Chapter 4

Neutralization effect of *Lactobacillus* strains on enteropathogenic *E.coli*-induced epithelial barrier dysfunction

"Whatever the mind can conceive and believe, it can achieve"

-Napoleon Hill

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4.1 Introduction

The epithelial cell lining of the intestine constitutes a major barrier which separates the internal environment of the gut from the external environment. It prevents pathogenic bacteria and the harmful substances from entering into the body. Besides acting as a protective barrier, the intestinal epithelium is also involved in nutrient absorption and waste secretion which require a selective permeable barrier. Infection by various pathogenic bacteria such *Escherichia coli* (*E. coli*) (Sherman et al., 2005) and *Salmonella typhi* (Kops et al., 1996) causes the intestinal barrier disruption and consequent enhancement in the permeability. Such enteric pathogens cause barrier disruption either by directly binding to the epithelial cells, or activating subsequent signalling cascades which regulate the barrier function, or through the secretion of toxins. Increased epithelial permeability or "leaky gut" is also associated with many gastrointestinal tract (GIT) disorders (Clayburgh et al., 2004). The intestinal permeability and the barrier function is mainly regulated by the intercellular junctional complexes which seals the gap between the adjacent epithelial cells (Marchiando et al., 2010). The intercellular junctional complexes includes several complexes such as tight junctions (TJ), adherence junctions (AJ), gap junctions, and desmosomes (Groschwitz and Hogan, 2009). The paracellular permeability across the epithelium is regulated by the TJ complex which comprises of integral membrane proteins such as claudins,

occludin, and junction adhesion molecule (JAM) that interact with the zonulins, zonula occludens (ZO)-1, ZO-2, and ZO-3 that are in turn bound to the perijunctional ring of cytoskeletal actin (Paris et al., 2008; Schneeberger and Lynch, 2004). Claudin subfamily proteins claudin-1 and -4 are known to be associated with a decrease in the paracellular permeability (Bücker et al., 2010). Occludin is involved in the regulation of intermembrane diffusion as well as paracellular diffusion of small molecules (Balda et al., 1996). Expression of JAM-1 protein leads to a reduction in paracellular permeability (Bazzoni G., 2003).The TJ structures are continuously remodelled in response to various signals, such as nutrients, cytokines and immune cells (Karczewski and Groot, 2000; Nusrat et al., 2000).

Lactobacillus play an important role in maintaining the homeostasis of the gut flora by adhering to and colonizing the intestinal mucosa and competing with pathogenic bacteria, such as some strains of *E. coli* (Collado et al., 2007; Sherman et al., 2005). The enhancement of epithelial barrier function is one of the proposed mechanisms by which certain probiotic organisms like lactobacilli may confer health benefits. *Lactobacillus* interacts with the epithelium resulting in a modulation of epithelial physiology in relation to barrier function and associated cell signalling. Enhancement in barrier integrity is often 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 epithelial barrier by altering the expression or phosphorylation of TJ proteins (Karczewski J et al., 2010; Miyauchi E et al., 2009; Resta-Lenert and Barrett, 2003). Another study demonstrated the ability of *Lactobacillus plantarum* to reduce tumor necrosis factor (TNF)-α-induced barrier dysfunction and inflammation of Caco-2 (Ko et al., 2007). In a study employing

Lactobacillus acidophilus to ameliorate the effects of interferon (IFN)-γ on transepithelial electrical resistance (TEER), protective effects were abolished through pharmacologic inhibition of ERK, p38, and PI3K (Resta-Lenert S. and Barrett, 2006). In the present chapter, lactobacilli strains were assessed for their neutralizing effect on the enteropathogenic *E. coli* O26:H11-induced epithelial barrier dysfunction *in vitro*. EPEC infection is reported to increase the paracellular permeability and disturb the barrier integrity in the mouse intestines and intestinal cell-line T84 (Philpott et al., 1996; Shifflett et al., 2005). The study was carried out using intestinal epithelial cell-lines, Caco-2 and HT-29. Barrier dysfunction was measured by transepithelial electrical resistance (TEER) and paracellular permeability across the monolayers. TEER value is directly proportional to the barrier integrity. Increase in the TEER value indicates decrease in the permeability of the epithelium to ions (McCormick, 2003). Paracellular permeability was measured using FITC –labelled inulin and latex beads. Furthermore, the studies were extended to investigate the effects of lactobacilli strains on the gene expression of TJ proteins and their distribution in the disturbed epithelial barrier. Expression levels of genes specific for claudin-1, -4, occludin, ZO-1 and JAM-1 were analyzed using quantitative real-time PCR. To examine the TJ protein distribution within epithelial cells, immunofluorescence assay was used. *Lactobacillus rhamnosus* GG (LGG) was used as a positive control since the strain is already reported to have preventive effects in the epithelial barrier dysfunction (Johnson-Henry K.C. et al., 2008).

4.2 Materials and Methods

4.2.1 Bacterial strains

Lactobacilli strains used in the present study are described in table 4.1. Lactobacilli and EPEC cells were cultured routinely as described in chapter 3 (Materials and Methods:

section 3.2.2). Lactobacilli cells were used at a density of 1 X 10^8 CFU/ml resuspended in antibiotic-free DMEM media whereas EPEC was used at a density of 1 X 10^7 CFU/ml.

Table 4. 1 Strains used in the study

NO.	***LACTOBACILLUS* STRAINS**	
Strains from the present study		
1	*Lactobacillus fermentum* FA-1	
2	*Lactobacillus fermentum* FA-5	
3	*Lactobacillus helveticus* FA-7	
4	*Lactobacillus fermentum* GKI-1	
5	*Lactobacillus fermentum* IIS11.2	
6	*Lactobacillus fermentum* GPI-3	
Other strains from the laboratory		**Accession number**
7	*Lactobacillus salivarius* GPI-1(S)	JX118837
8	*Lactobacillus fermentum* GPI-7	JX118831
9	*Lactobacillus plantarum* GRI-2	JX118835

4.2.2 Intestinal epithelial cell-culture

Caco-2 and HT-29 cell-lines were maintained in standard T25 tissue culture flask (Corning Incorporated) as described in the hapter 3 (Materials and Methods: section 3.2.1). For permeability and TEER assay, the cells were seeded onto the 6.5 mm diameter Transwell (Costar; Corning) polyester inserts (3 μm pore size) at a density of 0.5 X 10^5 cells per insert and grown at 37 °C with 5% CO_2 and 95% air atmosphere. TEER of both the cell-lines was measured regularly with a Millicell-ERS (Millipore). Caco-2 cell-lines were maintained until a TEER of 800 Ω X cm^2 (for 20-22 days) was achieved. HT-29 cell-lines were grown for 7-8 days until 400 to 500 Ω X cm^2 TEER was obtained. The monolayers were then used for the experiments. For gene expression

study, HT-29 cells were seeded in the standard 24-well plates (Costar; Corning) at a concentration of 0.5 X 10^5 cells per well and grown until confluency (7-8 days). For immunofluorescence study, Caco-2 cells (0.5 X 10^4 cells per chamber) were grown on Lab Tek II Chamber Slides with Permanox coating (Nalge Nunc International) for 20-22 days until confluent. Before experiments, the monolayers were pre-incubated with antibiotic-free DMEM media for 90 mins in all the assays.

