Indigenous lactobacilli strains of food and human sources reverse enteropathogenic *E. coli* O26:H11-induced damage in intestinal epithelial cell lines: effect on redistribution of tight junction proteins

Ruchi Jariwala, Hemanti Mandal and Tamishraha Bagchi

**Abstract**

The aim of the study was to investigate the neutralizing effect of lactobacilli isolated from indigenous food and human sources on enteropathogenic *Escherichia coli* (EPEC) O26:H11-induced epithelial barrier dysfunction in vitro. This was assessed by transepithelial electrical resistance (TEER) and permeability assays using intestinal cell lines, HT-29 and Caco-2. Furthermore, the expression and distribution of tight junction (TJ) proteins were analysed by qRT-PCR and immunofluorescence assay, respectively. The nine strains used in the study were from different species viz. *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus salivarius* and *Lactobacillus plantarum*. All strains were able to reverse the decrease in TEER and corresponding increase in permeability across *E. coli*-infected monolayers. Maximum reversal was observed after 18 h (up to 93.8±2.0 % by *L. rhamnosus* GG followed by *L. fermentum* IIs11.2 (92.6±2.2 %) and *L. plantarum* GRI-2 (91.9±0.9 %)) of lactobacilli exposure following EPEC O26:H11 infection. All strains were able to redistribute the TJ proteins to the cell periphery either partially or completely. Moreover, *L. helveticus* FA-7 was also able to significantly increase the mRNA expression of ZO-1 and claudin-1 (2.5-fold and 3.0-fold, respectively; *P<0.05*). The rapid reversal observed by these strains could be mostly because of the redistribution rather than increased mRNA expression of TJ proteins. In conclusion, *L. helveticus* FA-7, *L. fermentum* FA-1 and *L. plantarum* GRI-2 were good in all the aspects studied, and the other strains were good in some aspects. *L. helveticus* FA-7, *L. fermentum* FA-1 and *L. plantarum* GRI-2 can therefore be used for potential therapeutic purpose against intestinal epithelial dysfunction.

**INTRODUCTION**

The intestinal epithelium acts as a protective barrier against entry of most environmental antigens including microbial and dietary food antigens. The barrier function is maintained by the mucosal layer as well as junctional complexes such as adherence junctions and tight junctions (TJ) [1]. Enteropathogens such as *Escherichia coli* cause imbalance in the intestinal microenvironment thus disturbing the gut homeostasis. The effects of such pathogens on cellular junctions leads to intestinal barrier dysfunction. The TJ is responsible for maintaining paracellular permeability across the epithelial barrier [2], and an increase in paracellular permeability is known to be associated with alterations in TJ proteins [3, 4]. The large submembrane component of the TJ comprises a complex of integral membrane proteins which includes occludins, claudins and junction adhesion molecules (JAM) that interact with the zonula occludens (ZO) proteins, which are in turn bound to the perijunctional ring of cytoskeletal actin [5, 6]. These structures are continuously remodelled depending upon the interaction between the epithelium and the luminal agents including pathogens and commensal bacteria, or various physiological stimuli [7–9, 3]. Abnormalities of intercellular TJs are observed in a variety of intestinal disorders, including acute diarrhoeal illness, gluten-sensitive enteropathy (coeliac disease) and chronic inflammatory bowel disease [10].

Probiotics of the genus *Lactobacillus* play an important role in maintaining the homeostasis of gut flora by adhering to and colonizing the intestinal mucosa and competing with pathogenic bacteria, such as some strains of *E. coli* [11, 12].
The enhancement of epithelial barrier function is one of the proposed mechanisms by which certain probiotic organisms like lactobacilli may confer health benefits. Enhancement in barrier integrity is associated with changes in the TJ structure via alteration in TJ protein expression and distribution. They have also been shown to enhance the TJ integrity between intestinal epithelial cells that are not weakened. Some probiotics are known to improve the intestinal epithelium via alteration in TJ protein expression and distribution.

