Therapeutic Effect of Combinatorial Probiotics to Protect Intestinal Barrier Function

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Abstract

Aims: Inflammatory bowel diseases (IBD) are characterized by chronic inflammation of the gastrointestinal tract. The aim was to study the effect of combinatorial probiotics in the transwell co-culture model to ameliorate IBD.

Main Methods: Caco-2 cells co-cultured with RAW cell. LPS was added to induce inflammation. Probiotics were used to treat LPS induced inflammation on Caco-2 cell. TER (transepithelial electrical resistance) was measured in various experimental conditions. NO (Nitric oxide) and NBT (nitroblue tetrazolium) assays were performed to measure anti-oxidative property of probiotics and MTS assay for cell viability.

Key Findings: At 6 hours of incubation, TER of Caco-2 cell was decreased significantly by 15.17% in comparison to control when RAW 264.7 cell treated with LPS. Interestingly, TER was increased by 11.42% after addition of probiotics in Caco-2 cell under same experimental condition. Concentration of NO production in Caco-2 cell was increased by 3.64 fold when RAW 264.7 cells were stimulated with LPS and was decreased by 2.10 fold with addition of probiotics. Generation of superoxide in caco2 cell significantly increased by 158.33% when RAW cell stimulated with LPS and it decreased by 54.84% in addition of probiotics. Viability of Caco-2 cell was increased when probiotics were added.

Significance: Our combinatorial probiotics improved intestinal barrier function by increasing TER, might be through up-regulation of tight junction proteins. There might be soluble inducers which secreted by this combinatorial probiotics and improved intestinal epithelial cell homeostasis. It also showed NO and O^-_2 scavenging activities as a result it improved health of intestinal epithelial cell by anti-oxidant properties.

Keywords: Chronic Inflammation, Probiotics, Transepithelial Electrical Resistance, Intestinal Barrier Function, Anti-Oxidant

Introduction

Inflammatory bowel diseases (IBD) are mainly characterized by chronic and unregulated inflammation of the gastrointestinal (GI) tract comprises two diseases such as Crohn's disease (CD) and ulcerative colitis (UC) [1-4]. IBD causes in genetically susceptible people with chronic and relapsing inflammatory intestinal immune response of the intestinal microbiota [5,6]. Unregulated inflammation of the GI tract causes an increased permeability of the epithelium which leads to deterioration of the epithelial barrier function [7]. Goblet cells, found in the intestinal tract express rod-shaped mucins localized to the cell membrane to form the mucous layer [8,9].

Pathogens must penetrate the mucus which is the first barrier in the intestine to reach the epithelial cells during infecting the host. Different microbes have developed various methods to degrade mucus, such as reduction of mucin disulfide bonds, protease activity, and glycosidase activity for invasion of mucus [10-14]. The cellular barriers can be modulated by exposure to specific external stimuli and it effectively provides a selective permeable barrier that limits the permeation of different pathogens and toxins. Tight junction (TJ) struc-

tures are responsible for this barrier. Determining the transepithelial electrical resistance (TER) is a method for understanding the membrane permeability *in vitro* [15,16]. Various studies demonstrated that weakening of TJ associated with increased epithelial permeability [16-19]. Oxidative stress also is a potent cause of barrier integrity loss of intestinal epithelial cells [20]. Nitric oxide (NO) and reactive oxygen species (ROS) are important mediators in the pathogenesis of inflammatory bowel disease.

Probiotics, according to the World Health Organization and the Food and Agriculture Organization of the United Nations are 'living microorganisms which, when administered in adequate amounts, confer a health benefit to the host'. Probiotics are able to reach the intestines to confer benefit to the host [21-24]. Probiotics are beneficial to improve human health by improving the epithelial and mucosal barrier function, inhibition of pathogenic gut bacteria, and changing the host's immune response [25]. Previous studies described that, some beneficial bacteria are able to enhance intestinal barrier function using the TER assay, which is a measure of the integrity of the TJ between intestinal epithelial cells [19,21].

Transwell co-culture model is an indispensable tool for the IBD research, as this co-culture model mimic the human gut. Direct co-culture can be performed by layering two cell types one on top of the other. Transwell co-culture system is composed of human intestinal epithelial Caco-2 cells and murine macrophage RAW264.7 cells.

The aim of this study was to demonstrate the potential of combinatorial probiotics to improve epithelial integrity in the intestinal epithelial cells on LPS treated *in vitro* co-culture model. In this study, probiotic strains were evaluated in a model using a Transwell co-culture system.

