in cultured epithelial cells infected with EHEC O157:H7 compared with uninfected cells. In contrast, tyrosine phosphorylation of STAT-1 in Int 407 cells treated with IFN- γ remained intact when cells were infected with either EPEC strain E2348/69 (serotype O127:H6), or a non-pathogenic laboratory *E. coli* strain, HB101 (Fig. 1a). Quantification of immunoblots by densitometry showed that STAT-1 tyrosine phosphorylation was reduced to $20.6 \pm 5.6\%$ ($n=10$; $P<0.05$, ANOVA) in EHEC-infected epithelial cells, compared with uninfected epithelial cells (Fig. 1b). Levels of STAT-1 phosphorylation in EPEC-infected and HB101-infected epithelial cells were $122.7 \pm 35.0\%$ ($n=3$; $P>0.05$) and $135.3 \pm 51\%$ ($n=3$; $P>0.05$), respectively, relative to uninfected epithelial cells (Fig. 1b). Collectively, these findings are consistent with previous reports using other epithelial cell lines (Ceponis *et al.*, 2003; Jandu *et al.*, 2006, 2007).

L. helveticus R0052 protects against EHEC O157:H7-induced disruption of STAT-1 signalling

As shown in Fig. 2(a), tyrosine phosphorylation of STAT-1 in response to IFN- γ remained intact in Int 407 cells pre-treated (3 h) with *L. helveticus* R0052 followed by infection with EHEC O157:H7 (6 h). By contrast, co-incubation of Int 407 cells with *L. helveticus* R0052 and EHEC O157:H7 (6 h) did not prevent pathogen-mediated inhibition of STAT-1 activation. As shown by densitometry, pre-incubation of epithelial cells with the probiotic *L. helveticus* R0052 maintained STAT-1 activation in EHEC-infected epithelial cells: $100.7 \pm 34.3\%$, relative to uninfected epithelial cells ($n=6$; $P<0.05$, ANOVA) (Fig. 2b). Co-incubation of Int 407 cells with *L. helveticus* R0052 and EHEC did not prevent microbial inhibition of STAT-1 activation ($24.7 \pm 6.3\%$, relative to uninfected cells; $n=6$, $P>0.05$) (Fig. 2b). Incubation of *L. helveticus* R0052 alone with Int 407 cells did not alter IFN- γ -stimulated STAT-1 tyrosine phosphorylation ($82.1 \pm 9.8\%$ and $92.5 \pm 18.7\%$, for co-incubation and pre-incubation conditions, respectively; $n=4$, $P>0.05$) relative to uninfected cells. Taken together, using a STAT-1 tyrosine phosphorylation assay as the outcome measure, these findings indicate that pre-incubation, but not co-incubation, with probiotics is required to effectively protect epithelial cells from EHEC-mediated subversion of innate immunity.

As shown in Fig. 3(a), and as in our previous report (Jandu *et al.*, 2007), IFN- γ -stimulated tyrosine phosphorylation of STAT-1 was reduced after EHEC O157:H7 infection of HEp-2 cells ($13.6 \pm 5.5\%$, compared with uninfected epithelial cells; $n=9$, $P<0.05$). Pre-incubation (3 h) of HEp-2 cells with *L. helveticus* R0052 followed by EHEC O157:H7 infection (6 h) resulted in protection from pathogen-mediated disruption of IFN- γ -stimulated STAT-1 activation (Fig. 3b, $86.2 \pm 17.2\%$, relative to uninfected epithelial cells; $n=7$, $P=0.008$, paired Student's *t* test). *L. helveticus* R0052 alone did not reduce STAT-1 tyrosine

Fig. 2. Pre-incubation with *L. helveticus* R0052 protects Int 407 cells against EHEC O157:H7 inhibition of IFN- γ -induced STAT-1 activation. Int 407 cells were either pre-treated (3 h) with *L. helveticus* R0052 (m.o.i. 100:1 for 3 h at 37 °C in 5% CO₂) prior to EHEC O157:H7 infection (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂) or co-treated with *L. helveticus* R0052 and EHEC O157:H7. Cells were then incubated in the absence or presence of IFN- γ (50 ng ml⁻¹) for 30 min at 37 °C in 5% CO₂. Whole-cell protein extracts were collected and immunoblots probed with anti-latent-STAT-1 (I-STAT-1), anti-phospho-STAT-1 (pY-STAT-1) or anti- β -actin primary antibodies, followed by their respective secondary antibodies. Positively stained bands were detected by using an IR imaging system. (a) Lanes: 1 and 2, uninfected Int 407 epithelial cells in the absence and presence of IFN- γ , respectively; 3, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 4 and 5, *L. helveticus* R0052 alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 6, co-treatment with *L. helveticus* R0052 did not reverse EHEC O157:H7 inhibition of STAT-1 signalling, but pre-incubation of epithelial cells with *L. helveticus* R0052 prevented EHEC O157:H7 disruption of STAT-1 activation in Int 407 cells (lane 7). (b) STAT-1 phosphorylation was quantified by image-intensity analysis using software embedded in the IR imaging system (three to five separate experiments; * $P<0.05$, ANOVA).