4.2.3 TEER and permeability assay

The Caco-2 monolayers, grown as described above, were first infected with 1 X 10^7 CFU/ml of EPEC O26:H11 at the apical compartment of the insert and incubated at 37℃ in 5% CO_2/95% air atmosphere for 4 h. The monolayers were then washed twice with DPBS following which they were treated with 1 X 10^8 CFU/ml of different lactobacilli for 18 h. Caco-2 monolayers cultured under the same conditions, but in the presence and absence of pre-infection with EPEC O26 :H11 served as EPEC O26 :H11 control and absolute control, respectively. At the time of addition of lactobacilli, a 10 µl aliquot of fluorescent Red-labelled carboxylate-modified polystyrene latex beads (2.0 µm) (Sigma-Aldrich) was added to the apical compartment of the Caco-2 monolayers. At 2, 4, 16 and 18 h of lactobacilli treatment, aliquots were taken out from the basolateral compartment. The translocation of latex beads across the monolayers was estimated by counting under a CX41 fluorescent microscope (Olympus) and permeability for the same was depicted as relative to control. The TEER of the monolayers were measured before addition of EPEC O26:H11 (considered as -4 h) and at 0, 2, 4, 16 and 18 h following lactobacilli exposure. The percentage change in the TEER value after lactobacilli treatment was calculated with respect to the TEER values before addition of EPEC O26:H11 (i.e. -4 h). The results are expressed as a mean value of three independent experiments performed in duplicate.

In the HT-29 monolayers, the same protocol was followed except fluorescein isothiocyanate (FITC)-inulin (at a final concentration of 100 μg/ml; Sigma-Aldrich) was used to measure the paracellular permeability instead of latex beads. The fluorescence intensity was measured using a Synergy-HT multiplate reader (BioTek). The values obtained for the monolayers in each well was deducted from that of the control monolayer and 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.

4.2.4 Expression analysis of mRNA encoding TJ proteins

The effect of lactobacilli strains on the expression levels of mRNA encoding TJ proteins, claudin-1, -4, occludin, JAM-1, and ZO-1, in the EPEC O26:H11 infected HT-29 cells was carried out using quantitative real-time PCR (qRT-PCR). HT-29 monolayers grown in the standard 24-well plates were pre-infected with 1 X 10^7 CFU/ml of EPEC O26:H11 for 2 h following which the monolayers were washed twice with DPBS. Different lactobacilli (1 X 10^7 CFU/ml) were then added to the respective wells and incubated for 2 h followed by two washes with DPBS to remove lactobacilli cells. The monolayers were then incubated in antibiotic-containing medium for 20 h following which, total RNA was isolated. The HT-29 monolayers without any bacterial treatment was used as an absolute control whereas EPEC-infected monolayers without lactobacilli treatment served as EPEC O26:H11 control.

RNA isolation

Total RNA was isolated from HT-29 cells using total RNAiso Plus (Takara) reagent. The plastic ware and glassware used for RNA isolation were treated with chloroform to

destroy any RNase present. The reagents were prepared in diethyl pyrocarbonate (DEPC) treated autoclaved distilled water.

To the each well, 500µl of RNAiso Plus reagent (Takara) was added and mixed thoroughly. The suspension was then transferred to a microfuge tube and incubated at room temperature for 10 min for lysis. Following lysis, 0.1 ml of chloroform was added and mixed until the solution turned milky. The mixture was then left at room temperature for 5 min and centrifuged at 12,000 $\times g$ for 15 min at 4ºC. The supernatant was then transferred into a fresh tube and equal volume of 100% isopropanol was added. The content was mixed and kept at -20ºC for 10 min for precipitation, followed by centrifugation at 12,000 $\times g$ for 10 min at 4ºC. The supernatant was carefully removed without touching the pellet. 250 µl of ethanol (75%) was added to the pellet and centrifuged at 7,500 $\times g$ for 5 min at 4ºC and the supernatant was discarded. The pellet was dried at 42ºC for 10 min and dissolved in 50 µl DEPC treated water. The RNA samples were quantified using the NanoPhotometer (IMPLEN).

cDNA synthesis and confirmation of cDNA quality

cDNA was prepared using a Verso cDNA kit (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, the one µg of each RNA sample was mixed with anchored oligo-dT, RT enhancer which contains DNAse I, dNTP mix, Verso enzyme mix and cDNA synthesis buffer, followed by incubation at 50°C for 60 min and then at 95°C for 5 min to inactivate the enzyme in a thermal cycler (Eppendorf). Each of the cDNA preparations was then amplified for 35 cycles in a thermal cycler with β actin-specific primers (FP: 5'-AGCGGGAAATCGTGCGTGACA-3'; RP:5'-CGCAACTAAGTCATAGTCCG-3') by taking 2 µl of cDNA in a 25 µl system. Amplification conditions were as follows: initial denaturation at 94ºC for 6 min,

followed by 34 cycles of denaturation at 94ºC for 30 sec, annealing at 66 ºC for 30 sec, followed by extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 min. PCR products were separated on a 0.8% agarose gel and stained with ethidium bromide.

Table 4. 2 Reaction system for semi-quantitative reverse transcriptase-PCR

COMPONENTS	**VOLUME (µl)**
5X cDNA buffer	4
dNTP mix (5mM)	2.0
RNA primer (500 ng/µl)	1.0
RT enchancer	1.0
Verso enzyme mix	1.0
RNA template	x (corresponding to 1 µg)
Autoclaved MilliQ water	Upto 20 µl
Total volume	**20**

Table 4. 3 RT-PCR conditions for cDNA synthesis

TEMPERATURE	**TIME**
Take the template RNA	
70ºC	5 min
Add the other components to it	
50ºC (cDNA preparation)	60 min
95ºC (inactivation)	5 min

Table 4. 4 Reaction system for β-actin PCR

COMPONENTS	VOLUME (µl)
Autoclaved MilliQ water	17.0
cDNA	2.0
10X Buffer for Taq DNA Polymerase	2.5
dNTP mix (10mM)	0.5
Forward primer (10 mM)	1.25
Reverse primer (10 mM)	1.25
Taq DNA polymerase (2.0 U/µl) (Sigma-Aldrich)	0.5
Total volume	**25.0**

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR amplifications were 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 4.7). The amplification system and conditions were as follows:

Table 4. 5 Reaction system for qRT-PCR

COMPONENTS	VOLUME (µl)
DNA	0.5
Forward Primer (10mM)	0.5
Reverse Primer (10mM)	0.5
2x SYBr mix	5.0
Autoclaved MilliQ water	3.5
Total volume	**10.0**

Table 4. 6 Conditions for qRT-PCR analysis of TJ proteins.

STEPS		TEMPERATURE (°C)	TIME	NO.OF CYCLES
1	Initial denaturation	94	3 min	1

2	Denaturation	94	10 seconds	45
3	Primer annealing and extension	60	30 seconds	
4	Final Extension	72	1 min	1

Table 4. 7 Primers used for qRT-PCR analysis

GENE	PRIMER SEQUNECE
GAPDH	FP: 5'-TGAGCACCAGGTGGTCTCC-3' RP: 5'- TAG CCA AAT TCG TTG TCA TAC CAG-3'
Occludin	FP: 5'-CCAATGTCGAGGAGTGGG-3' RP: 5'- CGCTGCTGTAACGAGGCT-3'
Claudin-1	FP: 5'-AAGTGCTTGGAAGACGATGA-3' RP: 5'- CTTGGTGTTGGGTAAGAGGTT-3'
Claudin-4	FP: 5'-ACCCCGCACAGACAAGC-3' RP: 5'- TCAGTCCAGGGAAGAACAAAG-3'
ZO-1	FP: 5'-ATCCCTCAAGGAGCCATTC-3' RP: 5'- CACTTGTTTTGCCAGGTTTTA-3'
JAM-1	FP: 5'-AGCCTAGTGCCCGAAGTG-3' RP: 5'- TGTGGGGTGTAGAAGACAAATAA-3'

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. The product specificity was confirmed by single peak in melt curve analysis (from 65ºC to 95ºC in 0.5ºC/5s increments).