Materials and Methods

Materials

RAW 264.7 macrophage cells were used in this experiment. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin Streptomycin (Pen-Strep), and Nitro blue Tetrazolium chloride (NBT) were bought from Himedia, India. Sulfanilamide, N-1-naphthyl ethylene diamine dihydrochloride (NED) and dimethyl sulfoxide (DMSO) were obtained from Sisco research Laboratories (SRL), India. Ortho phosphoric acid, sodium nitrite ($NaNO_2$), methanol and potassium hydroxide (KOH) were bought from Merck, India. Bacterial lipopolysaccharide (LPS) was obtained from Sigma Aldrich. 1X phosphate buffered saline (PBS; pH 7.4) was prepared using 137 mM sodium chloride (NaCl; Merck, India), potassium chloride (KCl; Himedia India), disodium hydrogen phosphate (Na_2HPO_4; Qualigens, India), and potassium dihydrogen phosphate (KH_2PO_4; Himedia India). 96 well plates were obtained from Nest Biotech Co. Ltd., China, and CO_2 incubator (Thermo Fisher). Cells scrapers from Himedia, India. Multiplate reader (Thermo Fisher Multiskan EX), acetonitrile (HPLC gradient, Merck, India) and HPLC grade water (Spectrochem, India). All cell-culture work was done inside the biosafety cabinet.

Preparation of probiotics

Milk was boiled at 72°C for 1 - 2 minutes and then cooled to 42° - 45°C. Little powder culture of combinatorial probiotics was added to the cooled milk in sterilized cotton plugged conical flask, and incubated at 37°C in incubator for 8 - 9 hours. Resulted whey water was used for experiment.

Culture of RAW 264.7 and Caco-2 Cell

RAW 264.7 macrophages were cultured in DMEM, with 10% FBS and 1% Pen strep, and incubated at 37°C with 5% CO_2, till confluent. The cells were scraped off using scrapers, collected, washed in 1X PBS, and re-suspended in DMEM. Approximately 100 µl of 1×10^5 cells/ml were plated in the wells of a 24 well plate, so there were 10^4 cells per well.

Caco-2 cells were purchased from NCCS Pune and cultured at 37⁰C in a CO_2 incubator, culture medium composed of minimal essential medium (MEM) with 2Mm L- glutamine, 1Mm sodium pyruvate, non-essential amino acid and 1.5 gm /lt sodium bicarbonate 4.5 mg/mL glucose 20% fetal bovine serum and 1% pen-strep. Caco-2 cells were used between passages 40 and 46 in this study.

Experimental Design

The Caco-2 cells were placed on the apical side of the well and the RAW264.7 cells were placed on the basolateral side. Caco-2 cells were seeded at 1×10^5 cells/well onto Transwell insert plates (0.33 cm^2, 0.4 μm pore size, Milicell, India). RAW264.7 cells were seeded at 1×10^5 cells/well into the 24 well cell culture receiver plate (Milicell, India) and incubated overnight to completely adhere to the well. After replacing all media with MEM, the Transwell insert on which Caco-2 cells had been cultured were added into multiple plate wells preloaded with RAW264.7 cells. Transwell insert considered as apical side and receiver plate as basolateral side. cells were stimulated with LPS (1μg/ml) and combinatorial probiotics (10^8 CFU/ml) were used as therapeutic agent. Treatments were done in different combinations (Table 1).

	1	2	3	4	5
Apical	Caco-2	Caco-2	Caco-2+Pro	Caco-2+LPS	Caco-2+LPS
Basolateral	Raw 264.7	Raw 264.7+LPS	Raw 264.7+LPS	Raw 264.7	Raw264.7+Pro

***Table 1:** Treatment of probiotics and LPS in co-culture system in various combinations.*

TER Assay

Functional polarity was developed when electrical resistance between the apical and basolateral surfaces of the monolayers of Caco-2 and Raw264.7 cell, respectively was measured 380 Ω /cm^2 by the Millicell ERS-2 (Millipore, USA). TER was measured before the addition of LPS and the probiotics in co-culture model represented as control and it was also measured after treatment of LPS and probiotics in various combinations as described in Table 1 at 2 hrs, 4 hrs and 6 hrs intervals and expressed as Ω /cm2. Significance was determined using the two tailed Student t test, and significance results are denoted as *($P < 0.05$) **($P < 0.01$).

