Development and evaluation of *in vitro* intestinal epithelial models to study ameliorative effects of probiotics on inflammation induced barrier dysfunction

Thesis submitted in partial fulfillment of the requirements of

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Indian Institute of Science Education and Research, Pune

By

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CERTIFICATE

This is to certify that this dissertation entitled "**Development and evaluation of *in vitro* intestinal epithelial models to study ameliorative effects of probiotics on inflammation induced barrier dysfunction"** towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by **Mr. Mallikarjuna Mullangi,** at **Unilever R&D, Bangalore** under the supervision of **Dr. Gautam Banerjee, Unilever R&D, Bangalore.**

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DECLARATION

I hereby declare that the matter embodied in the thesis entitled "**Development and evaluation of *in vitro* intestinal epithelial models to study ameliorative effects of probiotics on inflammation induced barrier dysfunction"** are the results of the investigations carried out by me at the Immunology lab, Unilever R&D, Bangalore under the supervision of **Dr. Gautam Banerjee,** R&D, Bangalore and the same has not been submitted elsewhere for any other degree.

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ABSTRACT

The human intestinal epithelium constitutes major component of the body's interaction with the external environment. In addition to performing physiological functions such as digestion and absorption of nutrients, this epithelium provides a defensive barrier from the microflora colonizing the lumen. Inhabitation of the lumen with pathogenic bacteria causes inflammation and compromise in integrity of this barrier. A compromised intestinal barrier is observed in numerous patho-physiological disorders such as Crohn's disease, Inflammatory Bowel Disease, Type 2 Diabetes, etc. and in addition, alteration in the profile of the microflora is observed in various diseases. To understand the role of these bacteria in modulating inflammations and barrier function we aimed to develop an *in vitro* model. CaCO2 cell line based 5 day and 21-day monolayers are used to study the permeability characteristics of the intestine with good correlation to intestinal epithelium. Using these 5-day and 21-day monolayers we tried to study the effect of inflammations on the integrity of the barrier. Although it is reported in the literature that short term monolayers are suitable to study the permeability in the intestine, they are unsuitable for studying inflammation induced barrier dysfunction in the epithelium as explored by us. The 21-day CaCO2 monolayers remain a stable option for studying intestinal barrier characteristics and inflammations from cytokines and immune system can affect the barrier integrity. These compromised barriers can be restored by using metabolites of probiotics *Bifidobacterium lactis Bb12*; however supernatant of commercially available *L casei shirota* did not alleviate the inflammation induced barrier dysfunction indicating that this is a species specific phenomenon.

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Chapter 1: Introduction:

1.1 The Intestinal epithelium: the semi permeable barrier

Human body is being continuously exposed to outside environment and the specialized epithelial cells constitute the first line of defense that separate the host from external insults. The gastrointestinal tract is one of the main barriers of this defense mechanism and is specially adapted to colonization by commensal bacteria that aid in digestion and influence development and function of the mucosal immune system. Maintenance of homeostasis in this co-existence of microbes and immune cells is carried out by the intestinal epithelium by forming a physical and biochemical barrier and segregating between host and microorganism. In addition to being a barrier, intestinal epithelial cells (IECs) sense and respond to microbial stimuli to reinforce their barrier function and to participate in the coordination of appropriate immune responses from immune cells, ranging from tolerance to commensals and anti-pathogen immunity.

The intestinal barrier's role in digestion is selective absorption of nutrients, water and ions. Para cellular pathway consists of transport in intercellular space between adjacent epithelial cells. The trans-cellular pathway consists of absorption of nutrients such as sugars, amino acids, minerals and vitamins through specific transporters and channels found on the cell membrane (Camilleri et al., 2012) and is regulated by the apical junction complex, composed of Tight Junctions (TJs) and adherens junctions (AJs). The TJs, composed of integral membrane proteins have dual functionality of working as a barrier to harmful molecules and pores to ions and nutrients. The size and charge selective pores present in the TJs provide for selective diffusion of ions, water and solute. This selective permeation of nutrients through paracellular and trans-cellular pathways render the intestine its epithelial barrier function. A functioning epithelial barrier is essential for maintaining the homeostasis of the body, and its malfunction has been implicated in gut disorders such as celiac disease (CD), colorectal cancer, Irritable bowel syndrome (IBS) and systemic diseases like Type 2 diabetes mellitus (T2D) (Arrieta, Bistritz, & Meddings, 2006). Though diseases associated with increased

permeability vary in manifestation and symptoms, they have a common underlying factor i.e., the altered barrier function.