4.2.5 Immunofluorescence assay

As described earlier in section 4.2.2, Caco-2 monolayers were grown in Chamber slides and then the monolayers were infected with EPEC O26:H11 followed by lactobacilli treatment as described above in section 4.2.4. Distribution of TJ proteins in the Caco-2 cells following lactobacilli treatment was analyzed using antibodies specific for

claudin-1, -4, occludin, JAM-1 and ZO-1. Briefly, following the bacterial treatments, the 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 specific for (5 μg/ml rabbit anti-occludin, 10 μg/ml rabbit anti-claudin-1, 5 μg/ml rabbit anti-JAM-1, 2.5 μg/ml mouse anti-claudin-4, and 5 μg/ml mouse anti-ZO-1; Zymed, Invitrogen) overnight at 4 °C followed by washing twice with PBS. The cells were then incubated with secondary antibody (1/1000 dilutions of Alexa Fluor 488 donkey anti-rabbit for occludin, JAM-1 and claudin-1, and 1 μg/ml Alexa Fluor 488 goat anti-mouse for claudin-4 and ZO-1; Zymed, Invitrogen) at room temperature for 1 h in dark. 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.

4.2.6 Statistical analysis

All the analyses were conducted using Graph Pad Prism 6.01. Significant ANOVA was followed by Dunnett's test to compare the mean values of each lactobacilli group with EPEC O26:H11 group ($P<0.05$). 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 permeability assay using FITC-inulin, experiments were performed in duplicate with samples in triplicate from each experiment.

4.3 Results

4.3.1 Effect of lactobacilli on TEER of the EPEC O26:H11-infected monolayers

The TEER of Caco-2 (figure 4.1) and HT-29 (figure 4.2) monolayers was measured before addition of EPEC O26:H11 (indicated as -4 h) and 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 at -4 h of the respective monolayers. In EPEC O26:H11-infected Caco-2 and HT-29 monolayers, a significant decrease was observed in TEER with time, compared to uninfected monolayers ($P<0.05$). After 18 h of incubation, the TEER of the Caco-2 monolayer was reduced up to 35.7 ± 0.9% and that of HT-29 was reduced to 43.7 ± 2.1 %. However, when the monolayers were treated with the different lactobacilli strains, the reduction in the TEER was reversed. In the TEER of Caco-2 monolayers, significant % reversal in the 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. In the case of 18 h incubation with Caco-2 monolayers, 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 %).

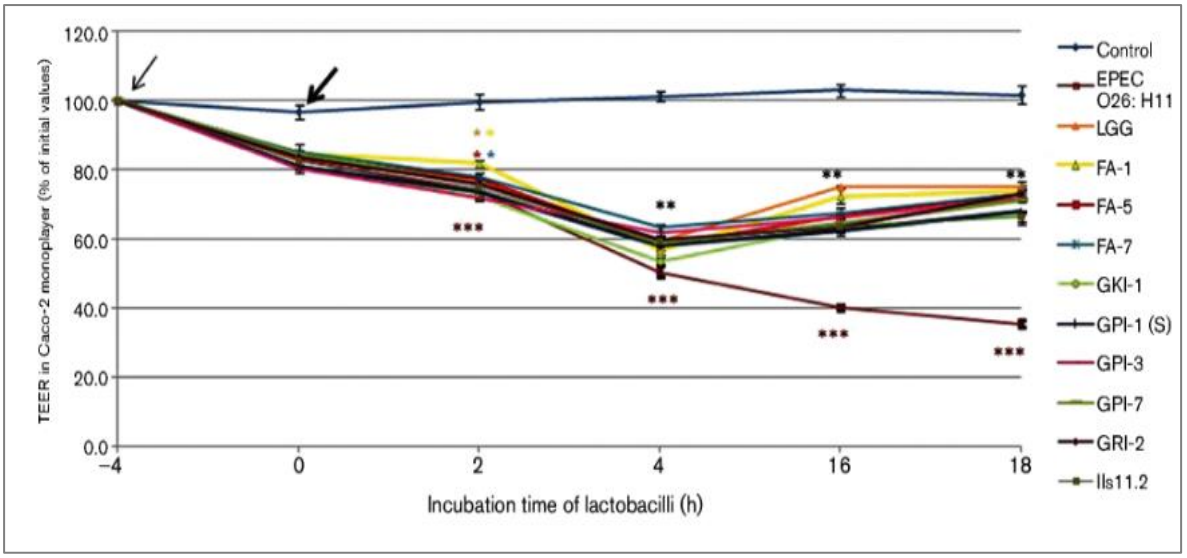

Figure 4. 1 TEER assay with Caco-2 cell-lines.
Effect of lactobacilli strains on TEER of EPEC O26:H11 infected Caco-2 monolayers. Each line represents the mean value and error bar as standard deviation of three independent experiments. The thin lined arrow indicates the addition of EPEC O26:H11 and the thick lined arrow indicates addition of lactobacilli. '*' in different colors indicate significant difference in TEER of monolayers treated with respective strains compared to EPEC O26:H11 infected monolayer. The color code of the asterisk (*) matches with the color code of strain showing significant difference. The sign '**' indicates significant difference in TEER of monolayers treated with all the strains compared to EPEC O26:H11 infected monolayer at respective time point. The sign '***' indicates significant difference in TEER of EPEC O26:H11infetced monolayer compared to control monolayer. Significant ANOVA was followed by Dunnett's test for multiple comparisons vs. the EPEC O26:H11 group ($P<0.05$).

Interestingly, in the HT-29 monolayers (figure 4.2), none of the lactobacilli strains were able to reverse the reduction in TEER induced by EPEC infection following 2 h of incubation. However, after 4 h of treatment with lactobacilli, this reduction in TEER of HT-29 monolayers infected with EPEC was significantly reversed by most of the strains except *L. helveticus* FA-7 and *L. fermentum* strains GPI-7 and FA-5 ($P<0.05$). Moreover, significant reversal in the TEER reduction of EPEC-infected HT-29 monolayers was observed following 16 and 18 h of exposure with each lactobacilli strains ($P<0.05$). When the HT-29 monolayers were treated with *L. fermentum* IIS11.2, *L. plantarum* GRI-2 and LGG for 18 h, maximum reversal was observed (92.6 ± 2.2 %, 91.9 ± 0.9 %, and 93.8 ± 2.0 %, respectively).

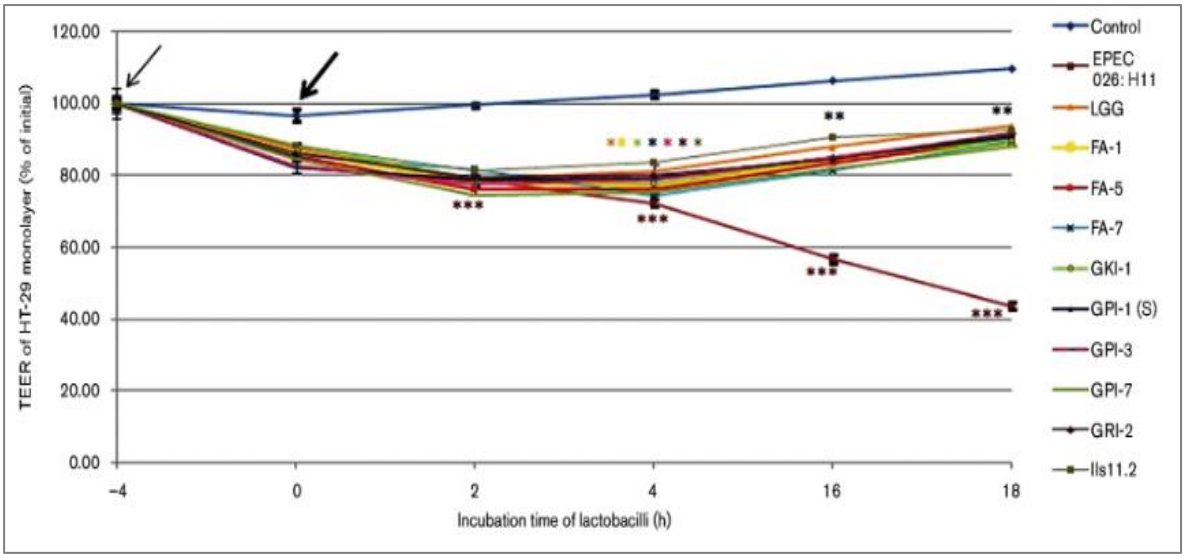

Figure 4. 2 TEER assay with HT-29 cell-lines. Effect of lactobacilli strains on TEER of EPEC O26:H11 infected HT-29 monolayers.