NO assay

NO assay was done according to the protocol used in the previous publication (4). Briefly, 100 μl of sulfanilamide solution (1% Sulfanilamide in 5% ortho phosphoric acid) was added to each well, and incubated at room temperature for 5 minutes in dark. 100 μl of NED solution (0.1% NED in distilled water) was then added, and incubated at room temperature for 5 minutes in dark. Absorbance was measured in a plate reader at 540 nm. Using the $NaNO_2$ standard curve, the absorbance values of the samples were plotted to get the concentrations of NO produced. The experiment was repeated thrice. Significance was determined using the two tailed Student t test, and significance results are denoted as *($P < 0.05$) **($P < 0.01$).

NBT assay

40 μl of NBT solution (0.5 mg/ml NBT in 1X PBS) was added to the cells in the 96 well plate, and incubated for 1 hour in a CO_2 incubator at 5% CO_2, 37°C. After 1 hour, the cells were washed twice with 1X PBS, then with methanol and air dried. 40 μl of 2M KOH was added, followed by 60 μl of DMSO, and incubated at room temperature for 10 mins, with shaking. Absorbance was measured immediately in a plate reader at 620 nm. The absorbance at 620 nm was plotted. Taking the absorbance of the samples to be directly correlated to the amount of intracellular –superoxide anion produced and the changes were calculated. Significance was determined using the two tailed Student t test, and significance results are denoted as *($P < 0.05$) **($P < 0.01$).

In-vitro cell proliferation studies

RAW macrophages and Caco-2 cells were treated with inflammatory agents (LPS) in various combinations as described in Table 1 and the effect of probiotics on cell viability was assessed using MTS assay test. The assay was performed using the Promega CellTiter 96® AQueous Non- Radioactive Cell Proliferation Assay Kit. 100 μl of cells from all the samples were added to the wells of a 96-well plate, and incubated for 1 hour in a CO_2 incubator at 5% CO_2, 37°C. 20 μl of MTS/PMS (phenazine methosulfate) solution was added to each well, and incubated in a CO_2 incubator for 1-4 hours. Absorbance was measured immediately in a plate reader at 492 nm. Cell viability for cells

without treatment (control) was taken to be 100%, and the cell viability of the experimental groups calculated accordingly. Significance was determined using the two tailed Student t test, and significance results are denoted as *(P < 0.05) **(P < 0.01).

Statistical analysis

The comparisons between experimental groups were performed using unpaired two-tailed Student's t-tests. The statistical analysis was performed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). A P value less than 0.05 were considered statistically significant.

HPLC Analysis

For HPLC analysis all the soups of apical compartment of co-culture were centrifuged and filtered by 0.22 μm syringe filter (Millipore, Germany) and 50 μl of filtrate administered as sample for HPLC analysis. HPLC was done by waters 2695 equipped with waters 1525 binary pump and waters 2487 DAD. The analytical column was a reversed-phase water C18 column (4.5 × 250 mm, Ireland). Eluent A was 0.1% (v/v) TFA in deionised water and eluent B was 0.09% (v/v) TFA in a mixture of 60:40 acetonitrile (HPLC gradient, Merck, India) and HPLC grade water (Spectrochem, India). Separation was conducted at room temperature (200C) at a flow rate of 0.8 ml/min with eluent A for 10 mins and a linear gradient, from 0% to 80% of eluent B, for 80 min. The column was finally eluted with 100% eluent B for 10 mins. The absorbance of the eluate was monitored at 214 nm.