1.1.1 Intestinal Tight Junctions

The semi permeable nature of the intestinal epithelium is attributed to by the multi-protein Tight Junction complexes in the intercellular space. Tight Junctions are assembled by homophilic and heterophilic interactions of integral and peripheral membrane proteins. The major integral tight junction membrane proteins are claudins, occludins, tricellulin, and Junctional adhesion molecules. These transmembrane proteins are anchored to the actin cytoskeleton by a cytoplasmic plaque consisting of adaptor proteins Zonula Occludens (ZO -1, -2, and -3).

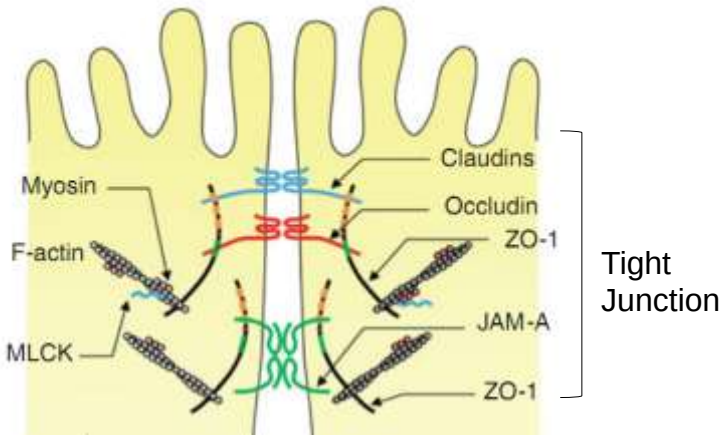


Figure 1: Intestinal Tight Junctions: Assembly of tight junction proteins at the apical end of the lateral surface of the intestinal epithelial cells. The figure shows the intercellular attachment of tight junction proteins such as Claudins, Occludins, Junctional Adhesion Molecules and the anchoring of TJ complexes to actin cytoskeleton through cytoplasmic adaptor proteins Zonula Occludens. (Adapted from (Sciences, 2013)**).**

Table 1: Structure, Function, Localization and major signalling pathways of the molecular components of intestinal epithelial Tight Junctions. (Aijaz, Balda, & Matter, 2006; Hossain & Hirata, 2008; Sciences, 2013)

protein	Protein Types	Structure and Function	Expression in the intestine
Claudins		Transmembrane proteins Cell-cell adhesion Regulation of paracellular permeability	Differential expression of Claudins along the intestine
	Claudin -1,-3,-4,-5,-8,-9,-11 and -14	Barrier forming proteins	Claudin 8 expressed in distal colon, minimal expression in Duodenum, Jejunum and Ileum
	Claudin -2,-7,-12 and -15	Pore forming proteins	Claudin 15 expressed in duodenum and jejunum, decreased expression in distal colon
Junctional Adhesion Molecules		Transmembrane Proteins Cell-cell adhesion	
	JAM-A	Regulation of junction assembly	Expressed in Duodenum and Ileum, higher expression in colon
	CAR	Regulation of leukocyte transmigration	
Occludin		Transmembrane Protein Cell-Cell Adhesion	
		Regulation of paracellular permeability	Expressed in Duodenum and Ileum, higher expression in Colon
		Modulation of signalling pathways: Raf-1, RhoA, TGF-β	
Zonula Occludens	ZO1	Cytoplasmic adaptor protein. Regulation of junction assembly, gene expression and cell proliferation	
	ZO-2	Cytoplasmic adaptor protein Regulation of gene expression Binds and inhibits viral oncogenes	Ubiquitous expression in the intestine
	ZO-3	Cytoplasmic adaptor protein	

1.2 Challenges to Intestinal Epithelial Barrier - Modulation of barrier function by gut microflora

The gastrointestinal tract faces the unique challenge of constant exposure to diverse community of microorganisms. This amplifies the chances of opportunistic breaching of the barrier and the intestinal epithelium has a major role in providing the defensive barrier from pathogenic bacteria. A symbiotic association between intestinal epithelium and the gut microbiota accounts for the tolerogenic and proinflammatory responses altering the composition and spatial localisation of the gut microbiome.

