Chapter 5

Studies on the ability of *Lactobacillus* strains to reverse the Dextran Sodium Sulphate (DSS)-induced colitis symptoms in mice

"The two most powerful warriors are patience and time"

-Leo Tolstoy

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5.1 Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) which typically involves inflammation of the intestine caused by an abnormal activation of the gut-associated immune system. One of the predominant symptoms of colitis includes diarrhea, which is caused by impaired absorptive transport processes and epithelial barrier dysfunction (Gitter et al., 2001; Schmitz et al., 1999). Impaired epithelial barrier function is characterised by an increased paracellular permeability of the intestine resulting in the passive loss of water and electrolytes into the lumen. The paracellular permeability is regulated by tight junction (TJ) proteins which mainly includes claudins, occludin and zonula occludens (ZO)-1 (Fanning A.S. et al., 1998; Itoh et al., 1999). Expression of the TJ proteins expression and subsequent complex formation are affected by external stimuli including cytokines (Nusrat et al., 2000). The pro-inflammatory cytokines produced under colitic conditions alter the expressions of TJ proteins and distribution in the epithelial cell causing impaired barrier function (Schulzke et al., 2009). For example, treatment of intestinal epithelial cell-line with TNF-α induces impairment of intestinal barrier by cytoskeleton rearrangement and TJ expression (Mankertz et al., 2009). Induction of colitis in mice by administration of 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) lead to decrease in the expression of genes encoding claudin-1, ZO-1 and occludin (Liu W. et al., 2013). Colitis induction by

Dextran Sodium Sulphate (DSS) caused decrease in the expressions of mRNA encoding ZO-1, claudin-1 and occludin in the colonic samples of mice (Zhao et al., 2012). Moreover, enhanced uptake of luminal antigens may exacerbate the pathologic condition of mucosal inflammation and barrier dysfunction (Swidsinski et al., 2002). The barrier disturbances in UC have some structural correlation with the histological changes in the colon (Glickman, 1998). Epithelial defects such as micro-erosions to gross ulcer-type lesions have been observed. Additionally, absence of crypts or crypt abscesses i.e. neutrophil infiltration of crypts are also characteristic features associated with epithelial destruction (Jadhav et al., 2013; Nanda Kumar et al., 2008). The other common symptoms observed in the patients suffering from colitis includes loss of body mass and shortening of colon. These are often directly correlated with the severity of inflammatory conditions (Okayasu et al., 1990; Yan Y. et al., 2009).

Various medication options are available to treat colitis of different stages. The most common includes oral sulfasalazine with combination of topical medications (Klotz et al., 1980). In case this medication has no effect, immunosuppressive biological agents are given (Rutgeerts et al., 2005; Sandborn W.J. et al., 2012). However, these conventional medications are associated with risks of cancer development and other side effects (Axelrad et al., 2016). In the past few decades, certain probiotics are known to be associated with improvement in the colitic symptoms. Different *in vivo* models support the involvement of lactobacilli in reversing mucosal inflammation and alteration of the intestinal barrier function in colitis. The probiotic compound VSL#3 has been reported to exhibit direct effect on the epithelial barrier function in the colitis model of IL-10 gene-deficient mice. Moreover, the enhancement of barrier integrity by secretory soluble factors of VSL#3 was also associated with a significant improvement in the histological damage (Madsen K. et al., 2001; Sood et al., 2009). Treatment of

DSS-induced colitis model of mice with *L. casei* DN1114001 lead to a decrease in the severity of colitic symptoms (Kokesova et al., 2006; Zakostelska et al., 2011). Some reports suggest improvement in the expression levels of mRNA encoding TJ proteins in the colitis mice model following lactobacilli treatment.

The present chapter includes the screening of lactobacilli strains for their potential to improve the colitis conditions in the mice model. Colitis was induced chemically by administration of DSS. DSS based colitis model of mice are most commonly used since this method provides ease in the induction of colitis, moreover, the duration required for colitis induction is very short and controllable. Additionally, clinical symptoms of DSS-induced colitis resembles the human ulcerative colitis (Okayasu et al., 1990). The strains were assessed for their potential ability to improve the symptoms associated with colitis, such as weight loss, colon shortening, histological damage in the colonic tissue, and altered gene expression of TJ proteins.

5.2 Materials and methods

5.2.1 Lactobacilli strains

Lactobacilli strains described in table 5.1 were used for the present *in vivo* study. *Lactobacillus rhamnosus* GG (LGG) was used as a standard probiotic strain. Lactobacilli cells were grown in de Man, Rogosa and Sharpe (MRS) broth (Himedia) at 37°C for 16-18 h. The cells were harvested by centrifugation at 10,000 ×*g* and 4°C for 2 min. The cells were then washed twice with phosphate buffered saline (PBS; pH-7.4) to remove traces of MRS media followed by resuspension in PBS. The cell density was adjusted to 1 X 10^{10} CFU/ml in PBS and 100 µl of the cell suspension was used for administration in mice.