Results

We found that, TER of Caco-2 cell was decreased by 19.66%, 8.63% and 15.17% in 2hrs, 4hrs and 6 hrs, respectively when RAW264.7 cell was treated with LPS. But it was increased by 1.94%.1.5% and 11.42% with addition of probiotics in Caco-2 cell under same experimental condition. Another experimental condition where apical Caco-2 cell stimulated with LPS and TER value of caco-2 cell decreased by 14.70%, 2.58 %, 11.56%, respectively in 2 hrs,4 hrs and 6 hrs incubation period with respect to control and under same condition TER value increased by 9.08%, 3.08% and 5.39% in 2hrs, 4 hrs and 6 hrs time interval, respectively when basolateral RAW cell treated with probiotics. But when Caco-2 cell treated with probiotics without any stimulation on RAW cell upto 2 hrs there was no change in TER and 1.51% and 8.52% decreased in respect to control in 4 hrs and 6 hrs interval respectively (Figure 1).
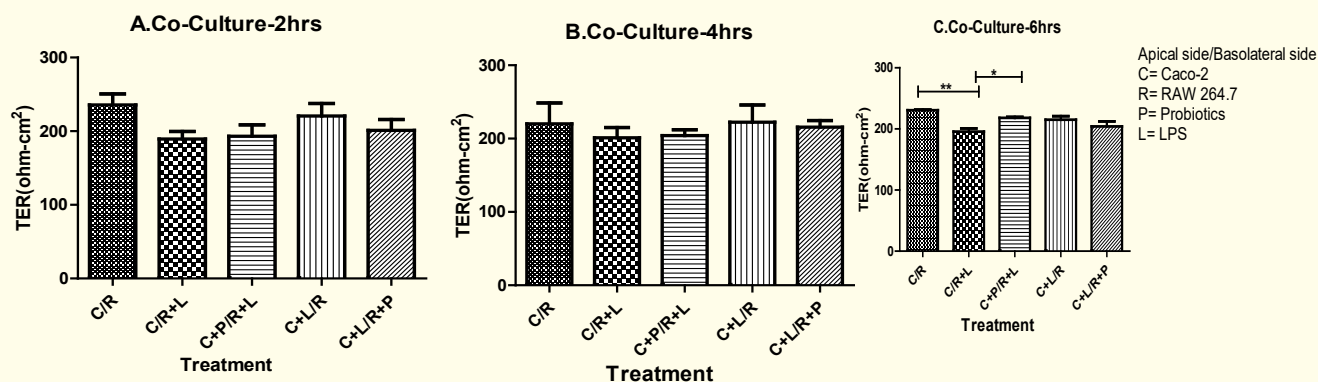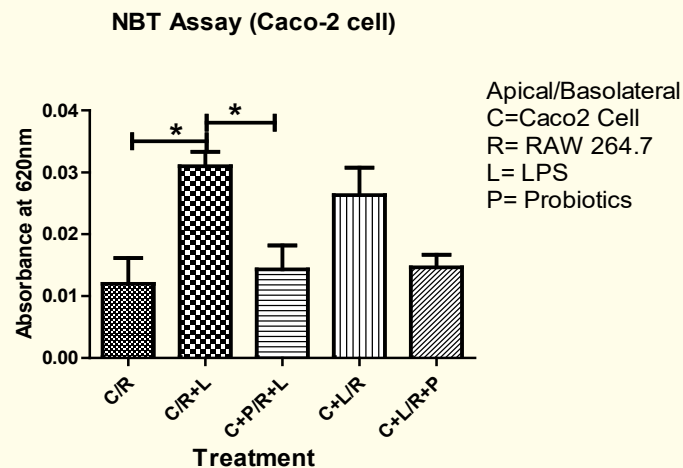

Figure 1A,1B,1C: *TER value of Caco-2 cell (apical side) in co-culture with RAW 264.7 cell (basolateral side) in various treatments with LPS and probiotics in time dependent manner.*

Figure 2 demonstrated that, concentration of NO of Caco-2 cell was increased by 3.64 fold when RAW 264.7 cells were stimulated with LPS where it was decreased by 2.10 fold with addition of probiotics in Caco-2 cell under same experimental condition. NO concentration of Caco-2 cell was increased by 2.88 fold when LPS was added to the apical caco-2 cell compression to control. And NO concentration reduced by 2.13 fold when probiotics was added to basolateral RAW 264.7 cell.
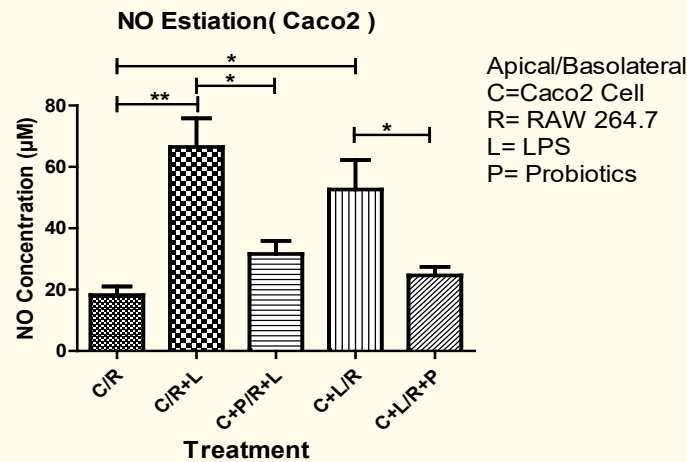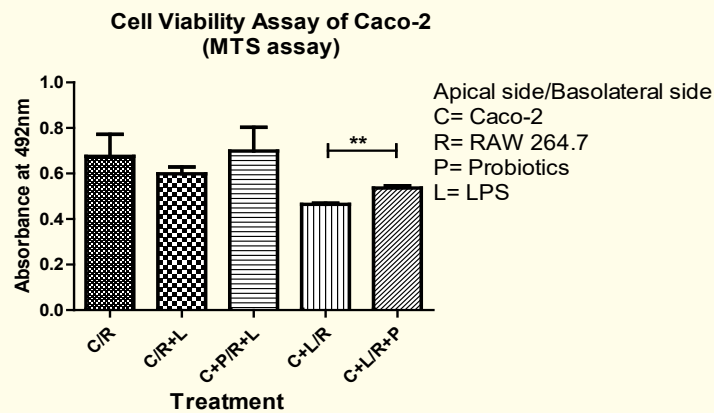
Figure 2: *NO concentration of Caco-2 cell (apical side) in co-culture with RAW 264.7 cell (basolateral side) in various treatment with LPS and probiotics.*