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>GenBank accession no. of 16S–23S sequence</th>
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<tr>
<td>Lactobacillus fermentum FA-1</td>
<td>Fermented bamboo shoot (Iku)</td>
<td>KT337434</td>
</tr>
<tr>
<td>Lactobacillus fermentum FA-5</td>
<td>Fermented soybean seeds (Agya)</td>
<td>KT337435</td>
</tr>
<tr>
<td>Lactobacillus helveticus FA-7</td>
<td>Fermented rice (Nyogrin)</td>
<td>KT337436</td>
</tr>
<tr>
<td>Lactobacillus fermentum KGI-1</td>
<td>Adult human gut origin</td>
<td>JX118832</td>
</tr>
<tr>
<td>Lactobacillus salivarius GPI-1S</td>
<td>Adult human gut origin</td>
<td>JX118837</td>
</tr>
<tr>
<td>Lactobacillus fermentum GPI-3</td>
<td>Adult human gut origin</td>
<td>JX118834</td>
</tr>
<tr>
<td>Lactobacillus fermentum GPI-7</td>
<td>Adult human gut origin</td>
<td>JX118831</td>
</tr>
<tr>
<td>Lactobacillus plantarum GRI-2</td>
<td>Adult human gut origin</td>
<td>JX118835</td>
</tr>
<tr>
<td>Lactobacillus fermentum Ilx11.2</td>
<td>Child gut origin</td>
<td>KT337437</td>
</tr>
</tbody>
</table>

Lactobacillus rhamnosus GG (LGG) is one of the clinically best-studied probiotic strains and has been widely used as a standard probiotic strain [16]. An earlier report also confirmed the preventive effect of LGG on changes in epithelial barrier function [13], prompting its use in the present study as a positive control.

In the present study we used various indigenous lactobacilli strains to assess their neutralization effect on enteropathogenic E. coli O26 : H11-induced epithelial barrier dysfunction using Caco-2 and HT-29 cell lines. The strains have been isolated from indigenous sources (local fermented foods and human stool samples) and already examined for their morphological and biochemical properties along with their adhesion ability on the intestinal epithelial cell lines either alone or in the presence of EPEC O26 : H11 and compared with LGG in our previous study [17]. Thus we believe that the strains isolated from the local fermented foods and human stool samples might serve as better probiotics for the local population. Improvement in the barrier function was assessed by measurement of transepithelial electrical resistance (TEER) and permeability across HT-29 and Caco-2 monolayers. Further study was conducted to verify the effects of these lactobacilli strains on EPEC O26 : H11-infected monolayers by analysing mRNA levels of various TJ proteins and also the distribution of these proteins in the epithelial cells.

**METHODS**

**Bacterial strains and culture conditions**

A total of nine different lactobacilli strains from human stool samples and food samples were used in this study as given in Table 1. Lactobacillus rhamnosus GG (LGG) (generously provided by Dr Shira Doron, MD, Department of Medicine, Tufts Medical Center, Boston, MA, USA) was used as the standard strain in the study. Lactobacilli were grown in de Man, Rogosa and Sharpe (MRS) broth (Himedia) at 37°C for 16–18 h. Preliminary growth studies revealed that the strains used in the present report showed best adhesion to intestinal cell monolayers when harvested after about 16–18 h of growth and they were in the late log phase and showed good viability (unpublished data). Overnight-grown Lactobacillus was harvested, washed with Dulbecco’s phosphate-buffered saline (DPBS), pH 7.0 (Sigma-Aldrich), resuspended in Dulbecco’s Modified Eagle Media (DMEM), pH 6.5 (Sigma-Aldrich) without antibiotic and used at a density of 1×10⁸ c.f.u. ml⁻¹ in all experiments. Enteropathogenic Escherichia coli (EPEC) O26 : H11 (obtained from the departmental culture collection facility) was grown overnight in Luria broth (LB; Himedia) at 37°C. Similarly, E. coli cells were resuspended in DMEM without antibiotic and adjusted to 1×10⁷ c.f.u. ml⁻¹. (The generation time for lactobacilli is longer at approx. 40 min, while E. coli requires roughly 20 min as doubling time, thus E. coli to lactobacilli was used at a 1:10 ratio).