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 colors indicate significant difference in TEER of monolayers treated with respective strains compared to EPEC O26:H11 infected monolayer. The color code of the asterisk (*) matches with the color code of strain showing significant difference. The sign '**' indicates significant difference in TEER of monolayers treated with all the strains compared to EPEC O26:H11 infected monolayer at respective time point. The sign '***' indicates significant difference in TEER of EPEC O26:H11 infetced monolayer compared to control monolayer. Significant ANOVA was followed by Dunnett's test for multiple comparisons vs. the EPEC O26:H11 group ($P<0.05$).

4.3.2 Effect of lactobacilli on the paracellular permeability across the EPEC O26:H11-infected monolayers

The permeability of the Caco-2 monolayers was analysed by monitoring the translocation of latex beads across the Caco-2 monolayer whereas with HT-29 cells, the permeability for FITC-inulin across the monolayers was examined (figure 4.3). In the EPEC O26:H11-infected monolayers, a continuous increase in the permeability across both the monolayers was observed throughout the time course of study. As Caco-2 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 EPEC-infected Caco-2 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).

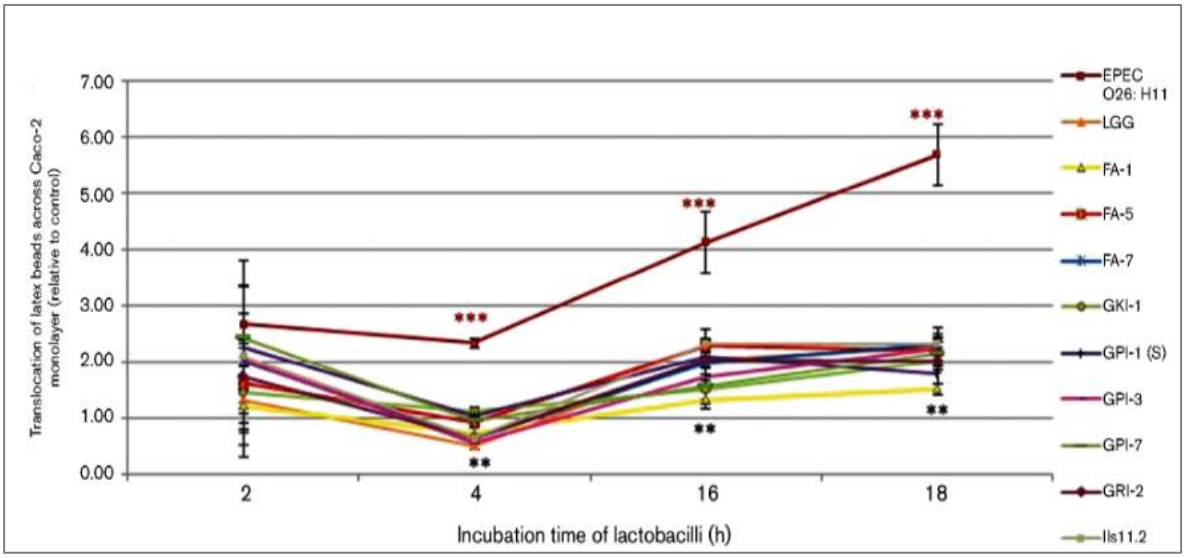

Figure 4. 3 Effect of lactobacilli strains on permeability of latex beads across EPEC O26:H11 infected Caco-2 monolayers.
Each line represents the mean value and error bar as standard deviation of three independent experiments. '**' indicates significant difference number of latex beads translocated across the Caco-2 monolayers treated with all the strains separately compared to EPEC O26:H11 infected monolayer at respective time point. '***' indicates significant difference in number of latex beads translocated across the EPEC O26:H11infetced monolayer compared to control monolayer. Significant ANOVA was followed by Dunnett's test for multiple comparisons vs. the EPEC O26:H11 group ($P<0.05$).

When EPEC-infected HT-29 monolayers were treated with lactobacilli strains, 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 (figure 4.4).

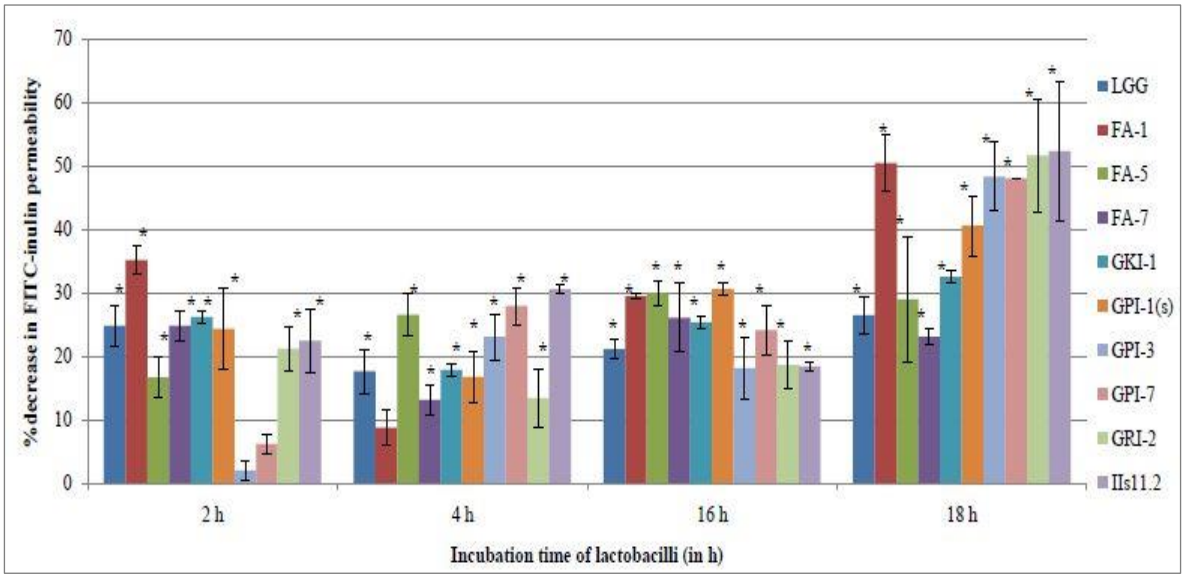

Figure 4. 4 Effect of lactobacilli strains on the permeability of FITC-inulin across EPEC O26:H11-infected HT-29 monolayers.

Each bar represents the mean value and error bar as standard deviation of two independent experiments. The values obtained for the monolayers each well deducted from that of the control monolayer. The permeability of 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. '*' indicates significant difference in FITC-inulin permeability across monolayers treated with respective strains compared to EPEC O26:H11-infected monolayer. Significant ANOVA was followed by Dunnett's test for multiple comparisons versus the EPEC O26:H11 group ($P<0.05$).