The microflora shares a mutualistic relationship with the host and contributes to the digestion of food and also to the prevention of propagation of pathogenic microorganisms (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). An alteration of this micro flora by diet, drug or environmental factors leads to selective propagation of pathogenic bacteria in the intestinal lumen (Ayres, Trinidad, & Vance, 2012). The Pathogen associated Molecular patterns (PAMPs) expressed on pathogenic or gram negative bacteria are recognised by the Pattern Recognition Receptors (PRR) on the intra-epithelial lymphocytes (IEL). This leads to initiation of an inflammatory cascade, causing destruction of the intestinal epithelium by the cytokines released by the lymphocytes.

1.2.1 Cytokines affect the permeability of the intestinal barrier

The cytokines released by activated IELs cause aberrant expression of claudins and increase the intestinal permeability. Alterations in the composition of TJ and actin rearrangement mediated endocytosis are the major pathways through which cytokines cause this. Exposure of intestinal epithelial cells to cytokines changes the Claudin complement of the TJ, which affects the size and charge selectivity of the paracellular pathway. For e.g., TNF-α causes decrease of barrier forming Claudin1 in the TJ complex and increases the pore forming Claudin 2 and this modulation of signalling pathways independent of their cytotoxic effects (Li et al., 2008).

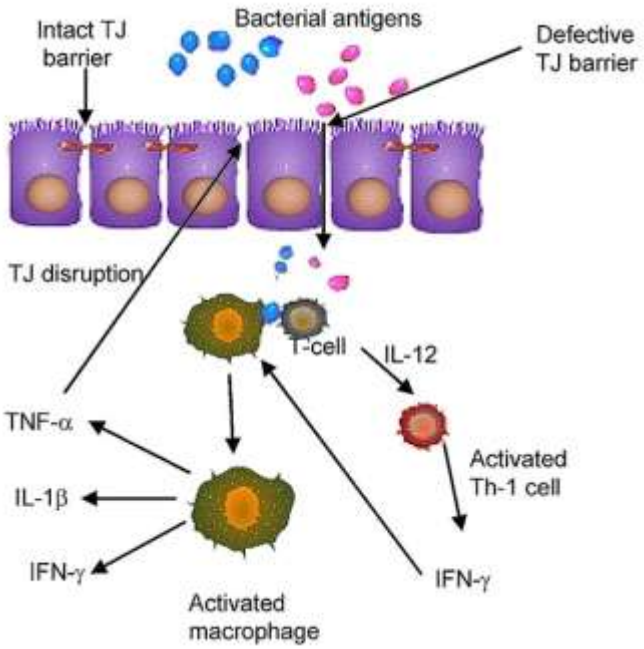


Figure 2: Altered gut microbiota affects the function of the intestinal barrier. Colonization of the intestinal lumen leads to increased permeability and activation of the mucosal immune system. This leads to further damage to the integrity of the barrier.

1.2.2 Cytokines affect the turnover of Tight Junction proteins

The claudins localized in the TJs are considered to be constitutively internalised and recycled. Cytokine mediated changes in the TJ could be explained by increased turnover rate at the expense of integration. The TJ proteins claudins are classified as barrier forming (1, 4, 5, 8, 11, 14 and 19) and pore forming (2, 7, 10, 15, 16) (Krause et al., 2008). Cytokine regulation of the transmembrane protein transcription is a powerful mechanism through which the TJ are remodelled and the major pathway followed is dependent on the NFκB signalling. Exposure to cytokines such as TNF-α and IL-1β activates the NFκB, leading to subsequent disruption of Claudin and Occludin protein levels and decrease in the Trans Epithelial Electrical Resistance (TEER) (Capaldo & Nusrat, 2010a). The change in localization of barrier forming and pore forming proteins as an effect of cytokine exposure can influence the permeability of the intestinal epithelium, thus altering the barrier function.

1.3 Experimental methods used for evaluating integrity of barrier

The permeability of the intestinal barrier is measured in both *in vivo* experimental animal models and *in vitro* cell culture models. Intestinal permeability in *in vivo* animal models is measured by oral administration of non-metabolizable sugars lactulose, sucralose, mannitol, cellulalose, etc and their excretion in urine. The ratio of sugars in urine to orally administered quantities provides a measure of the intestinal permeability. To achieve better compartmentalisation of the properties of the intestinal epithelium, *in vitro* cell culture based models are employed. One such model is the colorectal cancer cell line CaCO2 which differentiates into a polarised epithelium on culturing on a semipermeable support and correlates well with the human intestine.

The relative quantification of permeability characteristics of the *in vitro* models is carried out by measuring the transport of Polyethylene Glycol (PEG 400), Fluorescein Isothiocyanate labelled Dextran (FD4) or non-metabolized sugars and by measuring the Trans Epithelial Electrical Resistance (TEER).