**Epithelial cell culture**

The human colonic adenocarcinoma cell lines HT-29 and Caco-2 were obtained from National Centre for Cell Science (NCCS), Pune, India, and were routinely cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Sigma-Aldrich) and maintained at 37°C with 5% CO₂ and 95% air atmosphere. The medium was supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma-Aldrich), 10 mM non-essential amino acids, 1 mM Na-pyruvate and 50 µg ml⁻¹ gentamicin. The media lacked gentamicin whenever antibiotic-free medium was used.

**Measurement of transepithelial electrical resistance (TEER) and latex bead translocation across Caco-2 monolayers**

Caco-2 cells (0.5×10⁵ per insert) were seeded onto 6.5 mm diameter Transwell (Costar; Corning) polyester inserts (3 µm pore size) and grown until a TEER of 800 Ω·cm² (for 20–22 days) was achieved. The TEER of the Caco-2 cells was measured with a Millicell-ERS (Millipore). The Caco-2 monolayers were incubated in antibiotic-free media for 90 min before
addition of bacterial cells. The monolayers were pre-infected with $1 \times 10^7$ c.f.u. ml$^{-1}$ of EPEC O26 : H11 at the apical compartment of the insert and incubated at 37°C in 5% CO$_2$/95% air atmosphere for 4 h following which they were treated with $1 \times 10^8$ c.f.u. ml$^{-1}$ of different lactobacilli for 18 h. A 10 µl aliquot of fluorescent red-labelled carboxylate-modified polystyrene latex beads (2.0 µm) (Sigma-Aldrich) was added simultaneously with the addition of lactobacilli. The TEER of the monolayers was measured at 0, 2, 4, 16 and 18 h following lactobacilli exposure. Aliquots from the basolateral compartment were also simultaneously taken and the translocation of latex beads was estimated by counting under a CX41 fluorescent microscope (Olympus). Caco-2 monolayers cultured under the same conditions, but with and without pre-infection with EPEC O26 : H11 served as EPEC O26 : H11 control and absolute control, respectively. The percentage change in the TEER value after lactobacilli treatment was calculated with respect to the TEER values before addition of EPEC O26 : H11 (indicated by $-4$ h in Fig. 1a). Translocation of latex beads across the monolayers at each time interval was calculated and the permeability for the same was depicted as relative to control. Three independent experiments were performed in duplicate.

**Measurement of TEER and permeability to inulin across HT-29 monolayers**

HT-29 cells were seeded onto 6.5 mm diameter Transwell (Costar; Corning) polyester inserts (3 µm pore size) at a

![Graph](image.png)

**Fig. 1.** Effect of lactobacilli strains on TEER (a) and translocation of latex beads (b) across EPEC O26 : H11-infected Caco-2 monolayers. Each line represents the mean value and error bar as standard deviation of three independent experiments. The thin-lined arrow indicates the addition of EPEC O26 : H11 and the thick-lined arrow indicates addition of lactobacilli. ** in different colours indicate significant difference in TEER (a)/permeability (b) of monolayers treated with respective strains compared to EPEC O26 : H11-infected monolayers. The colour of the asterisk matches with the colour of the strain showing significant difference. *** indicates significant difference in TEER (a)/permeability (b) of monolayers treated with all the strains compared to EPEC O26 : H11-infected monolayer at respective time point. **** indicates significant difference in TEER (a)/permeability (b) of EPEC O26 : H11-infected monolayer compared to control monolayer. The permeability of monolayers was calculated as number of latex beads translocated into the basolateral chamber, and the values were expressed on the y-axis as translocation of beads across the bacteria-treated monolayers relative to control monolayer. Significant ANOVA was followed by Dunnett’s test for multiple comparisons versus the EPEC O26 : H11 group ($P$<0.05).
density of $0.5 \times 10^5$ cells per insert and were allowed to differentiate to form a monolayer for 7–8 days (approximately TEER was between 400 to 500 $\Omega \cdot \text{cm}^2$). The HT-29 monolayers were treated similar to Caco-2 monolayers as described above. Fluorescein isothiocyanate (FITC)-inulin (Sigma-Aldrich) at a final concentration of $100 \mu g \text{ ml}^{-1}$ was added to the apical compartment of the transwell chamber at the time of lactobacilli addition. HT-29 cells without any bacterial exposure were used as absolute control whereas HT-29 cells infected with EPEC O26: H11 but without any lactobacilli treatment were used as EPEC O26: H11 control. TEER was also measured at 0, 2, 4, 16 and 18 h following lactobacilli exposure. Percentage change in the TEER was calculated as described above. To check the permeability for FITC-inulin (excitation at 490 nm and emission at 520 nm) across HT-29 monolayers, aliquots from the basolateral compartment were collected at the 2, 4, 16 and 18 h of treatment and the fluorescence intensity was determined using a Synergy-HT multiplate reader (BioTek). The permeability for FITC-inulin in the lactobacilli-treated HT-29 monolayers was estimated by calculating the percentage decrease in the fluorescence intensity of FITC-inulin in the basolateral chamber compared to that of the EPEC O26: H11 control.