phosphorylation in HEp-2 cells ($106.4 \pm 35\%$ relative to untreated controls; $n=4$). Pre-incubation (3 h) of Caco-2 cells with *L. helveticus* R0052 also prevented EHEC O157:H7-mediated inhibition of IFN- γ -induced tyrosine

Fig. 3. *L. helveticus* R0052 prevents EHEC O157:H7-induced inhibition of STAT-1 phosphorylation in HEp-2 cells. HEp-2 cells were pre-treated with *L. helveticus* R0052 (m.o.i. 100:1 for 3 h) and then infected with EHEC O157:H7 (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂). Cells were then incubated in the absence or presence of IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5% CO₂. (a) Lanes: 1 and 2, uninfected HEp-2 epithelial cells in the absence and presence of IFN- γ , respectively; 3 and 4, *L. helveticus* R0052 alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 5 and 6, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 7 and 8, pre-treatment of epithelial cells with *L. helveticus* R0052 prevented EHEC O157:H7 disruption of STAT-1 activation in HEp-2 cells. (b) Densitometry of immunoblots was performed to quantify the integrated intensities of positively staining bands. STAT-1 tyrosine phosphorylation remained intact in epithelial cells pre-incubated with *L. helveticus* R0052 prior to EHEC O157:H7 infection (five to seven separate experiments; * $P < 0.05$, ANOVA).

phosphorylation of STAT-1 in polarized Caco-2 intestinal epithelial cells ($87.5 \pm 8.0\%$ relative to uninfected controls; $n=4$) (data not shown as a figure).

***L. rhamnosus* R0011 does not protect epithelial cells against EHEC O157:H7-mediated disruption of IFN- γ -Jak1,2-STAT-1 signalling**

To determine species and strain specificity, *L. rhamnosus* R0011 (Sherman *et al.*, 2005) was then tested. As shown in Fig. 4(a, b), STAT-1 tyrosine phosphorylation was reduced

Fig. 4. *L. rhamnosus* R0011 does not protect Int 407 cells against EHEC O157:H7 disruption of IFN- γ -induced STAT-1 activation. Int 407 cells were either pre-treated with *L. rhamnosus* R0011 (m.o.i. 100:1 for 3 h at 37 °C in 5% CO₂) prior to EHEC O157:H7 infection (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂) or co-treated with *L. rhamnosus* R0011 and EHEC O157:H7. Cells were then stimulated with IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5% CO₂. Whole-cell protein extracts were collected and immunoblots were probed with anti-latent-STAT-1 (I-STAT-1), anti-phospho-STAT-1 (pY-STAT-1) or anti- β -actin primary antibodies, followed by their respective secondary antibodies. β -Actin was used as a protein loading control. (a) Positively stained bands were detected using an IR imaging system. Lanes: 1 and 2, uninfected epithelial cells in the absence and presence of IFN- γ , respectively; 3, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 4 and 5, *L. rhamnosus* R0011 alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 6 and 7, *L. rhamnosus* R0011 lacked the ability to protect Int 407 cells from EHEC O157:H7-disrupted STAT-1 signalling. (b) STAT-1 phosphorylation was quantified using image-intensity analysis (three separate experiments; $P > 0.05$).

($22.7 \pm 14.2\%$ of the value for untreated controls; $n=3$, $P < 0.05$) in Int 407 cells pre-incubated (3 h) with *L. rhamnosus* R0011 followed by EHEC O157:H7 infection (6 h). Phosphorylated STAT-1 levels were also reduced ($24.3 \pm 11.3\%$ of the value for untreated controls; $n=3$, $P > 0.05$) in Int 407 cells co-incubated with *L. rhamnosus* R0011 and EHEC. Tyrosine phosphorylation of STAT-1 in

response to IFN- γ was not reduced when Int 407 cells were incubated with *L. rhamnosus* R0011 alone ($144.9 \pm 53.6\%$ of value for uninfected controls, $n=3$, $P>0.05$).