4.3.3 Levels of mRNA expression specific for TJ protein in EPEC-infected HT-29 cells

The levels of mRNA expression encoding 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 analyzed by qRT-PCR; results are given in figure 4.5. The expression of these genes was normalized to that of the reference gene, GAPDH. HT-29 monolayers without any bacterial exposure was used as a control. Treatment of EPEC-infected HT-29 monolayers with *L. helveticus* FA-7 exhibited significant increase in the levels of mRNA expression specific for ZO-1 and claudin-1 compared to only EPEC-infected

monolayers (P<0.05). However, the levels of mRNA expression specific for the other TJ proteins (claudin-4, occludin and JAM-1) in EPEC-infected HT-29 monolayers were not affected by the treatment with FA-7. The level of mRNA expression specific for claudin-1 was 3-fold higher in FA-7 treated cells compared to the EPEC-infected cells (1.10±0.04 in *L. helveticus* FA-7 and 0.33±0.08 in EPEC), whereas the levels of mRNA expression specific for ZO-1 was 2.5-fold higher in the in *L. helveticus* FA-7-treated HT-29 cells compared to EPEC O26:H11-infected cells (1.13±0.20 in *L. helveticus* FA-7 and 0.45±0.11 in EPEC). The other strains were not able to significantly increase levels of mRNA expression specific for any TJ proteins studied in HT-29 cells compared to the EPEC O26:H11-infected cells ($P<0.05$).

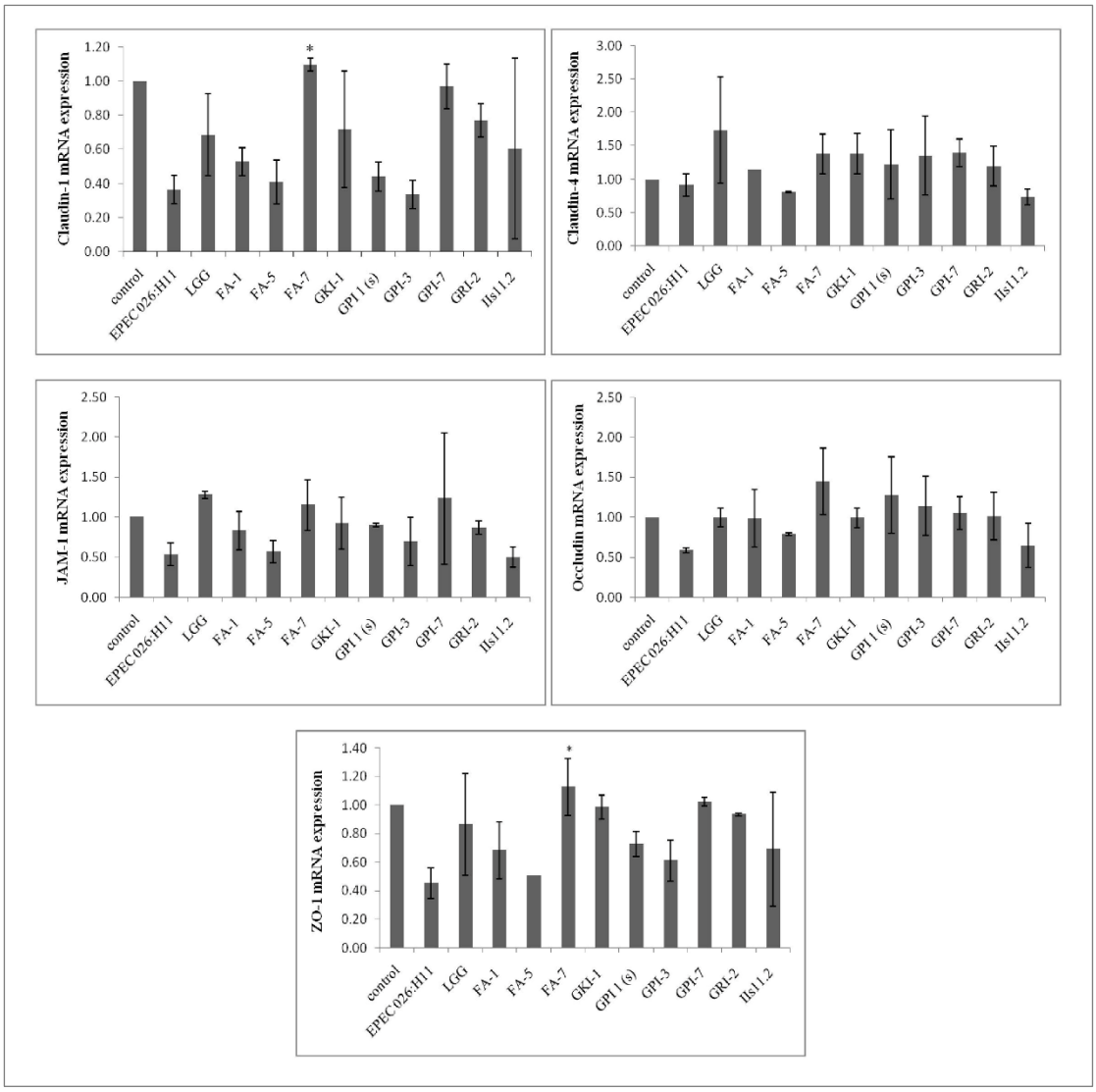

Figure 4. 5 Fold expression of mRNA specific for tight junction proteins in EPEC O26:H11-infected HT-29 cells with and without lactobacilli treatment.
HT-29 cells without any bacterial exposure served as a control. Asterisk (*) denotes that values are significantly different from that of EPEC O26:H11 ($P<0.05$). Each bar represents the mean value and error bar as standard deviation of real-time PCR performed in triplicate from two independent experiments.

4.3.4 Distribution of TJ proteins in Caco-2 cells

The distribution of various TJ proteins (claudin-1, claudin-4, ZO-1, JAM-1 and occludin) was analyzed 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; figure4.6, claudin-4; figure 4.7, JAM-1; figure 4.8, occludin; figure 4.9, and

ZO-1; figure 4.10). This altered distribution of TJ proteins was however ameliorated when EPEC O26:H11-infected cells were treated with various lactobacilli. When EPEC-infected 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. Similar results were obtained with LGG. 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 EPEC-infected Caco-2 cells, the other TJ proteins were redistributed to the cell periphery. Redistribution of ZO-1 proteins to the cell borders was observed when Caco-2 cells were treated with *L. helveticus* FA-7, and *L. fermentum* strains GKI-1, GPI-3, and IIS11.2 (figure 4.10 (H, I, K and L)). Immunofluorescence staining of Caco-2 cells with antibody specific for occludin revealed complete redistribution of protein to the cell-periphery when monolayers were treated with *L. fermentum* strains FA-5 and GPI-3. Treatment of Caco-2 monolayers with *L. fermentum* FA-5 also exhibited the complete redistribution of claudin-1 and JAM-1 proteins 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.