1.3.1 Trans Epithelial Electrical Resistance (TEER)

Differentiated *in vitro* cell monolayers form an epithelial barrier through the apical junctional complexes in the intercellular space. This hampers the free movement of water and ions and is gradually restricted. The TEER is used to quantify the permeability of these ions electrodes across the Trans well permeable inserts on which the cells are grown. Differentiated monolayers with intact intercellular attachments give a high and stable resistance measurement over a period of time.

1.4 Compromise in epithelial barrier function.

Various disease conditions like colitis, crohns disease, type 2 diabetes, have been known to compromise the intestinal barrier function. In this report, our focus is restricted to type 2 diabetes.

Type 2 Diabetes (T2D) is characterized by insulin resistance; compensatory hyperinsulinemia and pancreatic beta cell failure, leading to diminished glucose transport to liver, muscle cells, and fat cells (Fujioka, 2007). Increased intestinal permeability has been observed in patients suffering from T2D (Shakil, Church, & Rao, 2008). Although diabetes can affect intestinal barrier function, clinical evidence suggests that altered barrier function has an underlying role in pathogenesis of diabetes (Arrieta et al., 2006). Analysis of diabetic subjects without Crohn's disease revealed an increased lactulose/mannitol permeability ratio (L/M ratio), and high resolution microscopy revealed altered TJ structure, duodenal mucosa and aberrant microvilli structures, though histological pattern remained unaffected. In addition pre diabetic patients had increase in the intestinal permeability compared to the control patients (Secondulfo et al., 2004).

An increased systemic circulation of inflammatory cytokines observed in T2D, desensitizes insulin receptor and thus insulin resistance. One of the hypothesis suggested for this increased systemic circulation of pro inflammatory cytokines is increased intestinal permeability (Hotamisligil & Erbay, 2008).

Increased circulating endotoxin levels in the serum has been observed in type 2 diabetes patients (Miller et al., 2009) and one of the reasons cited is increased intestinal permeability for triggers of inflammation i.e. LPS from gram negative gut microbiota (Creely et al., 2007). The other reason being increased absorption of LPS molecules by chylomicrons during uptake of fats because of high-fat diet. The difference in gut bacteria composition in healthy and diseased gut indicates that there is some association between the gut bacteria and metabolic diseases but cause and effect relationship needs studies in cohorts followed for a long duration of time.

1.5 Approaches to restore the integrity of compromised intestinal epithelial barriers

Therapeutic approaches aimed at gut barrier correction focus on several modes such as altering exposure to nutrients, altering gut microbial composition, modification of gut

barrier proteins, and restraining the intestinal inflammation (Hering & Schulzke, 2009). Incretin hormone GLP-1 secreted by the intestinal L cells enhances the barrier function. The secreted GLP-1 is constantly degraded by the DPP-IV peptidase. Therapeutic approaches aim at enhancing the secretion rate of GLP-1 and stabilising the secreted GLP-1(Mannucchi, Ognibe, Cremasco, Bardini, & Mencucci, 2001). Barrier enhancing bacterial metabolites such as the small chain fatty acids (SCFAs) butyrate and propionates is another therapeutic tool to correct barrier function. *In vitro* experiments show a dose dependent increase in the TEER when supplemented with butyrate.

1.5.1 **Probiotic approaches**

Probiotics are viable non-pathogenic microorganisms that are able to colonize the gut and render benefit to the host. Recent studies have found that probiotics counter gastrointestinal inflammation in Inflammatory Bowel Diseases and reduce cytokine secretion (Boirivant & Strober, 2007). Commonly used bacterial probiotics include *Lactobacillus* species, *Bifidobacterium species, Escherichia Coli*, *and Streptococcus species*. The probiotic and pathogenic bacteria cannot be distinguished experimentally and colonisation in healthy and diseased human and animal models is the sole criterion to identify them. The constructive effect of the probiotics on the intestine is rendered by numerous action mechanisms:

- Excluding pathogenic bacteria through increased mucin secretion.
- Intestinal epithelium generates a defensive barrier by secreting antimicrobial peptides and probiotic bacterial metabolites re-inforce the integrity of the barrier by inducing secretion of these anti-microbial peptides. For E.g., Butyrate secreted by *Bifidobacterium* induces increased production of antimicrobial peptide cathelicidin (Schauber et al., 2003).
- Some probiotics express antimicrobial factors which directly inhibit the growth or kill pathogenic bacteria. Bacteria from *Lactobacillus* and *Bifidobacterium* strains can directly kill *Salmonella typhimurium in vitro* (Fayol-messaoudi, Berger, Moal, & Alain, 2005).