As shown in Fig. 5(a), pre-incubation of HEp-2 cells with *L. rhamnosus* R0011 (3 h) followed by EHEC infection (6 h) resulted in STAT-1 tyrosine phosphorylation levels comparable with values observed in HEp-2 cells incubated with EHEC O157:H7 alone ($7.5 \pm 2.4\%$ of the value for uninfected epithelial cells; $n=6$, $P<0.05$) (Fig. 5b). As with Int 407 cells, *L. rhamnosus* R0011 incubated with HEp-2 epithelial cells alone did not alter STAT-1 signalling ($101.7 \pm 1.7\%$; $n=3$ of value for uninfected controls, $P>0.05$).

Surface-layer proteins derived from *L. helveticus* R0052 do not prevent EHEC O157:H7 disruption of IFN- γ -Jak1,2-STAT-1 signalling

Differences in the outer cell surface composition of various probiotic strains could explain observed differences between the two probiotics tested (Johnson-Henry *et al.*, 2007). To determine whether surface-layer proteins afford protection against EHEC infection, epithelial cells were pre-incubated (3 h) with an extract of surface-layer

proteins (0.14 mg ml^{-1}) followed by *E. coli* O157:H7 infection for 6 h. As shown by immunoblotting (Fig. 6a) and by densitometry (Fig. 6b), pre-incubation of epithelial cells with surface-layer protein extracts did not protect epithelial cells against EHEC O157:H7-induced inhibition of STAT-1 tyrosine phosphorylation ($15.0 \pm 8.6\%$ of value for uninfected controls; $n=3$, $P<0.05$). Surface-layer proteins alone did not affect IFN- γ -stimulated STAT-1 tyrosine phosphorylation ($99.1 \pm 34.5\%$ relative to uninfected controls; $n=3$, $P>0.05$). Higher concentrations of surface-layer protein extracts were not employed due to problems with solubility in the tissue-culture medium (Johnson-Henry *et al.*, 2007).

In addition, live probiotics were required, because boiled *L. helveticus* R0052 did not restore STAT-1 activation in EHEC-infected epithelial cells ($21.8 \pm 9.0\%$, relative to uninfected cells; $n=3$, $P>0.05$) (data not shown as a figure). Neither conditioned medium nor culture supernatants prepared from *L. helveticus* R0052 resulted in the recovery of STAT-1 tyrosine phosphorylation in EHEC-infected epithelial cells, indicating that viable, intact *L. helveticus* R0052, rather than secreted products, is required to prevent EHEC subversion of epithelial cell signal transduction responses to infection.

Fig. 5. *L. rhamnosus* R0011 does not protect HEp-2 epithelial cells from EHEC O157:H7-induced inhibition of STAT-1 tyrosine phosphorylation. Cultured epithelial cells were pre-treated with *L. rhamnosus* R0011 (m.o.i. 100:1 for 3 h at 37 °C in 5% CO₂) and then infected with EHEC O157:H7 (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂). Cells were then stimulated with IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5% CO₂. Whole-cell protein extracts were collected for immunoblotting and densitometry of immunoblots was performed to quantify the integrated intensities of positively stained bands. (a) Lanes: 1 and 2, uninfected HEp-2 epithelial cells in the absence and presence of IFN- γ , respectively; 3 and 4, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 5 and 6, *L. rhamnosus* R0011 alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 7 and 8, neither co-treatment (0 h) nor pre-treatment (3 h) of epithelial cells with *L. rhamnosus* R0011 reversed EHEC O157:H7 inhibition of STAT-1 signalling in HEp-2 cells. (b) Densitometry of immunoblots was performed to quantify the integrated intensities of positively stained bands. Controls included uninfected epithelial cells in the absence and presence of IFN- γ (six separate experiments; $P>0.05$).