Table 5. 1 Strains used in the study

SUB-GRPOUP	LACTOBACILLI STRAINS
A	*Lactobacillus fermentum* FA-1
B	*Lactobacillus fermentum* FA-5
C	*Lactobacillus helveticus* FA-7
D	*Lactobacillus fermentum* GKI-1
E	*Lactobacillus salivarius* GPI-1(S)
F	*Lactobacillus fermentum* GPI-3
G	*Lactobacillus fermentum* GPI-7
H	*Lactobacillus plantarum* GRI-2
I	*Lactobacillus fermentum* IIS11.2
J	*Lactobacillus rhamnosus* GG

5.2.2 Experimental Animals

Female BALB/c mice were obtained from the Animal House Facility, Department of Biochemistry, M. S. University of Baroda and housed with ad libitum access to water and food in an environmentally controlled room (25°C) with a 12-h light-dark cycle each. Care and procedures adopted for the present investigation were in accordance with the approval of the CPCSEA or Institutional Animal Ethics Committee (938/PO/a/06/CPCSEA). Female mice of 6–8 weeks old and weighing between 20-21 g were reared in groups of 5 mice per cage. Mice were separated into three main group: groups 1: control group, group 2: DSS group, and group 3: lactobacilli treatment group, where group 3 was further divided into subgroup A-J, each subgroup for different lactobacilli (see table 5.1 for lactobacilli used in different sub-groups). For each group and subgroup (in group 3) 5 mice were used.

5.2.3 Induction of colitis in mice

Dextran sodium sulphate (DSS; MP Biomedicals) of 40 KDa molecular weight was used at concentration of 3% (w/v) in drinking water for colitis induction. The mice from

group 1 received normal drinking water and were given PBS (100 µl) intragastrically from day 8 to day 21. For Group 2 and 3, the normal drinking water was replaced with drinking water containing 3% DSS for initial 7 days to induce colitis. From day 8 to 21, group 2 mice received 100 µl of PBS intragastrically and were given normal drinking water. Mice from the subgroup A-J, of group 3, intragastrically received different lactobacilli (10^9 CFU) from day 8 to day 21 and were given normal drinking water. After 21 days of treatment, all the mice from different groups were sacrificed by cervical dislocation, the distal colon was excised and used for histological analysis and qRT-PCR analysis.

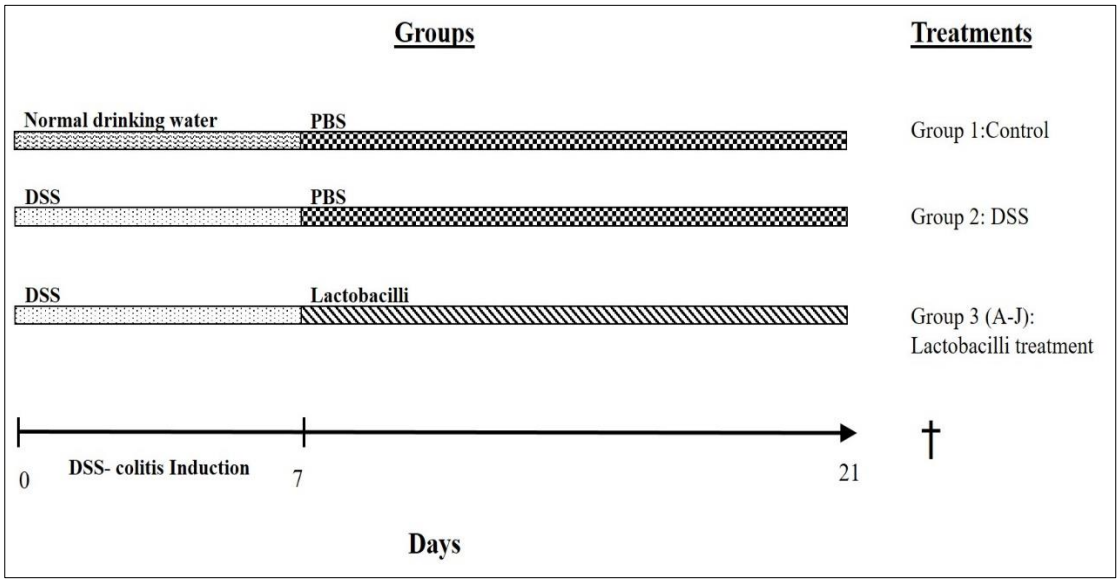

Figure 5. 1 Experimental design for colitis induction by DSS followed by lactobacilli treatment

5.2.4 Assessment of body weight and colon length

Body weight of all the mice was measured daily from day 1 to day 21 and the change in the weight of an individual mice was expressed as weight change on day 'x' by subtracting the weight on that particular day from that of the initial weight (i.e. day 1) of that mice. The mice were sacrificed after 21 days, the entire colon was removed and

the length was measured. Mean colon length of group 1 mice and of each sub-group (from group 3) of mice was compared with that of the DSS group of mice (group 2).