**Quantitative real-time polymerase chain reaction (qRT-PCR) for detecting the mRNA levels of TJ-specific proteins in HT-29 cells**

HT-29 cells were seeded in standard 24-well plates (Costar; Corning) at a density of $0.5 \times 10^5$ cells per well and maintained at 37°C in 5% CO$_2$/95% air atmosphere until confluence. Prior to any bacterial exposure, the monolayers were incubated with antibiotic-free media for 90 min. Monolayers were infected with $1 \times 10^8$ c.f.u. ml$^{-1}$ of EPEC O26: H11 cells for 2 h, washed twice with DPBS and then treated with $1 \times 10^8$ c.f.u. ml$^{-1}$ of different lactobacilli for 2 h. Lactobacilli were then removed by washing twice with DPBS followed by incubation with antibiotic-containing medium for further 20 h. The EPEC O26: H11-infected HT-29 monolayer without any lactobacilli treatment was used as EPEC O26: H11 control whereas a monolayer without any bacterial infection served as absolute control.

Following the treatment as described above, total RNA was isolated from HT-29 cells using RNAiso plus reagent (Takara) following which cDNA was prepared using a Verso cDNA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The quality of cDNA preparation was further confirmed by PCR amplification of the beta-actin gene. qRT-PCR amplifications were then performed using DyNAmo Flash SYBR Green qPCR Kit (Thermo scientific) in a CFX96 real-time thermal cycler (Bio-Rad) with primers specific for human claudin-1, claudin-4, occludin, JAM-1 and ZO-1 (Table 2). The amplification conditions were as follows: initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 10 s and annealing and extension for 30 s at 60°C, with the fluorescence was recorded after each cycle. Each sample was run in triplicate and cycle threshold (Ct) was used for gene expression analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Data was analysed using the $2^{-\Delta\Delta Ct}$ method.

**Analysis of TJ protein distribution in Caco-2 cells by fluorescence microscopy**

Caco-2 cells were grown on Lab Tek II Chamber Slides with Permanox coating (Nalge Nunc International) for 20–22 days until confluent. The monolayers were then treated as described earlier for the real-time PCR experiment. Following treatment, monolayers were rinsed twice with PBS, fixed in ice-cold 100% methanol for 20 min, and blocked with 5% (v/v) normal goat serum for 2 h at room temperature. The cells were then immunostained with primary antibodies (5 µg ml$^{-1}$ rabbit anti-occludin, 10 µg ml$^{-1}$ rabbit anti-claudin-1, 5 µg ml$^{-1}$ rabbit anti-JAM-1, 2.5 µg ml$^{-1}$ mouse anti-claudin-4 and 5 µg ml$^{-1}$ mouse anti-ZO-1; Zymed, Invitrogen) overnight at 4°C, followed by two PBS washes, and the secondary antibody (1/1000 dilutions of Alexa Fluor 488 donkey anti-rabbit for occludin, JAM-1 and claudin-1, and 1 µg ml$^{-1}$ Alexa Fluor 488 goat antimouse for claudin-4 and ZO-1; Zymed, Invitrogen) treatment for 1 h. The monolayers were washed twice with PBS to remove unbound antibodies and were then examined under a BX51 fluorescent microscope (Olympus). For each slide of a single treatment, at least 4–5 images were chosen randomly and captured from different regions. Representative images are shown in the Results.