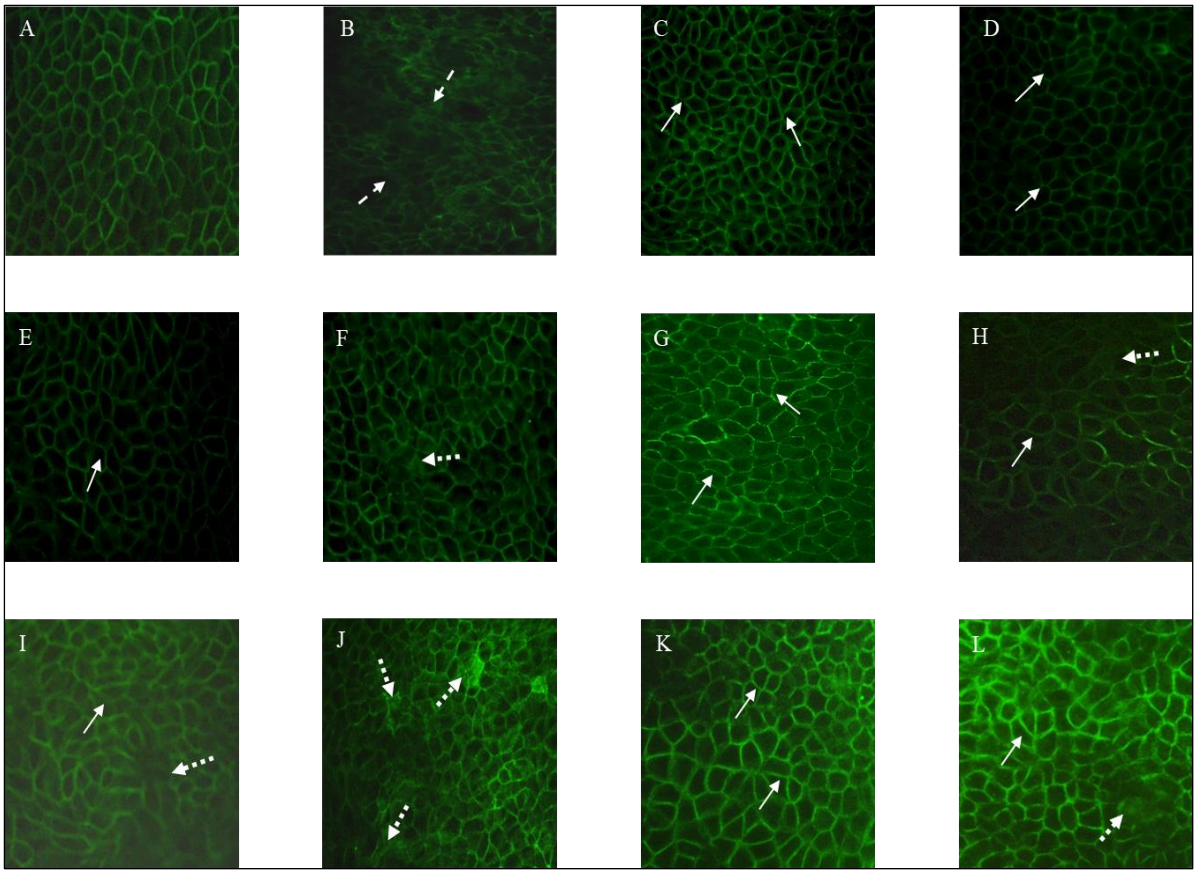

Figure 4. 6 Immunofluorescence staining of claudin-1 protein in Caco-2 cell line.
(A) Control Caco-2 cells, (B) EPEC O26 : H11-infected Caco-2 cells, (C–L) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: LGG (C), FA-1 (D), GRI-2 (E), GPI-1(S) (F), FA-5 (G), FA-7 (H), GKI-1 (I), GPI-7 (J), GPI-3 (K), and IIS11.2 (L). Arrows with dashed line indicate disrupted distribution of claudin-1 as represented by fluorescence in the cytoplasm. Normal arrows indicate the complete redistribution of claudin-1 protein from cytoplasm to periphery. Arrows with dotted line indicate the partial redistribution of claudin-1 proteins from cytoplasm to periphery.

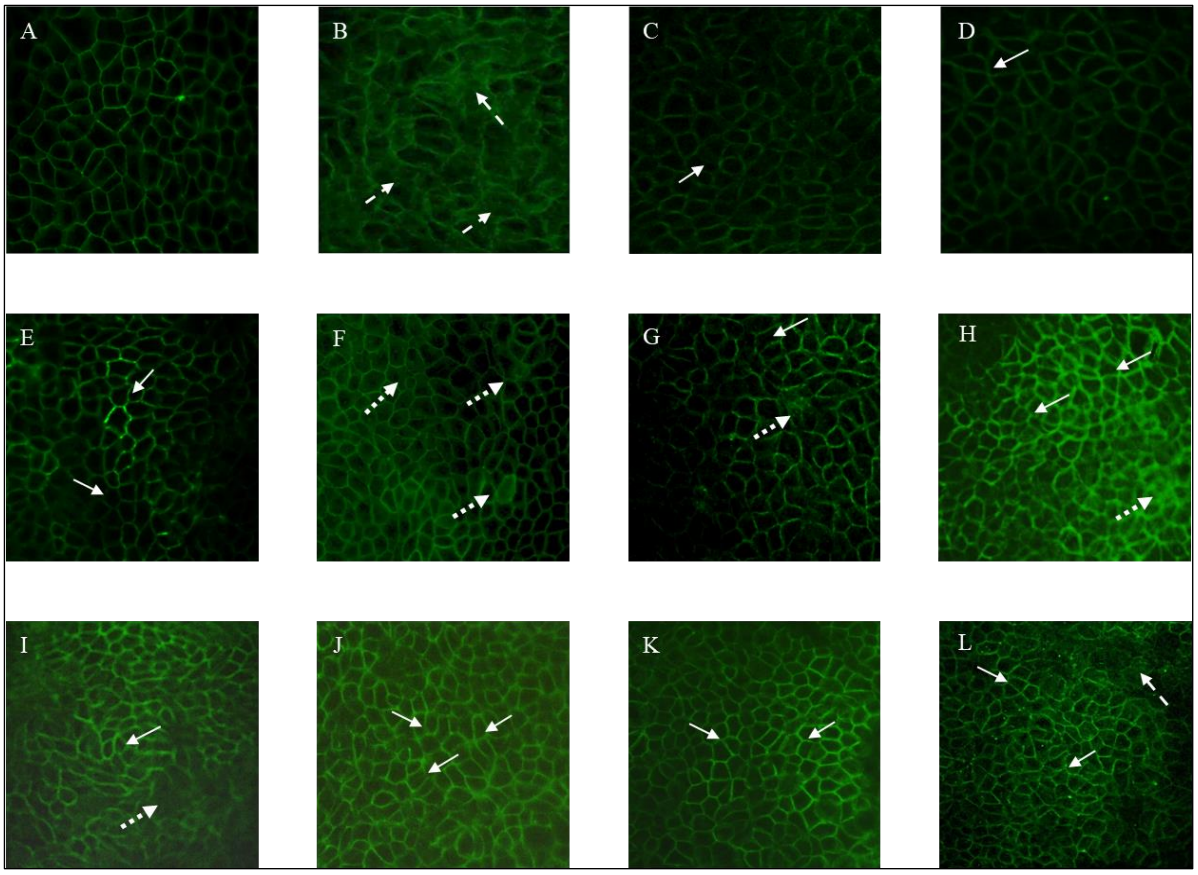

Figure 4. 7 Immunofluorescence staining of claudin-4 protein in Caco-2 cell line.
(A) Control Caco-2 cells, (B) EPEC O26 : H11-infected Caco-2 cells, (C–L) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: LGG (C), FA-1 (D), GRI-2 (E), GPI-1(S) (F), FA-5 (G), FA-7 (H), GKI-1 (I), GPI-7 (J), GPI-3 (K), and IIS11.2 (L). Arrows with dashed line indicate disrupted distribution of claudin-4 as represented by fluorescence in the cytoplasm. Normal arrows indicate the complete redistribution of claudin-4 protein from cytoplasm to periphery. Arrows with dotted line indicate the partial redistribution of claudin-4 proteins from cytoplasm to periphery.