5.2.5 Histological analysis

For assessment of histological changes, distal colon was used as changes are most prominent in the distal colon when DSS is used for colitis induction. The distal part of the colon was excised and washed with PBS. The tissue samples were then fixed in 10 % neutral buffered formalin solution for 24 h. After fixation, the tissues were embedded in paraffin embedding followed by sectioning and staining with haematoxylin and eosin (H&E). The sections were then examined for the histological changes and given the scores as described in table 5.2. For each mice, three different sections were studied for the histological scoring.

Table 5. 2 Histological scoring system for DSS-induce colitis.

HISTOLOGICAL CHANGES	**SCORE**			
	0	**1**	**2**	**3**
Loss of goblet cells	None	Mild/focal	Numerous and diffuse	Numerous and diffuse
Loss of Crypt/crypt abscesses	None	Few (1 in several fields)	A few (1-4 per field)	Many (≥5 per field)
Hyperaemia in the mucosa	None/not evident	Mild	Diffuse	Diffuse
Cellular infiltration in the lamina propria	None	A few	Moderate/focal	Numerous and diffuse
Elongation of colonic mucosa	Normal (≤100%)	Mild (101–150%)	Evident (≥150%)	Evident (≥150%)
Epithelial erosion	None	Mild/focal	Evident/diffuse	Evident/diffuse

5.2.6 Expression analysis of mRNA encoding TJ proteins

The levels of mRNA expression encoding TJ proteins (claudin-1, occludin and ZO-1) in the colonic tissue of each mice were assessed by RNA isolation followed by cDNA preparation and qRT-PCR using specific primers.

RNA isolation

The colonic tissues were excised, washed with PBS and then placed in RNAlater stabilization reagent (Sigma-Aldrich). Total mRNA was isolated using total RNA extraction kit (Bangalore Genei, Bangalore, India) following the manufacturer's instructions. Briefly, the samples were removed from the RNAlater reagent and centrifuged at 10,000 $\times g$ for 10 min at 4°C to remove any traces of RNAlater. Approximately 50 mg of tissue was homogenized in 0.5 ml of denaturing solution followed by addition of equal volume of acid phenol. To this, 200 µl of choloroform-isoamyl alcohol (mixed in the ratio of 49:1) was added and mixed thoroughly. The mixture was then incubated on ice for 15 min. Following this, the mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The upper aqueous phase was then transferred into another vial and 1ml of 100% isopropanol was added and incubated at -20°C for 30 min for precipitation of RNA. Following this, the mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in 300 µl of denaturing solution followed by addition of 100% isopropanol in equal volume. The suspension was mixed thoroughly and incubated at -20°C for 1 h followed by centrifugation at 10,000 rpm for 20 min at 4°C. The pellet was then rinsed with 600 µl of 75% ethanol to remove any residual amount of guanidine by incubating at room temperature for 10-15 min. Following this, the tubes were centrifuged at 10,000 rpm for 20 min at 4°C, the supernatant was discarded and the RNA pellet was allowed

to dry by incubating at 42°C for 10 min. The RNA was resuspended in 100 µl of DEPC water (preheated to 55°C) and was incubated at 55°C for 10 min to ensure complete solubilisation of RNA. The integrity of the samples was confirmed by inspecting the 28S and 18S rRNA bands following separation by electrophoresis on a 2% agarose gel. The RNA samples were quantified using the Nanophotometer (IMPLEN).

cDNA synthesis and qRT-PCR analysis

cDNA was synthesized using a Verso cDNA kit (Thermo Fisher Scientific) as described above in the Chapter 4 (Materials and methods: 4.2.4). The quality of cDNA preparation was confirmed by amplification of β-actin using gene specific primers. qRT-PCR amplifications were then performed in CFX96^{TM} real-time thermal cycler (Bio-Rad Laboratories) with primers specific for claudin-1, occludin and ZO-1 (see table 5.3 for primer sequences). The amplification conditions and system were as described in Chapter 4 (Materials and methods: 4.2.4). The transcript expressions were normalized to β-actin transcript expression. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$ method.

Table 5. 3 Primers used for qRT-PCR analysis of tight junction.

GENE	FORWARD PRIMER
β-Actin	FP: 5'-TGACAGGATGCAGAAGGAGA-3' RP: 5'-GCTGGAAGGTGGACAGTGAG-3'
Claudin-1	FP: 5'-ATCGTCAGCACTGCCCTGC-3' RP: 5'-TCAGATTCAGCAAGGAGTCGA-3'
Occludin	FP: 5'-AGTACATGGCTGCTGCTGATG-3' RP: 5'-CCCACCATCCTCTTGATGTG-3'
ZO-1	FP: 5'-TTATACAATGGAAAGCTGGGCT-3' RP: 5'- GATCACCACCCGCTGTCTTT -3'

5.2.7 Statistics

All statistical analysis was performed using GraphPad Prism software (version 5.0). All results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed followed by Dunnett's test to find out the statistical significance between DSS group and lactobacilli treatment group, and control group and DSS group ($P<0.05$).