Fig. 6. *L. helveticus* R0052-derived surface-layer proteins do not protect HEp-2 epithelial cells against EHEC O157:H7-induced inhibition of STAT-1 tyrosine phosphorylation. Cultured epithelial cells were pre-treated with surface-layer proteins (S-protein; 0.14 mg ml⁻¹ for 3 h at 37 °C in 5% CO₂) and then infected with EHEC O157:H7 (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂). Cells were stimulated with IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5% CO₂. Whole-cell protein extracts were collected and immunoblots probed with anti-latent-STAT-1 (I-STAT-1), anti-phospho-STAT-1 (pY-STAT-1) or anti- β -actin primary antibodies, followed by their respective secondary antibodies. (a) Positively staining bands were detected using a commercial IR scanner. Lanes: 1 and 2, uninfected epithelial cells in the absence and presence of IFN- γ , respectively; 3 and 4, surface-layer proteins alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 5 and 6, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 7 and 8, pre-treatment with surface-layer proteins did not prevent EHEC O157:H7-mediated disruption of STAT-1 activation in HEp-2 cells. (b) Positively stained bands on immunoblots were quantified using image-intensity analysis software (three separate experiments; $P>0.05$).

***L. helveticus* R0052 protection against EHEC O157:H7-induced disruption of STAT-1 tyrosine phosphorylation is independent of probiotic contact with epithelial cells**

To determine whether *L. helveticus* R0052 requires direct contact with epithelial cells, Transwell studies were conducted. As shown in Fig. 7(a), IFN- γ -stimulated STAT-1 tyrosine phosphorylation was inhibited only when

Fig. 7. Contact-independent protection of *L. helveticus* R0052 against EHEC O157:H7-mediated inhibition of STAT-1 activation. HEp-2 epithelial cells were grown in the upper chamber of a 12-well Transwell fitted with a 0.4 μ m pore-size filter membrane. Probiotics were added either to the upper chamber in direct contact with epithelial cells or to the lower chamber separated from cells by a filter membrane (3 h; m.o.i. 100:1 at 37 °C in 5% CO₂). EHEC strain CL56 serotype O157:H7 was then added to the upper chamber (m.o.i. 100:1 for 6 h at 37 °C in 5% CO₂). After washings, epithelial cells were stimulated with IFN- γ (50 ng ml⁻¹) for 0.5 h at 37 °C in 5% CO₂. Whole-cell protein extracts were then collected for immunoblotting, and blots were scanned using an IR scanner. (a) Positively staining bands were detected using a commercial IR scanner. Lanes: 1 and 2, uninfected epithelial cells in the absence and presence of IFN- γ , respectively; 3 and 4, EHEC O157:H7 disrupted IFN- γ -induced STAT-1 phosphorylation; 5 and 6, *L. rhamnosus* R0052 alone did not alter IFN- γ -stimulated STAT-1 phosphorylation; 7, pre-treatment of epithelial cells with *L. rhamnosus* R0052 followed by EHEC infection in the absence of IFN- γ . After IFN- γ stimulation, *L. rhamnosus* R0052 in either direct contact (lane 8) or indirect contact (lane 9) with epithelial cells prevented EHEC inhibition of STAT-1 activation. (b) Densitometry was performed to quantify the integrated intensities of positively staining bands (four separate experiments; $P>0.05$).

EHEC was in direct contact with epithelial cells ($32.0 \pm 11.7\%$ of value for uninfected controls; $n=4$). Probiotic amelioration of EHEC-induced inhibition of STAT-1 tyrosine phosphorylation occurred either when *L. helveticus* R0052 was in direct contact with epithelia ($83.9 \pm 8.7\%$ of value for untreated cells; $n=4$) or when it was separated from epithelial cells by a filter membrane ($73.6 \pm 4.4\%$ of value for untreated cells; $n=4$). *L. helveticus* R0052 alone did not alter STAT-1 tyrosine phosphorylation ($82.2 \pm 8.6\%$ of value for uninfected controls; $n=4$) (Fig. 7b).

DISCUSSION

Herein we show, for the first time to our knowledge, that the probiotic strain *L. helveticus* R0052 protects cultured epithelial cells against EHEC O157:H7 subversion of the IFN- γ -Jak1,2-STAT-1 signal transduction cascade. In contrast, another probiotic, strain *L. rhamnosus* R0011, did not protect host epithelial cells against EHEC-mediated inhibition of STAT-1 signalling. Probiotic protection of STAT-1 signal transduction required both pre-treatment time and viable probiotics, as neither co-treatment with *L. helveticus* nor boiled R0052 prevented EHEC inhibition of tyrosine phosphorylation of STAT-1. In addition, *L. helveticus* R0052 prevents EHEC pathogenesis irrespective of probiotic contact with epithelial cells.