NBT assay showed that, concentration of superoxide of Caco-2 cell was significantly increased by 158.33% with respect to control when RAW 264.7 cells were stimulated with LPS where it was decreased by 54.84% with respect to treatment with addition of probiotics in Caco-2 cell under same experimental condition. Superoxide concentration of Caco-2 cell was increased by 116.66% when LPS was added to the apical caco-2 cell compression to control. It was reduced by 42.31% when probiotics was added to basolateral RAW 264.7 cell (Figure 3).
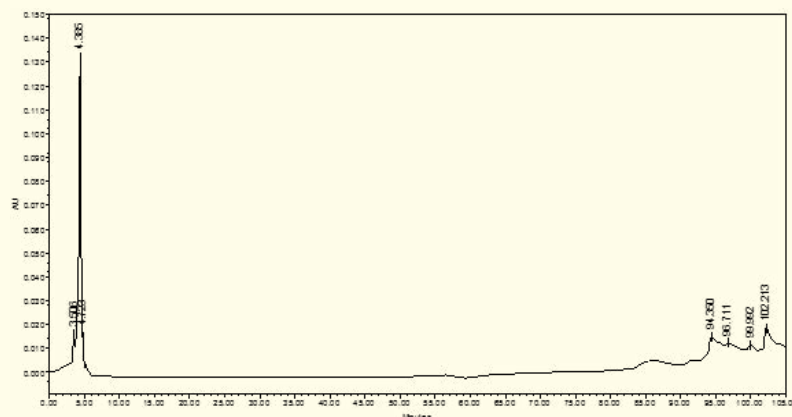

Figure 3: *Bar diagram demonstrates production of superoxide anion concentration of Caco-2 cell (apical side) in co-culture with RAW 264.7 cell (basolateral side) in various treatment with LPS and probiotics after 6 hrs.*

Figure 4 demonstrated viability of Caco-2 cell (apical side) in co-culture system with RAW 264.7 cell (basolateral side) in various treatment with LPS and combinatorial probiotics after 6 hrs. Viability of Caco-2 cell was significantly decreased by 11.4 % with respect to control when RAW 264.7 cells were stimulated with LPS where it was increased by 16.9 % with respect to treatment with addition of probiotics in Caco-2 cell under same experimental condition. Cell viability reduced by 31.26% when apical Caco-2 cell stimulated with LPS with respect to control and viability significantly increased by 15.52% after addition of probiotics in basolateral RAW cell and it was reduced by 42.31% when probiotics were added to basolateral RAW 264.7 cell.

Figure 4: *Bar diagram demonstrates viability of Caco-2 cell (apical side) in co-culture system with RAW 264.7 cell (basolateral side) in various treatments with LPS and probiotics after 6 hrs. Viability of Caco-2 cell was significantly decreased by 11.4 % with respect to control when RAW 264.7 cells were stimulated with LPS where it was increased by 16.9 % with respect to treatment with addition of probiotics in Caco-2 cell under same experimental condition. Cell viability reduced by 31.26% when apical Caco-2 cell stimulated with LPS with respect to control and viability significantly increased by 15.52 % after addition of probiotics in basolateral RAW cell and it was reduced by 42.31% when probiotics was added to basolateral RAW 264.7 cell.*