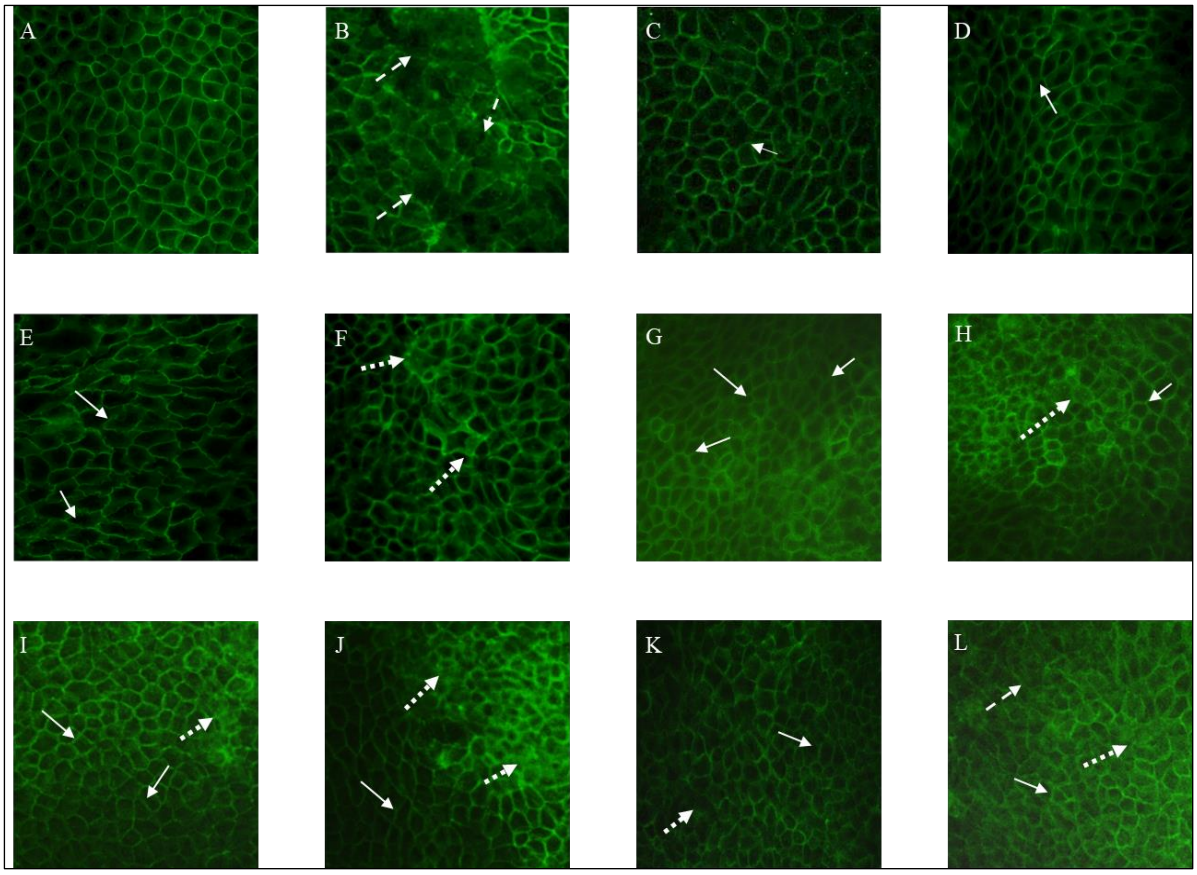

Figure 4. 8 Immunofluorescence staining of JAM-1 protein in Caco-2 cell line.
(A) Control Caco-2 cells, (B) EPEC O26 : H11-infected Caco-2 cells, (C–L) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: LGG (C), FA-1 (D), GRI-2 (E), GPI-1(S) (F), FA-5 (G), FA-7 (H), GKI-1 (I), GPI-7 (J), GPI-3 (K), and IIS11.2 (L). Arrows with dashed line indicate disrupted distribution of JAM-1 as represented by fluorescence in the cytoplasm. Normal arrows indicate the complete redistribution of JAM-1 protein from cytoplasm to periphery. Arrows with dotted line indicate the partial redistribution of JAM-1 proteins from cytoplasm to periphery.

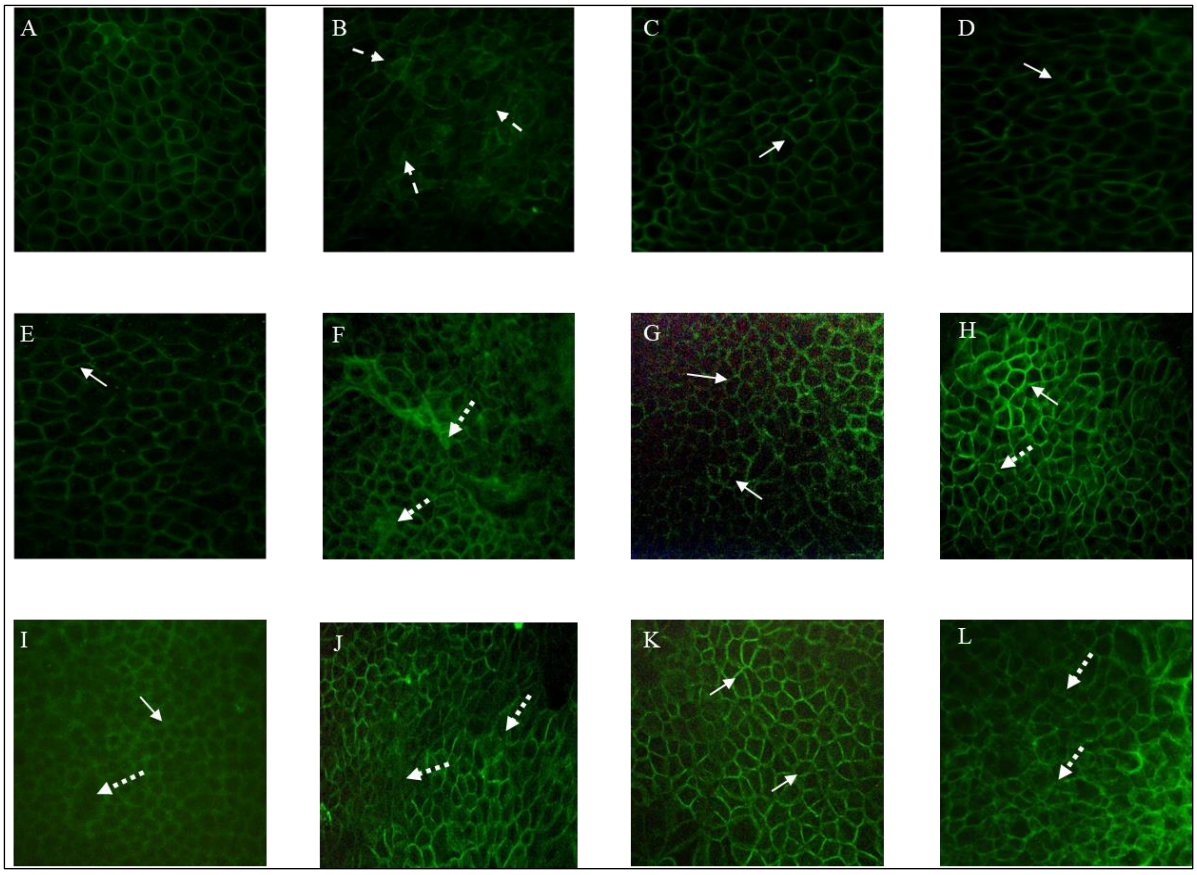

Figure 4. 9 Immunofluorescence staining of occludin protein in Caco-2 cell line.
(A) Control Caco-2 cells, (B) EPEC O26 : H11-infected Caco-2 cells, (C–L) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: LGG (C), FA-1 (D), GRI-2 (E), GPI-1(S) (F), FA-5 (G), FA-7 (H), GKI-1 (I), GPI-7 (J), GPI-3 (K), and IIS11.2 (L). Arrows with dashed line indicate disrupted distribution of occludin as represented by fluorescence in the cytoplasm. Normal arrows indicate the complete redistribution of occludin protein from cytoplasm to periphery. Arrows with dotted line indicate the partial redistribution of occludin proteins from cytoplasm to periphery.