**Statistical analysis**

Results are given as mean values and standard deviations of the same were calculated. For TEER assays, the permeability assay using latex beads and the immunofluorescence assay, three independent experiments were performed separately. For real-time PCR, results were obtained from two independent experiments performed in triplicate, and for the

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**Table 2. Primers used for real-time PCR amplification of genes encoding tight junction proteins and GAPDH (reference gene) in HT-29 cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
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<td>GAPDH</td>
<td>TGACACCAGGGTGCTCCG</td>
<td>TAGCCCTACAGCTGATCAAGC</td>
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<tr>
<td>Occludin</td>
<td>CCAATGTGCGAACGTTGG</td>
<td>CCAGTGTCGAAAGGCTT</td>
</tr>
<tr>
<td>Claudin-1</td>
<td>AAGTCGGTTGGAAGCAGTGA</td>
<td>CTTGAGTGGTGGTAAAGGTT</td>
</tr>
<tr>
<td>Claudin-4</td>
<td>ACCCGCGCAGAAGCAGC</td>
<td>TCAGCCACGAAAAGCAGC</td>
</tr>
<tr>
<td>ZO-1</td>
<td>ATCCCCGACAGGACATTC</td>
<td>CACTCTCATTTGCCAGGTTTT</td>
</tr>
<tr>
<td>JAM-1</td>
<td>AGCTAGTGGCGGAAATG</td>
<td>TGTGGGCTGAGACAAATAA</td>
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</table>
permeability assay using FITC-inulin, experiments were performed in duplicate with samples in triplicate from each experiment. Significant ANOVA was followed by Dunnett’s test to compare the mean values of each lactobacilli group with EPEC O26: H11 group (P<0.05). All analyses were conducted using Graph Pad Prism 6.01.

RESULTS

**Effect of lactobacilli on TEER and permeability to latex beads across Caco-2 monolayers infected with EPEC O26: H11**

The TEER of Caco-2 monolayers was measured at 0, 2, 4, 16 and 18 h following lactobacilli treatment and the percentage change in the TEER value after lactobacilli treatment was calculated with respect to the TEER values before addition of EPEC O26: H11 (indicated by −4 h in Fig. 1a). In EPEC O26: H11-infected Caco-2 monolayers, a significant decrease was observed in TEER with time, compared to uninfected monolayers. The TEER of the monolayer decreased up to 35.7±0.9 % after 18 h incubation with EPEC O26: H11. However, when the cells were treated with the different lactobacilli strains, the reduction in the TEER was reversed. Significant reversal in TEER reduction was observed with LGG (77.2±1.7 %), *L. helveticus* FA-7 (77.8±1.27 %) and *L. fermentum* strains FA-1 (81.8±1.0 %) and FA-5 (77.5±0.9 %) after 2 h incubation, whereas with the other strains, it was observed after 4 h incubation (P<0.05). The significant reversal continued at 16 and 18 h of lactobacilli incubation. For 18 h incubation, the highest reversal was observed when monolayers were treated with LGG (75.3±1.5 %) followed by *L. fermentum* FA-1 (74.0±1.6 %) and *L. plantarum* GRI-2 (73.1±1.1 %).