5.3 Results

5.3.1 Body weight

During the experimental period of 21 days, control group of mice (group-1) gained 3.2 g (16%) by weight (figure 5.2). Contrary to this, significant loss in the mean body mass was observed in the DSS group of mice (group-2) compared to the control group of mice ($P<0.05$). Compared to the initial weight, the mean weight loss in the DSS group of mice at the end of the experiment was 5.2 g (26%). However, when the mice were given different lactobacilli (group-3), recovery in weight loss was observed within 2-3 days of treatment. The mean change in the weight of mice treated with FA-7 and IIS11.2 compared to their initial weight was not statistically significant than that of the DSS group of mice ($P<0.05$). Whereas, the mice treated with other lactobacilli strains had significant difference in the mean weight change varying from -2.3 g (-11.5%) to 2.4 g (12%) compared with that of the DSS group of mice ($P<0.05$).

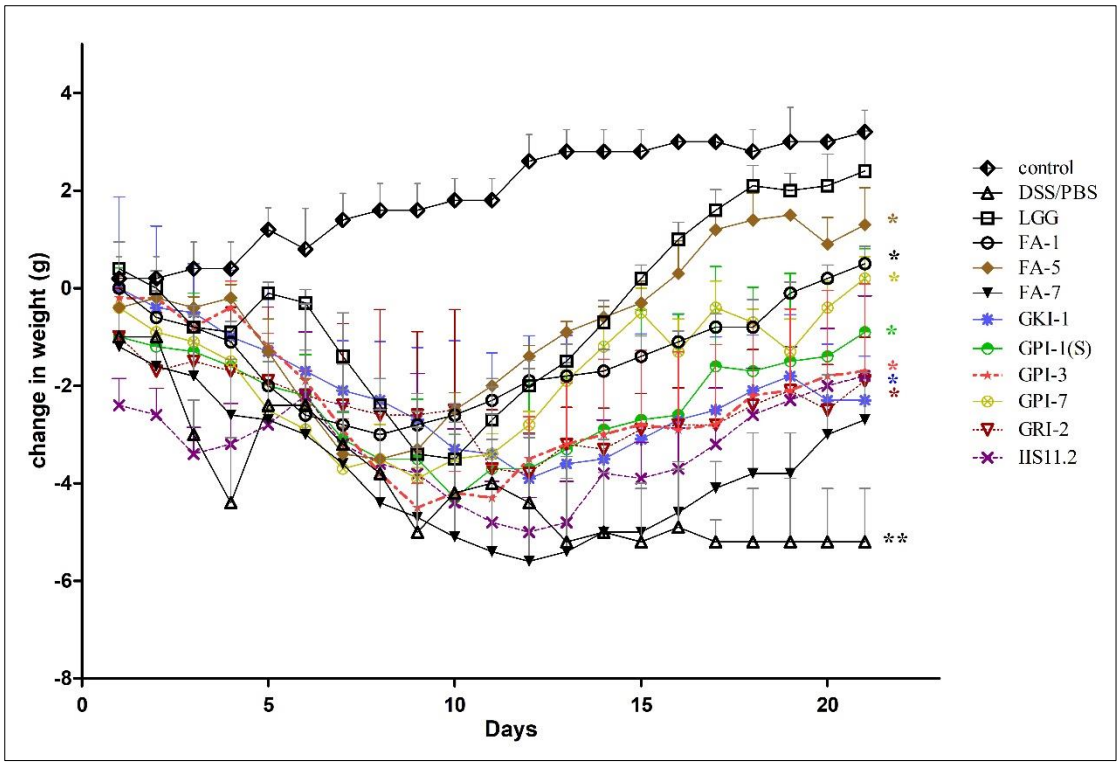

Figure 5. 2 Body weight changes in mice from different groups.
The body weight of mice on the first day was taken as the basal level. The body weight of mice each day minus the basal body weight was expressed as the body weight change. The negative value indicates the decreased weight, and the positive value indicates the increased weight. "*" in different colours indicates significant difference in the respective lactobacilli treated mice vs DSS group of mice ($P<0.05$). The colour code of the asterisk matches with the colour code of the strain showing significant difference. "**" indicates significant difference in the DSS group of mice vs control group ($P<0.05$).

5.3.2 Colon length

The mean colon length of group 1 mice was 11.2 cm, whereas the colon length of DSS treated mice (group-2) was significantly reduced to 7.8 cm ($P<0.05$). The colon of the mice treated with lactobacilli strains GKI-1, IIS11.2, GPI-7, GPI-3 and GPI-1(S), had longer colon than that of the DSS group of mice, although the difference was not statistically significant ($P<0.05$). However, the treatment with the other lactobacilli strains (LGG, FA-1, FA-5, FA-7 and GRI-2) significantly reduced the inflammatory colonic shortening in group-3 mice compared to the DSS treated mice ($P<0.05$) and the mean length ranged from 8.5 cm to 8.9 cm.