- Secreted metabolites such as SCFA can disrupt the outer membrane of gram negative bacteria and cause inhibition of pathogen growth (Alakomi et al., 2000).
- Competing for binding sites on the epithelial cells. *In vitro* culture experiments have shown *L. rhamnosus* and *L. acidophilus* can bind to epithelial cells and prevent binding and entry of pathogenic *E Coli* (EPEC) (H- et al., 2005).

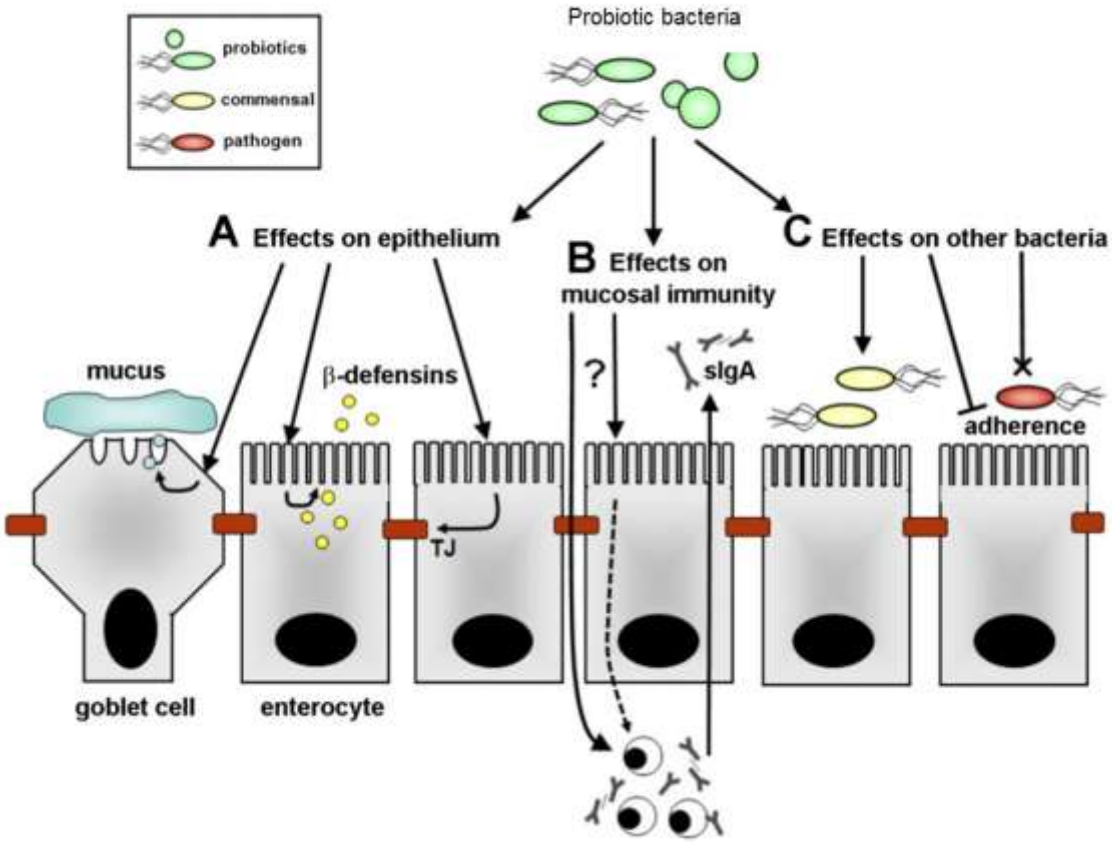


Figure 3: Effects of probiotics on intestinal barrier function. Probiotics affect the epithelial barrier in multifactorial ways. Of these the three major pathways are through a) Increased production of antimicrobial peptide β-defensins b) Increased levels of IgA producing cells and promoting IgA secretion c) Killing and inhibiting growth of pathogenic bacteria

1.5.1.1 Probiotics enhance barrier function by promoting Tight Junction assembly

Disruption in the tight junction assembly of the intestine is the main cause of increased permeability of the contents of the lumen into the circulatory system. There is enough evidence that probiotics modulate barrier function. Studies have shown that pre-treatment with probiotics can inhibit the TJ alteration caused by infection and or pro inflammatory cytokines (Ohland & Macnaughton, 2010) and that probiotics can directly alter the epithelial barrier function by influencing the Tight Junction assembly. Bacterial culture medium from probiotics such as *S. thermophilus L*. *acidophilus* enhance the barrier function and increase expression of tight junction proteins occludin and ZO-2 in *in vitro* models and expression of pore forming protein Claudin2 is reduced upon treatment with culture supernatant of *B*. *infantis* (Ewaschuk et al., 2008).