The permeability of Caco-2 monolayers was analysed by translocation of latex beads across the Caco-2 monolayer. The latex beads were added to the apical chamber of transwell inserts and translocation of the beads to the basolateral chamber relative to control was determined after 2, 4, 16 and 18 h of lactobacilli incubation (Fig. 1b). In EPEC O26: H11-infected monolayers, a continuous increase in the translocation of latex beads was observed throughout the time course. As monolayers were treated with lactobacilli strains, the translocation of beads to the basolateral chamber of the treatment groups decreased significantly after 4 h of treatment compared to EPEC-infected monolayers (P<0.05). Maximum reduction was observed when monolayers were treated with *L. fermentum* GPI-3 (4.1-fold) for 4 h and with *L. fermentum* FA-1 (3.7-fold) for 18 h which was similar to that of the LGG-treated monolayer (4.6-fold).

**Effect of lactobacilli strains on TEER and permeability to inulin across HT-29 cells infected with EPEC O26: H11**

When TEER was measured in the HT-29 monolayers infected with EPEC O26: H11, a significant decrease was observed compared to control monolayers (Fig. 2a) (P<0.05). This reduction in TEER continued further with increased incubation period. The TEER decreased to 43.7±2.1 % after 18 h incubation indicating a disruptive effect of EPEC O26: H11 on the integrity of the monolayers. This reduction in TEER was however significantly reversed by most of the lactobacilli strains except *L. helveticus* FA-7 and *L. fermentum* strains GPI-7 and FA-5 following their exposure to EPEC O26: H11-infected monolayers for 4 h (P<0.05). However, none of the strains were able to impact the TEER reduction following only 2 h of exposure. When TEER was measured at 16 and 18 h of lactobacilli exposure to infected monolayers, significant reversal in TEER reduction was observed with all strains studied (P<0.05). Maximum reversal was observed when monolayers were treated with *L. fermentum* IIIs11.2 (92.6±2.2 %), *L. plantarum* GRI-2 (91.9±0.9 %) and LGG (93.8±2.0 %) for 18 h.

The effect of lactobacilli strains on permeability of monolayers to FITC-inulin was checked following 2, 4, 16 and 18 h of exposure to infected monolayers and the values were deducted from that of control monolayers. The percentage reduction in the monolayer permeability to FITC-inulin with respect to that of EPEC O26: H11-infected monolayers without any lactobacilli treatment was calculated for each time point (Fig. 2b). It was observed that all the strains were able to reduce the permeability significantly at 16 and 18 h (P<0.05). At 2 h and 4 h a significant reduction in the permeability was also observed with all strains except for *L. fermentum* strains GPI-3 and GPI-7 (at 2 h), and *L. fermentum* FA-1 (at 4 h) (P<0.05). However, the highest reduction was observed after 18 h incubation with most of the strains except *L. fermentum* FA-5 and *L. helveticus* FA-7 which required 16 h incubation for maximum reduction (Fig. 2b).

**Effect of lactobacilli on expression of TJ protein-specific mRNA in E. coli-infected HT-29 cells**

The mRNA expression for TJ proteins (claudin-1, claudin-4, ZO-1, JAM-1 and occludin) in EPEC O26: H11-infected HT-29 cells, with/without lactobacilli treatment was analysed by qRT-PCR; results are given in Fig. 3. HT-29 monolayers in the absence of any bacterial infection served as control. The mRNA expression of ZO-1 and claudin-1 was significantly higher in *L. helveticus* FA-7-treated HT-29 cells compared to only EPEC O26: H11-infected cells (P<0.05). The mRNA expression of claudin-1 in HT-29 treated with *L. helveticus* FA-7 was 3-fold higher compared to *E. coli*-infected cells (0.33±0.08 in *E. coli* and 1.10±0.04 in *L. helveticus* FA-7), while the mRNA expression of ZO-1 was 2.5-fold higher in *L. helveticus* FA-7-treated HT-29 cells compared to EPEC O26: H11-infected cells (0.45±0.11 in *E. coli* and 1.13±0.20 in *L. helveticus* FA-7). No significant difference was observed in the mRNA expression of claudin-4, JAM-1 and occludin in *L. helveticus* FA-7-treated HT-29 cells compared to the EPEC O26: H11-infected cells. The other strains were not able to significantly increase mRNA expression of any TJ proteins in HT-29 cells compared to the EPEC O26: H11-infected cells (P<0.05).
**Effect of lactobacilli on distribution of TJ proteins in EPEC O26: H11-infected Caco-2 cells**