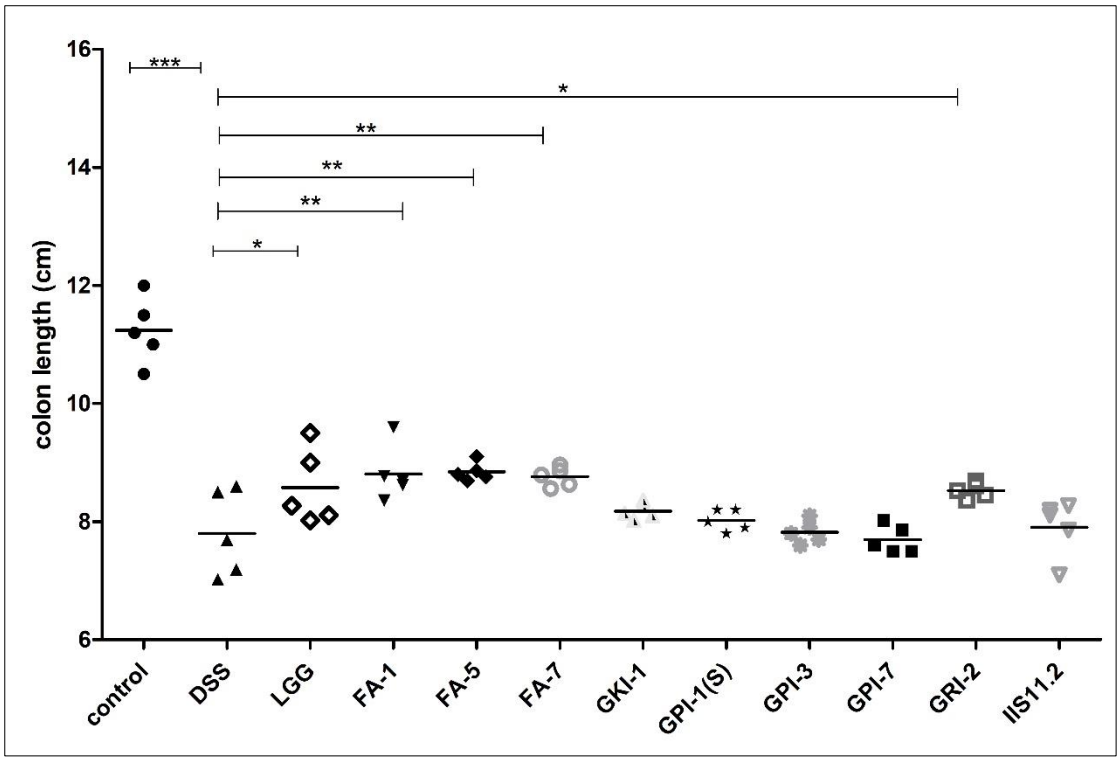

Figure 5. 3 Length of colon at the end of the experiment.
* P < 0.05 vs colitis group; **P < 0.01 vs colitis group; ***P < 0.001 vs control group.

5.3.3 Levels of mRNA expression specific for TJ proteins

The expression of mRNA specific for claudin-1, occludin and ZO-1 in the distal colon was analysed for each group of mice. When compared to the control group (group 1) of mice, significant reduction in the expression levels of mRNA encoding claudin-1, occludin and ZO-1 was observed in the colonic tissue of DSS group of mice (group 2). However, treatment of mice with FA-7 following DSS-induced colitis significantly improved the level of mRNA expression specific for claudin-1 and occludin, while it had no effect on the ZO-1 specific mRNA expression ($P<0.05$). On the contrary, treatment of DSS induced colitis mice with FA-1 significantly improved the levels of mRNA expression of ZO-1, while no effect was observed on the expression levels of mRNA specific for claudin-1 and occludin ($P<0.05$). Moreover, when the colitis induced mice were treated with LGG and GPI-3, significant improvement in the expression levels of mRNA specific for claudin-1 was observed ($P<0.05$). The other

lactobacilli strains had no significant improvement in the levels of mRNA expression of any of these TJ proteins compared to DSS group of mice ($P<0.05$).