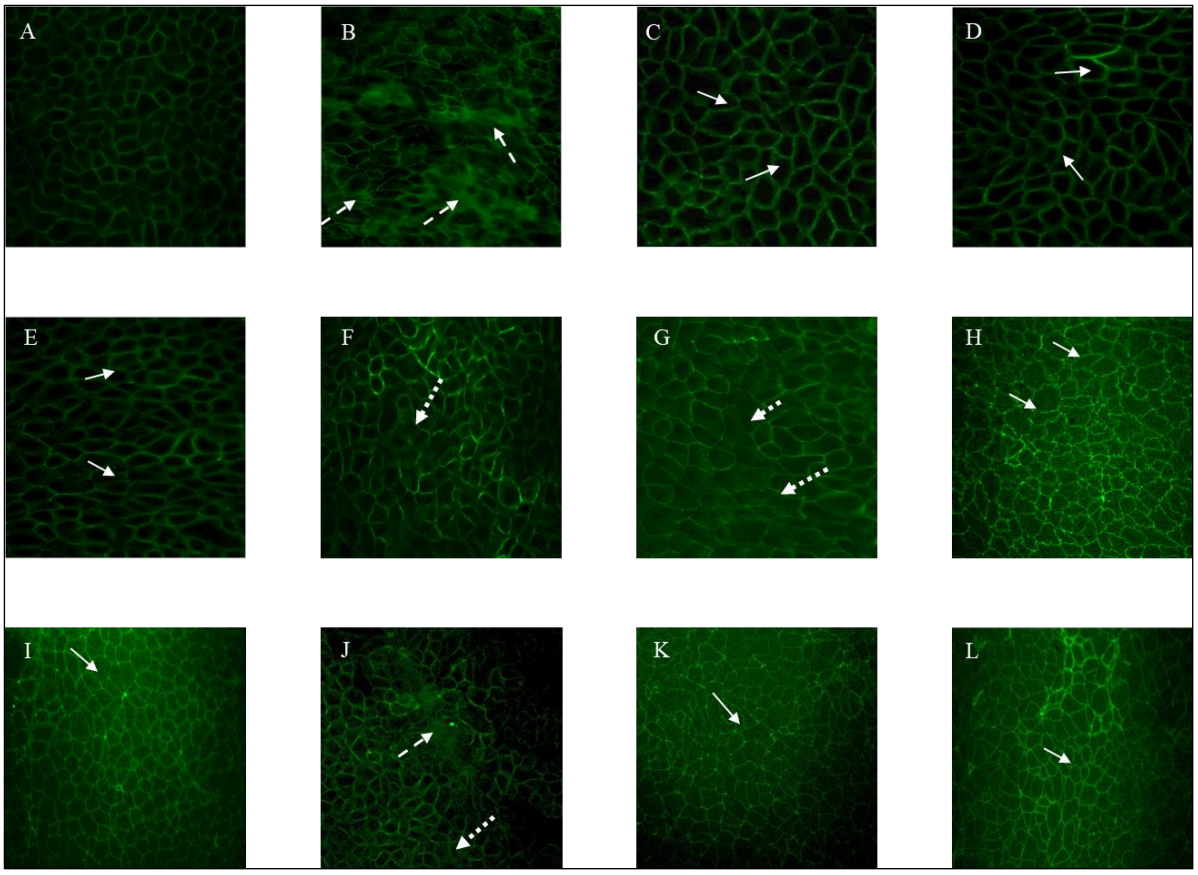

Figure 4. 10 Immunofluorescence staining of ZO-1 protein in Caco-2 cell line.
(A) Control Caco-2 cells, (B) EPEC O26 : H11-infected Caco-2 cells, (C–L) EPEC O26 : H11-infected Caco-2 cells treated with lactobacilli strains: LGG (C), FA-1 (D), GRI-2 (E), GPI-1(S) (F), FA-5 (G), FA-7 (H), GKI-1 (I), GPI-7 (J), GPI-3 (K), and IIS11.2 (L). Arrows with dashed line indicate disrupted distribution of ZO-1 as represented by fluorescence in the cytoplasm. Normal arrows indicate the complete redistribution of ZO-1 protein from cytoplasm to periphery. Arrows with dotted line indicate the partial redistribution of ZO-1 proteins from cytoplasm to periphery.

4.4 Discussion

Epithelia form barriers which are essential to life, in particularly, to the intestine. The intestinal barrier, which allows the absorption of nutrients, also prevents the microbial contamination of the intestinal tissues. Thus, maintaining barrier integrity is important to prevent the invasion of pathogenic bacteria into the underlying mucosa and immune cells. Lactobacilli under the category of probiotic bacteria are known to promote the intestinal barrier integrity by various mechanisms. In the present study, the lactobacilli

strains were *in vitro* assessed for their potential to improve/neutralize the epithelial barrier dysfunction caused by EPEC O26:H11. Barrier integrity are commonly measured by TEER of the monolayer and permeability of the monolayer across the barrier. The intestinal epithelial cell-lines, Caco-2 and HT-29, were infected with EPEC O26:H11 to induce barrier dysfunction. The infection with EPEC caused significant reduction in the TEER and increase in the macromolecule permeability across the monolayer with time, which served as an indication of the disruptive effect on the epithelial monolayer. 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 *L. fermentum* IIS11.2, *L. plantarum* GRI-2 and LGG in HT-29 monolayers. Similar results have been reported by Parassol et al., (2005) using T84 cells. Although all strains were able to reverse the reduction in TEER induced by EPEC O26:H11, the time required by the different strains varied. Most of the lactobacilli strains required 4 h to initiate significant reversal of TEER reduction in HT-29 cells except *L. helveticus* FA-7 and *L. fermentum* strains FA-5 and GPI-7 which required 16 h for the same. The reversal in Caco-2 monolayers required 2 h incubation with *L. fermentum* strains FA-1 and FA-5, *L. helveticus* FA-7 and LGG, whereas the other strains required 4 h. Previous studies by Anderson et al., (2010) and Yu et al., (2012) observed reversal in similar time intervals. Co-incubation of *L. plantarum* DSM 2648 with *E.coli* O127:H6 prevented decrease in TEER in Caco-2 monolayer up to 10 h (Anderson et al., 2010). Attenuation in the TEER reduction of Caco-2 monolayer was observed after 2 h of co-incubation of *L. amylophilus* D14 with pathogenic bacteria and this attenuation was even continued up to 12 h of co-incubation (Yu et al., 2012). Donato et al., (2010) and Yang et al., (2015) also reported such strain-dependent variation in the TEER recovery. Since different lactobacilli uses different

ways to modulate the barrier integrity, such variations may be observed. For example, in Caco-2 cells, treatment of *L. plantarum* in Caco-2 cells prevented TNF-α- and phorbol ester-induced dislocation of occludin and ZO-1, and associated reduction in TEER by suppressing the NF-κβ signaling pathway (Karczewski J. et al., 2010; Ko et al., 2007). In another report, *L. plantarum* DSM 2648 exhibited protection against enteropathogenic *E. coli* (EPEC)-induced TEER reduction in Caco-2 monolayer by reducing the EPEC adherence to Caco-2 cells (Anderson et al., 2010). In the present study, 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 (Neunlist et al., 2003; Seth et al., 2008) and latex beads (Schulte et al., 2000) 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., (2011) also reported a similar effect of *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 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 (Chantret et al., 1988; Grasset et al., 1984).

The barrier integrity and thus TEER is regulated mainly by the TJ proteins which seal the paracellular space between epithelial cells (Suzuki, 2013). 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 (Yang et al., 2015; Yu et al., 2012). 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., (2013). The immunofluorescence staining revealed that *L. fermentum* FA-1 and *L. plantarum* GRI-2 completely redistributed all the TJ proteins from the cytoplasm to the cell periphery whereas the other strains partially redistributed some or the other TJ proteins to the cell-periphery. However, the gene expression of TJ proteins were not significantly increased by these strains and thus no new proteins were being synthesized. These results therefore suggest 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 E. et al., 2012) also suggested that attenuation in H_2O_2-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 (Sultana et al., 2013).

Although the lactobacilli strains studied were able to neutralize the EPEC O26:H11-induced epithelial barrier dysfunction *in vitro*, 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.