The distribution of various TJ proteins (claudin-1, claudin-4, ZO-1, JAM-1 and occludin) was analysed in EPEC O26: H11-infected Caco-2 cells with or without lactobacilli treatment. In the EPEC O26: H11-infected Caco-2 cells, the fluorescence was discontinuous and dispersed in the cytoplasm for all the TJ proteins studied (i.e. claudin-1, claudin-4, JAM-1, ZO-1 and occludin). This altered distribution of TJ proteins was however ameliorated when EPEC O26: H11-infected cells were treated with various lactobacilli (Fig. 4). When Caco-2 cells were treated with *L. fermentum* FA-1 followed by staining with antibodies specific for various TJ proteins, a well-defined staining pattern at the cell periphery was observed. This redistribution of TJ proteins was also observed in *L. plantarum* GRI-2-treated Caco-2 cells except for JAM-1 protein. Nevertheless, while JAM-1 distribution was relatively less disturbed in *L. plantarum* GRI-2-treated cells compared to *E. coli*-infected Caco-2 cells, the other TJ proteins were redistributed to the cell periphery. Distribution of TJ proteins was relatively less disturbed when EPEC O26: H11-infected Caco-2 cells were treated with other lactobacilli strains.

**DISCUSSION**

The integrity of mucosal barriers is absolutely essential in order to defend against the invasion of pathogenic bacteria. In the present study, the neutralizing effect of various
lactobacilli strains on EPEC O26 : H11-induced epithelial barrier dysfunction was studied in vitro by TEER and permeability assays. Further, the effect of these strains on mRNA expression of TJ proteins and distribution of these proteins in EPEC O26 : H11-infected intestinal epithelial cell lines was studied. We used two human colonic adenocarcinoma cell lines, HT-29 and Caco-2, to study the neutralizing effect of lactobacilli on epithelial barrier dysfunction. HT-29 cells are fast growing, unlike Caco-2 cells, and require only 7–8 days to fully differentiate, whereas the latter take 20–22 days. Thus the expression of TJ proteins and their assembly into a TJ complex would also be rapid in HT-29 cells. Therefore we thought HT-29 cells would be better to use for studies related to the gene expression of TJ
proteins using qRT-PCR. Similarly, TJ protein distribution was studied using Caco-2 cells due to their ability to form very good junction complexes. Hence, any minor disruption could be detected using fluorescence microscopy which might be difficult to study using HT-29 cells where the TJs are not as compact. Epithelial barrier integrity was verified by measurements of TEER and permeability for macromolecules like latex beads and inulin. The decrease in the TEER of both Caco-2 and HT-29 cell lines upon infection with EPEC O26 : H11 indicates the disruptive effect of EPEC O26 : H11 on epithelial barrier with respect to time. When reversal of TEER reduction was studied at different time intervals following treatment with different lactobacilli strains, maximum reversal (up to 93.8±2.0 %) was observed after 18 h of treatment with \textit{L. fermentum} strains FA-1 and FA-5, \textit{L. helveticus} FA-7 and LGG, whereas the other strains required 4 h. Previous studies by Anderson et al. [19] and Yu et al. [20] observed reversal in similar time intervals. Such strain-dependent variation in the TEER recovery was also suggested by Donato et al. [21] and Yang et al. [22].