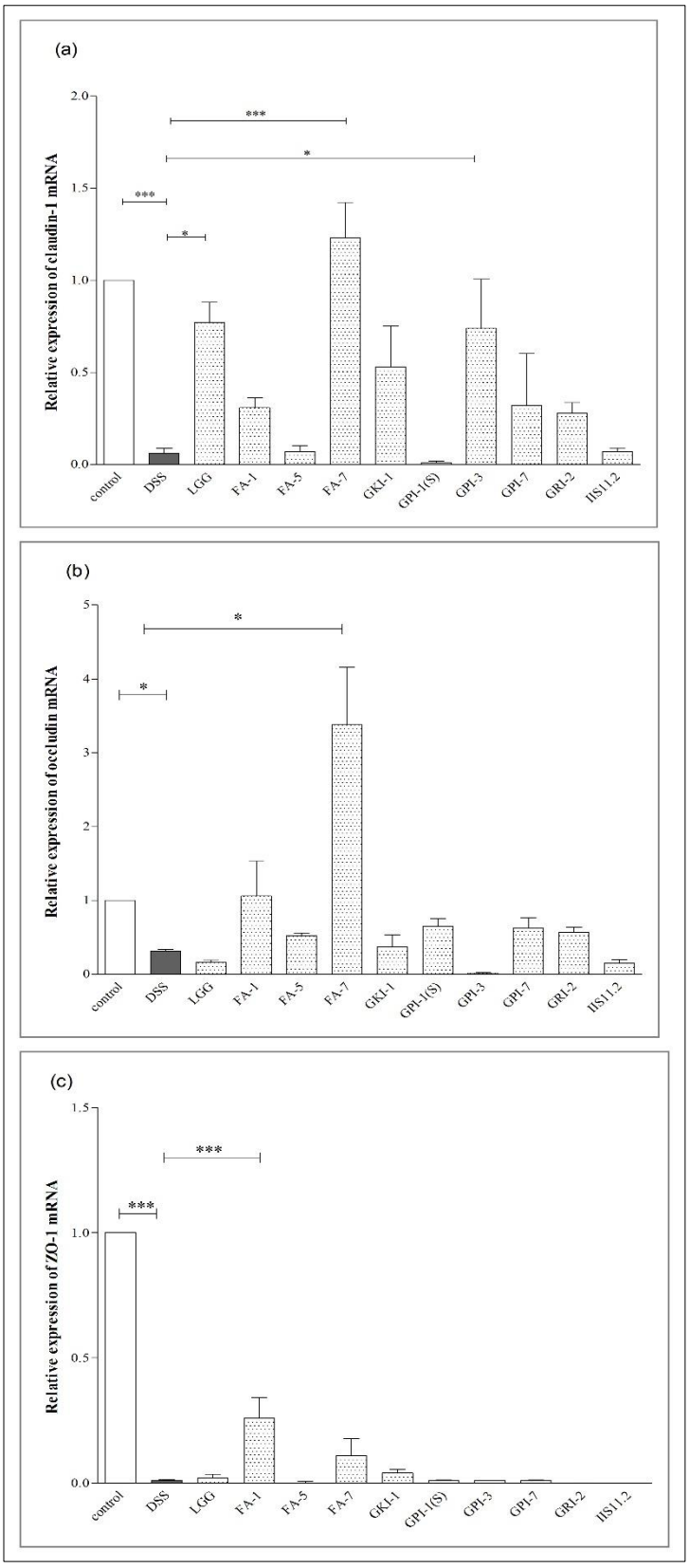

Figure 5. 4 Relative expression levels of mRNA encoding different tight junction (TJ) proteins.
((a) claudin-1; (b) occludin and (c) ZO-1) in the colon samples of different groups of mice. *P<0.05,**P<0.01,***P<0.001.

5.3.4 Histological analysis

Colonic sections of each mice were observed under microscope to investigate the histological changes in the colon during the course of experiments. Control group of mice exhibited a normal histological pattern whereas abnormalities were observed in the colonic sections of DSS group of mice. Microscopic examinations of sections from DSS group of mice revealed extensive loss of goblet cells, crypt disruption/abscesses, infiltration of inflammatory cells, elongation of mucosa and epithelial erosion. These sections were given scores based on the abnormalities observed as given in table 5.2. The mean score for DSS group of mice was significantly higher (10) than that of the control group of mice (group 1) ($P<0.05$). Lactobacilli treated colitis mice (group-3) exhibited an improvement in this altered colonic histology associated with colitis inflammatory process which resulted in the reduction in the histological scores (ranging from 2.6 to 9.5) compared to the colitis group of mice (10.0) (group-2). Although, mice treated with GKI-1, GPI-1 (S) and IIS11.2, separately, had lower histological scores (8.2, 9.2 and 9.5 respectively) compared with the DSS group of mice, the difference was not statistically significant ($P<0.05$). However, the mice treated with the other lactobacilli strains significantly reduced the histological scores (ranging between a minimum of 2.6 in LGG to a maximum of 7.4 in FA-5) compared to the DSS group of mice ($P<0.05$).