Figure 5: *HPLC chromatogram of soup of apical compartment of co-culture experiment C/R+L combination. One major peak was identified at 214 nm and its retention time and area percentage was 4.385 (22.51%).*
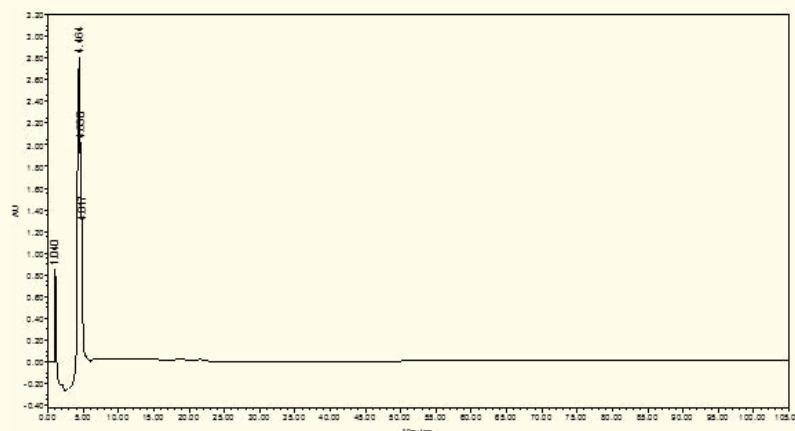
Figure 6: *HPLC chromatogram of soup apical compartment of co-culture experiment C+P/R+L combination. Three major peaks were identified at 214 nm and their retention time and area percentage were 1.040 min (22.07%), 4.464 min (75.94%) and 4.630 min (1.64%), respectively.*
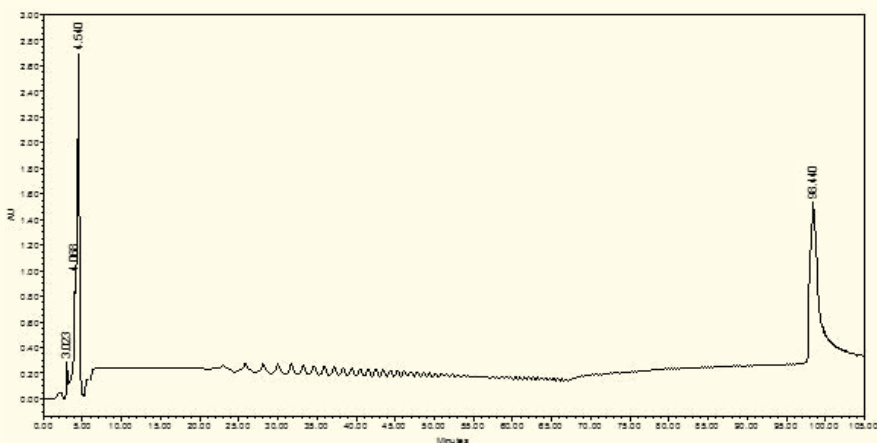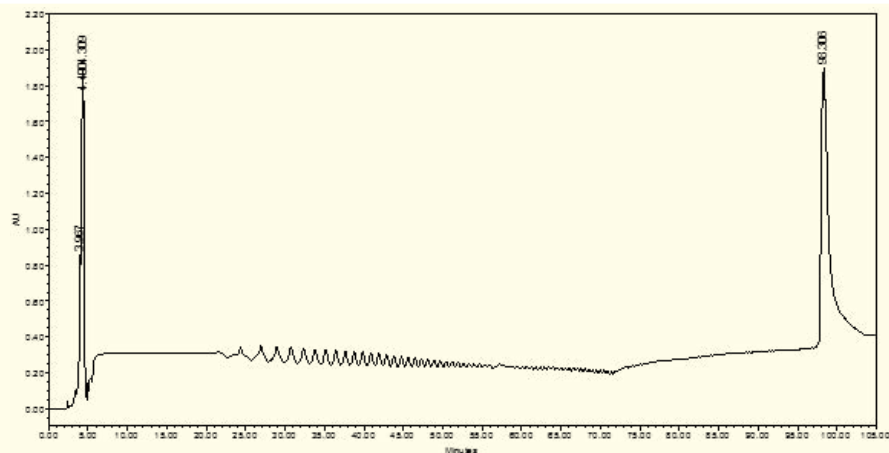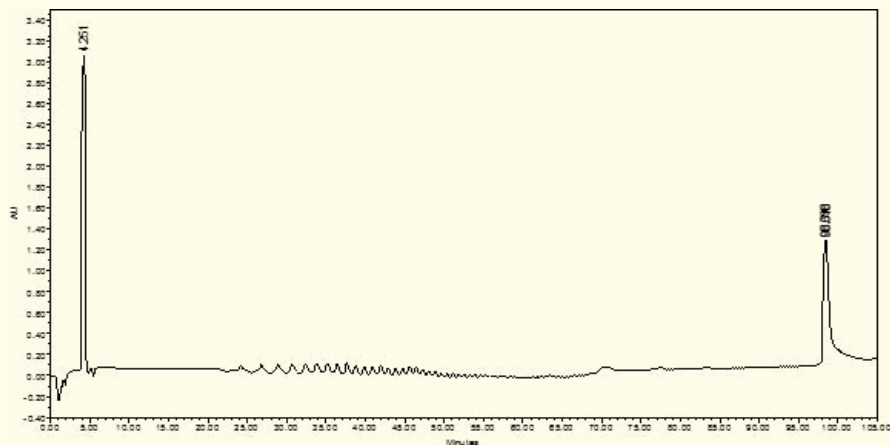
Figure 7: HPLC chromatogram of soup of apical compartment of co-culture experiment C+L/R combination. Four major peaks were identified at 214 nm and their retention time and area percentage were 3.023 min (0.81%), 4.068 min (0.61%), 4.540 min (20.76%) and 98.440 min (77.82 %) respectively.