The effect on TEER recovery was also corroborated by the effect of these strains on permeability to macromolecules on monolayers pre-infected with EPEC O26 : H11. In earlier studies, fluorescent-conjugated inulin [23, 24] and latex beads [25] have been used to validate the intestinal epithelial permeability to macromolecules. The disruptive effect of EPEC O26 : H11 on the monolayers as indicated by an increase in the permeability of monolayers for macromolecules at different time intervals was reversed following lactobacilli treatment. Eun et al. [26] also reported a similar effect of \textit{Lactobacillus casei} pre-treatment on the permeability for FITC-dextran in cytokine-induced epithelial barrier dysfunction. In the present study, differences were observed in the effectiveness and time taken by these strains to neutralize the EPEC O26 : H11-induced barrier dysfunction for both the cell lines. The differences in the polarity and TJ protein expression could be the reason for the differences observed in the effectiveness and incubation time required by the strains to reverse TEER reduction and increased permeability. Caco-2 cells form a monolayer of highly polarized

![Representative images of immunofluorescence staining of tight junction (TJ) proteins in Caco-2 cell line. (a) Control Caco-2 cells, (b) EPEC O26 : H11-infected Caco-2 cells, (c–f) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: \textit{Lactobacillus rhamnosus} LGG (c), \textit{Lactobacillus fermetum} FA-1 (d), \textit{Lactobacillus plantarum} GRI-2 (e) and \textit{Lactobacillus salivarius} GPI-1(S) (f). Arrows with dashed line (b) indicates disrupted distribution of tight junction as represented by fluorescence in the cytoplasm. Normal arrows (c, d, e) indicate the complete redistribution of TJ protein from cytoplasm to periphery. Arrows with dotted line (f) indicate the partial redistribution of TJ proteins from cytoplasm to periphery. Representative images from three independent experiments with all the strains used in this study.](image-url)
cells, joined by functional tight junctions, with well-developed and organized microvilli on the apical membrane whereas the HT-29 cell line is derived from human intestinal mucus-secreting goblet cells. HT-29 grow as a multilayer of non-polarized, undifferentiated cells under normal growth conditions with relatively less expression of tight junctions compared to Caco-2 cells [27, 28].

The barrier integrity and thus TEER is regulated mainly by the TJ proteins which seal the paracellular space between epithelial cells [29]. In the present study, reversal in TEER reduction was observed within 2–4 h of incubation with some of the strains. This reversal could be due to the increased expression of TJ proteins and/or re-distribution of TJ proteins towards the cell periphery by these strains [20, 22]. The results from qRT-PCR and immunofluorescence staining indicated that most of the strains had no effect on the transcription of TJ proteins. Only L. helveticus FA-7 significantly increased the mRNA expression of claudin-1 and ZO-1 and distributed it to the cell periphery while the rest of the strains redistributed the already existing proteins from the cytoplasm to the cell boundaries as observed by immunofluorescence assay. The reason behind this unresponsiveness on the transcription of TJ proteins may be linked to the divergence in the interaction of these lactobacilli strains with host-cell receptors and stimulatory components. Such strain-dependent effects could probably be attributed to the expression of different proteins and carbohydrates by individual strains of lactobacilli as hypothesized by Sultana et al. [30]. The immunofluorescence staining revealed that L. fermentum FA-1 and L. plantarum GRI-2 completely redistributed the TJ proteins from the cytoplasm to the cell periphery. These strains did not significantly increase the transcription of TJ proteins and thus no new proteins were being synthesized. From these results it can therefore be concluded that redistribution of already existing proteins from the cytoplasm to the cellular junctions could be the main reason for the rapid reversal observed in TEER and permeability assays. An earlier report by Miyauchi et al. [31] also suggested that attenuation in H₂O₂-induced TEER reduction by Lactobacillus strains is attributed to the redistribution of transmembrane TJ proteins. Moreover, from the present study it could also be concluded that the mechanism by which lactobacilli strains improve the barrier function varies from strain to strain which is similar to earlier reports [30].

In conclusion, all the strains studied were able to neutralize the EPEC O26: H11-induced epithelial barrier dysfunction in vitro. However, the effectiveness and the incubation period required by these strains for having the same effect varied. Moreover, the mechanism by which they improve the barrier function also differed amongst different strains. Most of the strains manifested their beneficial effect on the redistribution of TJ proteins, while only for L. helveticus FA-7 was the effect observed at both the mRNA level and the distribution of TJ proteins. So overall although L. helveticus FA-7, L. fermentum FA-1 and L. plantarum GRI-2 were good in all the aspects studied, the other strains were also good in some aspects.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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