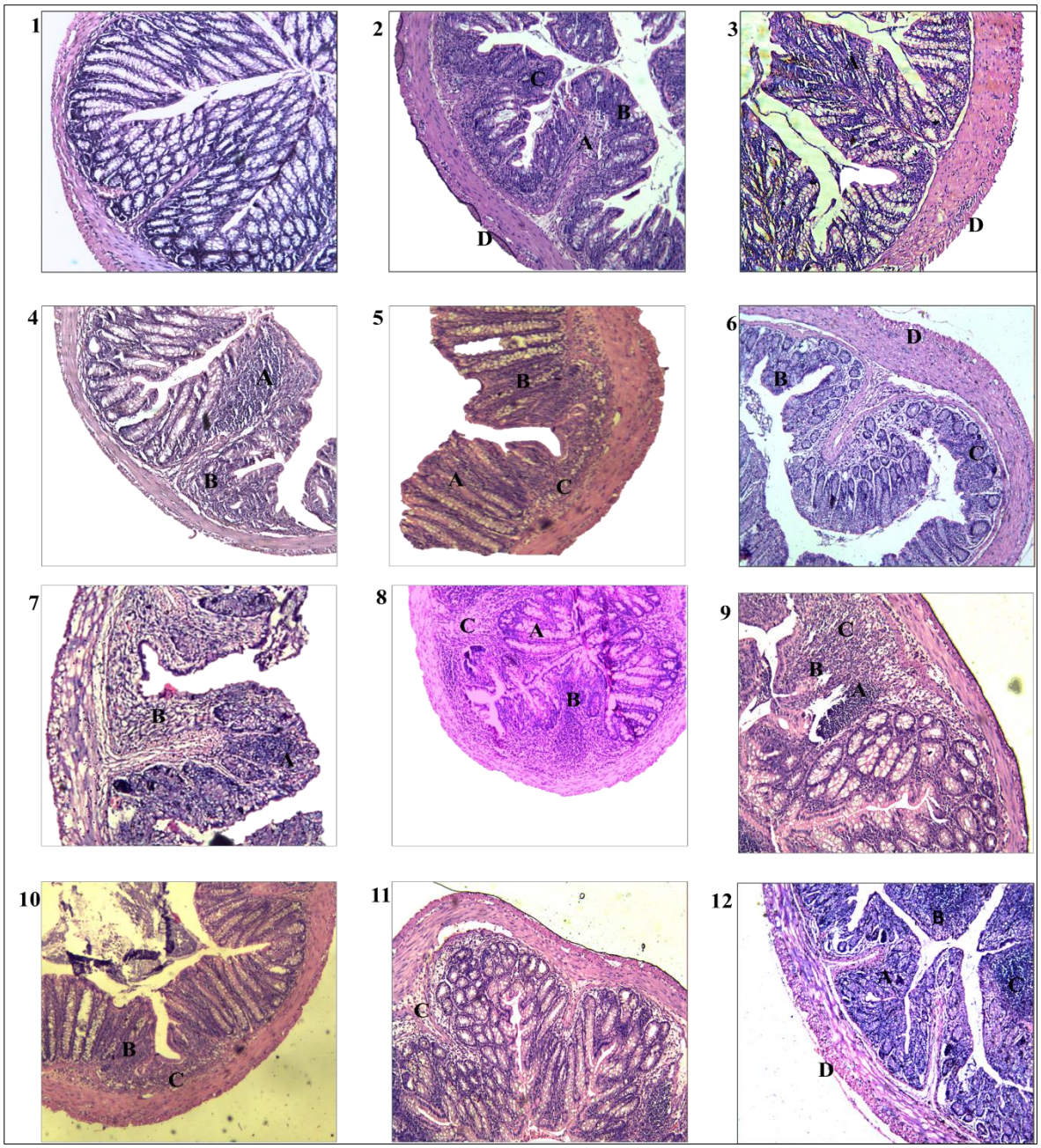

Figure 5. 5 Colon sections of mice from different groups.
1: Control, 2: DSS, 3: LGG, 4: FA-1, 5: FA-5, 6: FA-7, 7: GKI-1, 8: GPI-1(S), 9: GPI-3, 10: GPI-7, 11: GRI-2, 12: IIS11.2; A: loss of goblet cells, B: loss of crypts, C: cellular infiltration, D: epithelial erosion

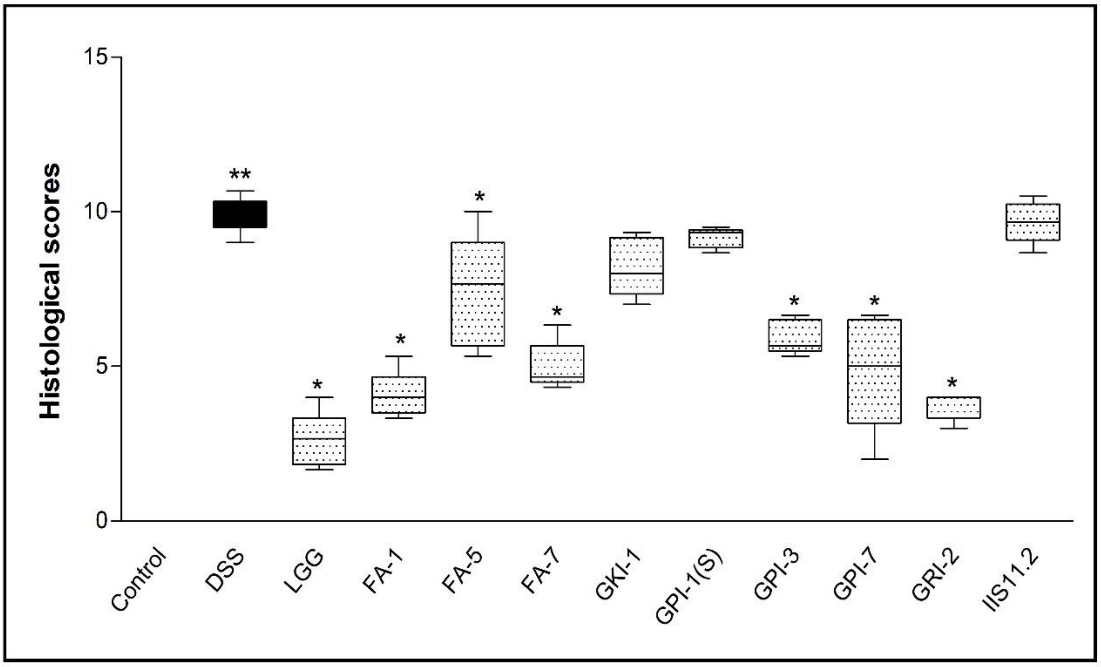

Figure 5. 6 Histological scores of mice in different groups.
*P<0.05 vs DSS group of mice, **P<0.05 vs control group of mice.