Figure 8: HPLC chromatogram of soup apical compartment of co-culture experiment C+L/R+P combination. Four major peaks were identified at 214 nm and their retention time and area percentage were 3.967 min (0.09%), 4.309 min (0.11%), 4.40 min (0.57%) and 98.306 min (97.99 %) respectively.

Figure 9: HPLC chromatogram of soup of apical compartment of co-culture experiment C+P/R combination. Only two major peaks were identified at 214 nm and their retention time and area percentage were 4.251 min (64.49%) and 98.39 min (35.47 %) respectively.

Discussion

In human, the mucosal barrier of the GI tract is composed of microbial flora of the intestine, the epithelial cells, the mucus layer, and the intercellular TJ. In epithelial cell TJs are the apical most component of junctional complex which regulates cell permeability of the critical components of TJs claudin-1, occluding and ZO-1 [41]. LPS insult down regulates the level of ZO-1 and ZO-2, and ZO-2 redistributed in human corneal epithelial cells [42]. There is a medical need to better understand the interactions of intestinal epithelial cells with intestinal microbes. In this study, we demonstrated that, the combinatorial probiotics used for the experiment significantly increased the TER in the co-culture system and as a result successfully decreased the membrane permeability in LPS challenged CaCo-2 cell. TER is a highly sensitive parameter for membrane permeability which reflects the tightness of the intercellular junctions. A decrease in TER value indicates an increase in the paracellular permeability, and vice versa. Different studies demonstrated that, commensal probiotics promote intestinal barrier integrity both *in vitro* and *in vivo*. Probiotics preserve the intestinal barrier in mouse models of colitis and reduce intestinal permeability in human patients with Crohn's Disease [26,27].

Dysregulation of intestinal permeability cause intestinal inflammation. Epithelium has developed some mechanisms to protect itself from inflammatory responses. Some of these are to prevent bacterial growth as well as to restrict the contact with the bacteria. Disruption of the intestinal barrier leads to loss of immune tolerance to the microflora [28,29]. Dysregulation of intestinal barrier function has been demonstrated as an important factor for IBD [30]. Administration of nonpathogenic bacterial species can decrease the paracellular permeability which provides defense against pathogenic bacteria which ultimately help protect against infections, prevent chronic inflammation by maintaining mucosal integrity [31]. Proinflammatory cytokines seen in IBD patients such as tumor necrosis factor (TNF) alpha, Interferon gamma, Interleukin 1 beta, and Interleukin 13 can increase epithelial permeability and as a result increase the inflammation [32-35].

In this study, Caco-2 cells were used as a model of the intestinal epithelial barrier. We found that, TER of Caco-2 cell was decreased when RAW264.7 cell treated with LPS. Administration of our combinatorial probiotics to the cell culture medium was able to reduce the LPS-induced inhibition of TER. A decreasing TER value can be used as an indicator of inflammation. On the other hand, TER was increased significantly after 6 hours of addition of probiotics in Caco-2 cell under same experimental condition. TER was increased by 1.94%, 1.5% and 11.42% in 2hrs, 4hrs and 6 hrs, respectively (Figure 1). Use of probiotics in this study led to an increase of the transepithelial electrical resistance which as a result decrease permeability. Our result supported by previous studies which showed that probiotics improve epithelial cell barrier function by regulation of TJs [37-39] and it have been shown that probiotics modulate various TJs protein like zonula occludens-1, or increased zonula occludens-1 [39,40]. This could be due to the soluble factors which are inducing cellular mechanism by eukaryotic host cell (patient of IBD) to counteract the effect of LPS and prevent leakage and inflammation. Recent study by Hsieh C., *et al*. [41] demonstrated that *Bifidobacterium* species restored the epithelial TJ barrier of Caco-2 cell layer, where TJ barrier integrity was damaged by pretreatment of TNF-α. Another study stated that, improvement of epithelial barrier function could be possible by live probiotics [42]. Probiotic bacteria compete with pathogenic microbes for binding sites to epithelial cells and the mucous layer and finally contribute to intestinal barrier function. Probiotics inhibits pathogenic adherence to prevent intestinal infection [23,43,44].