Previous studies have shown probiotic-mediated protection of cultured epithelial cells in response to EHEC O157:H7 infection. In a previous study, pre-treatment (3 h) of epithelial cell(s) with probiotics was required to preserve epithelial cell integrity during EHEC infection, whereas co-incubation of probiotics and the EHEC pathogen did not maintain epithelial cell integrity (Sherman *et al.*, 2005). In a subsequent study, *L. helveticus* R0052-derived surface-layer proteins were shown to preserve epithelial cell integrity during EHEC infection (Johnson-Henry *et al.*, 2007). However, in the present study, *L. helveticus* R0052-derived surface-layer proteins did not prevent EHEC O157:H7-induced subversion of STAT-1 activation by IFN- γ . Collectively, these results indicate that mechanism(s) by which *L. helveticus* preserves STAT-1 tyrosine phosphorylation in cultured epithelial cells during EHEC infection occur independently of surface-layer proteins.

Other probiotics can also preserve epithelial cell integrity and maintain intracellular signal transduction cascades in response to pathogens. For instance, Resta-Lenert & Barrett (2003) have shown that the probiotics *Streptococcus thermophilus* and *Lactobacillus acidophilus* protect epithelial cells (HT-29 and Caco-2) against enteroinvasive *E. coli*-mediated disruption of epidermal growth factor signalling. Consistent with our results, pre-treatment of epithelial cells with probiotics was required to provide protection against the effects of EHEC on host cell signal transduction. In addition, live probiotics are required to ameliorate the

adverse effects of pathogens, since neither antibiotic-killed (Resta-Lenert & Barrett, 2003) nor heat-killed (Sherman *et al.*, 2005; this study) probiotics prevent the pathogenic effects of *E. coli* enteropathogens on cultured epithelial cells.

Potential mechanisms of probiotic-mediated protection against pathogens include their ability to maintain intestinal epithelial integrity (Madsen *et al.*, 2001), production of bacteriocins (Corr *et al.*, 2007), increased mucin production and secretion from goblet cells (Mack *et al.*, 2003), and immunomodulatory effects (Boirivant & Strober, 2007). Colonization resistance refers to the ability of some probiotics to guard against pathogen binding via the formation of a protective barrier between epithelial cells and the infecting organism (Eutamene & Bueno, 2007; Fedorak & Madsen, 2004). To test this hypothesis, we employed a Transwell model of infection to evaluate the effects of direct and indirect contact of *L. helveticus* R0052 with cultured epithelial cells as a mechanism maintaining STAT-1 signalling in EHEC-infected cells. The results demonstrate that *L. helveticus* R0052 protects epithelial cells against EHEC subversion of STAT-1 tyrosine phosphorylation independent of probiotic contact with host cells.

Our study focuses on the ability of *L. helveticus* R0052 to retain the innate immune signalling cascade: inhibition of IFN- γ -stimulated STAT-1 tyrosine phosphorylation by EHEC O157:H7 infection. There are several implications arising from the results of this study. As a pro-inflammatory cytokine, IFN- γ is generated in response to infection by enteric pathogens. *In vivo* studies using *Citrobacter rodentium* infection in mice, as a model of EHEC infection (Mundy *et al.*, 2006), have shown that this results in IFN- γ recruitment into the lamina propria and increased transcript expression of this cytokine in infected mouse colonic mucosa (Higgins *et al.*, 1999). In addition, *C. rodentium* infection of IFN- γ gene knockout mice results in increased pathogen burden, enhanced gut injury and greater mortality (Bry & Brenner, 2004; Simmons *et al.*, 2002, 2003). Taken together, these *in vivo* studies and our *in vitro* results indicate that microbial evasion of the IFN- γ -Jak1,2-STAT-1 signalling cascade represents an immune evasion strategy employed by these non-invasive enteric pathogens.

In summary, we have shown that *L. helveticus* R0052 maintains IFN- γ -Jak1,2-STAT-1 activation following EHEC O157:H7 infection of multiple epithelial cell lines. Viable *L. helveticus* R0052 is required for protection, while contact with epithelial cells is not necessary. Future research efforts should focus on characterizing the molecular interactions between this probiotic and host epithelial cells. Also, studies should describe the influence of this probiotic on the expression of EHEC O157:H7 virulence factors.

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