5.4 Discussion

Colitis is characterized by impaired epithelial barrier function and tissue destruction with relapsing diarrhoea (Gitter et al., 2001; Schmitz et al., 1999). Oral treatment with probiotic bacteria has emerged recently as a potentially useful therapeutic strategy for colitis (Bibiloni et al., 2005; Kim M.S. et al., 2017; Kruis et al., 2004). However, different probiotic bacteria exhibit different effects on colitis because of the specific damage site of UC and the different colonisations of each bacterium (Chapman et al., 2007; M. S. Kim et al., 2017; Schultz, 2008). The present study was aimed to evaluate the effect of different lactobacilli strains on DSS-induced colitis in mice. The evaluation was done on the basis of improvement in the colitis symptoms in mice, such as weight loss, shortening of colon, impairments in colon histology and expression of mRNA encoding TJ proteins.

The administration of 3% DSS into mice for 7 days lead to significant weight loss. However, we observed that treatment of mice with lactobacilli strain following the

colitis induction promoted significant recovery of weight loss. The other important clinical parameter of DSS-induced colitis is the shortening of the colon (Yan Y. et al., 2009). In our study, colon length was significantly reduced in the DSS group of mice. This reduction in the colon length was because of the inflammation caused by administration of DSS and has also been associated with the severity of disease (Okayasu et al., 1990). Administration of mice with different lactobacilli strains resulted in the improvement in the shortening of colon length which was an indirect indication of improvement in the inflammatory condition of colitis mice.

Colitis has been reported to have association with impairment of epithelial barrier function which is regulated by TJ proteins. In the present study, colitis induction by DSS lead to significant reduction in the expression levels of mRNA encoding ZO-1 protein in the distal colon samples. Earlier studies of colitis induction by DSS also report decrease in the expression of mRNA specific for ZO-1, claudin-1 and occludin (Liu W. et al., 2013; Zakostelska et al., 2011; Zhao et al., 2012). In present study, it was observed that when colitic mice was treated with FA-1, reduction in the expression levels of ZO-1 transcript was significantly reversed. However, administration of colitic mice with FA-7 lead to improvement in the expression levels of mRNA specific for claudin-1 and occludin, expression level of ZO-1 encoding mRNA was unaffected. In the previous study using *E.coli* Nissle as a probiotic therapy simultaneously with colitis induction, elevation in the expression levels of ZO-1 mRNA was observed (Kokesova et al., 2006). In another study, the expression levels of mRNA encoding ZO-1 and occludin was significantly preserved following *L. casei* treatment in the colon of colitis mice (Zakostelska et al., 2011). Moreover, improvement in the expression levels of mRNA encoding claudin-1 by FA-7 was also observed in the *in vitro* study using epithelial cell-line (Chapter 4). Additionally, contrary to the present *in vivo* findings,

FA-7 also lead to the improvement in the expression levels of mRNA specific for ZO-1 in the *in vitro* study. In the *in vivo* study, treatment with FA-7 caused improvement in the expression of occludin encoding mRNA. Such strain dependent variation in the expression of mRNA encoding tight junction was also observed in *in vitro* study.

Colitis induction by DSS in mice lead to severe damage in the colonic tissue which lead to increase histological score. Lactobacilli administration improved the colonic tissue damage as indicated by significantly lower histological score of colonic tissue compared to the DSS colitic mice. We observed a severe loss of goblet cells and crypt abscesses, loss of surface epithelium, moderate to severe epithelial erosion and infiltration of inflammatory cells in untreated DSS colitic mice. Such changes in the colonic tissue were also reported by Nanda Kumar et al., (2008) and Jadhav et al., (2013) in their studies using DSS to induce colitis. Colitic mice treated with lactobacilli strains (FA-1, FA-5, FA-7, LGG, GRI-2, GPI-3 and GPI-7) had either no or moderate loss in goblet cells, less crypt abscesses, intact surface epithelium, and moderate or less infiltration of inflammatory cells. This caused significant decrease in the histological scores compared to the untreated DSS colitic mice tissue. Jadhav et al., (2013) also reported such ameliorative effect of *Lactobacillus* strain on the colon histology of colitis mice. Improvement in the histological scores by lactobacilli was also reported by Herías et al., (2005) in post-acute study of DSS-induced colitis. VSL#3 was also associated with a significant improvement in the histological damage (Madsen K. et al., 2001; Sood et al., 2009).

In the present study using DSS-induced colitis model of mice, lactobacilli strains exhibited differential efficacy in the improvement of colitis. Most of the strains improved the colonic tissue damage and other colitis symptoms, only FA-7, FA-1 and

GPI-3 had effect on the expression levels of mRNA encoding one or the other tight junction proteins.