Beneficial microbes are essential to normal gut physiology, as shown by Collins., *et al* [45] by altering intestinal function in germ-free mice. Thus, in our study, friendly bacteria and their products (combinatorial probiotics) was considered when evaluating intestinal barrier function. A study by Resta-Lenert and Barrett [18] found that *S. thermophilus* and *L. acidophilus* independently increased TER and decreased permeability of HT-29 and Caco-2 cells. Epithelial barrier integrity could be increased by administration of different probiotic species which are capable of modifying specific TJ proteins.

Increased synthesis of nitric oxide is another characteristic of inflammatory bowel disease. The action of NO depends on its enzymatic sources such as inducible NOS (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial NOS (eNOS). These isoforms have been localized in the GI tract. Constitutive synthesis of NO by these isoforms is involved in the maintaining of the GI mucosal integrity through modifying epithelial secretion, gastric mucosal blood flow, and barrier function [46,47]. One of the main results of an inflammatory reac-

tion in a body is the overproduction of pro-oxidative agents, like nitric oxide. This has been demonstrated clearly in our experiment and probiotics successfully reduced the NO content. Increased iNOS expression is found in chronic ulcerative colitis and peptic ulcer patients in the study by Rachmilewitz D., *et al*. [48]. In this study, concentration of NO of Caco-2 cell was measure and it has been found that, it increased by 3.64 fold when RAW 264.7 cells were stimulated with LPS whereas it was decreased by 2.10 fold with addition of probiotics in Caco-2 cell under same experimental condition (Figure 2). Similarly, by NBT (nitroblue tetrazolium) assay our result demonstrated that, concentration of superoxide of Caco-2 cell was decreased after probiotic use. Viability of Caco-2 cell was significantly decreased by 11.4 % with respect to control when RAW 264.7 cells were stimulated with LPS where it was increased by 16.9 % with respect to treatment with addition of probiotics in Caco-2 cell under same experimental condition. Cell viability reduced by 31.26% when apical Caco-2 cell stimulated with LPS with respect to control and viability significantly increased by 15.52% after addition of probiotics in basolateral RAW cell (Figure 3). The MTS assay is self-explanatory as to the cytotoxicity generated and the amount reversed by probiotics treatment. Previous study demonstrated the anti-inflammatory effect of probiotics which play an important role in treatment of enterocolitis [49]. The findings in this study using co-culture system confirmed that our combinatorial probiotics can reduce the inflammation in IBD patients.

HPLC analysis was done to identify major peptide from apical soup (Figure 5-9). On preliminary investigation HPLC data revealed that, there may be some soluble factor which produce by live probiotics during co-culture system and help to protect from LPS induced inflammation on Caco-2 cell. Further investigation is going on to identify each HPLC peaks by MS and NMR. Our future interest is to identify some interesting pro- and anti- inflammatory cytokines which get activated or suppressed by probiotics.

Conclusion

The combinatorial probiotics used in this study increased the TER in the co-culture system and as a result successfully decreased the membrane permeability. The concentration of NO and super oxide was decreased after adding probiotics in the co-culture system. The treatment of IBD with probiotics may prevent or reverse increased permeability of the epithelium. Inhibition of iNOS may afford a potential therapeutic way to the treatment of IBD. Understanding the cellular and molecular mechanisms are critical to developing future therapies for GI diseases mediated by oxidative stress. It may be acting via mitochondrial NADPH oxidase pathway. Mitochondrial derived oxidative reactive pathway may have obstructed by crosslinking of the receptor expressed by probiotics directly with the intestinal host cells or interfering intermediate pathways by biochemical moieties secreted by quorum sensing mechanism of bacteria in combinatorial culture. Future studies should investigate the effects of various probiotics on TJ structure and function.

Contribution of Authors

RD did most of the preliminary experiments, collated and performed detailed analyses of all data SK performed all microbiological experiments. SB contributed intellectually and participated in writing the manuscript. UPS has mentored all the proteomic analyses pertaining to this project and helped in the acquiring of data and all final analyses. ERB conceptualized the project, designed all experiments and analyzed the data and wrote this manuscript.

Acknowledgement

Conflict of Interest

The authors have declared that no conflict of interest exists.

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