Probiotic approaches to restore barrier function aim to promote colonisation of the intestine by health promoting bacteria. In this direction, evidence suggests that probiotics can restore TEER compromised by cytokines TNF-α and IFN-γ. Commensal or probiotic bacteria mediated stimulation of TLR signalling may have a role in restoring inflammation affected barrier compromise. Studies have found *L. rhamnosus* mediated prevention of cytokine induced apoptosis in *in vitro* and *ex-vivo* experiments. In vivo studies by (Ukena et al). reveal that dextran sodium sulphate (DSS) mediated barrier compromise and disease progression can be alleviated by pre incubating with *E coli* strain Nissle (EcN). The microbial composition of the intestine has a major role to play in determining the immune response to low grade inflammations. Moreover it is found that promoting the growth of *Bifidobacterium spp*. and *Lactobacillus spp*. leads to enhanced barrier function(Griffiths et al., 2004). Thus inclusion of prebiotics in diet that promote growth of barrier promoting probiotics is a suitable therapeutic approach to cure metabolic disorders originating because of altered barrier function. For eg. Administration of Hydrolysed casein to diabetes prone Bio Breeding rats leads to an improved intestinal barrier function(Visser et al., 2010)

1.6 Other benefits of probiotics for diabetes patients

Hyperglycaemia and impaired glucose tolerance are common occurrences in patients suffering from Type 2 Diabetes. Sustained glucose release instead of sudden glucose surge post meal is preferred in these patients. Alpha-glucosidase inhibitors (AGI) are prescribed to this effect. AGI such as Miglitol, Acarbose and Voglibose, inhibit a number of α-glucosidase enzymes and consequently delay the absorption of glucose.

The role of bacterial metabolites in regulation of glucose absorption in the intestinal lumen and regulation of α-glucosidases activity has not been explored in much detail. Here the possibility of probiotic metabolites in regulating the activity of α-glucosidase is analysed by an *in vitro* α-Glucosidase activity assay.

1.7 The Objectives of the project

To understand the ameliorative effects of metabolites of probiotics on inflammation induced damage of the integrity of the epithelial barrier, we aim to develop a short term CaCO2 monolayers based *in vitro* procedure. To this effect the following objectives are to be realized.

- To develop a CaCO2 cell line based *in vitro* intestinal epithelial barrier model.
- To evaluate the effect of endotoxins and inflammatory cytokines on the integrity of this *in vitro* model of intestinal barrier.
- To investigate the ameliorative effects of probiotics, if any, on compromised barrier function.
- To investigate the effect of probiotic metabolites on alpha-Glucosidase activity.

Chapter 2: Materials and Methods

2.1. Cell culture

The human colorectal cancer cell line CaCO2 (obtained from the ATCC) was maintained in DMEM high glucose medium supplemented with 10% Fetal Calf Serum, 1mM Sodium Pyruvate and 1% Non-Essential Amino Acids. The cells were grown at low density and sub-cultured regularly at 50 % density and maintained at 37^0C and 5 % CO_2 incubator. Cells were sub cultured with 0.25% trypsin/EDTA and counted using a haemo-cytometer.

2.2. Trans-well hanging inserts

Millicell trans-well hanging inserts with a semipermeable Bio pore membrane (PTFE, Cat. PICM01250) or polyethylene terephthalate (PET, Cat. PIHT12R48) were obtained from Millipore.

2.3. Short term CaCO2 monolayers with modified culture medium

CaCO2 cells were seeded in the transwell inserts at desired densities and incubated at 37^0C and 5 % CO_2. Post incubation for 48hrs, they were treated with differentiation medium with low serum. This differentiation medium contained DMEM high glucose medium supplemented with 0.2 % Fetal Calf Serum, 1 mM Sodium Pyruvate and 1% non-Essential Amino Acids.

2.4. Short term CaCO2 monolayers with Enterocyte Differentiation Medium

CaCO2 cells were seeded in the transwell inserts at desired densities and incubated at 37^0C and 5 % CO_2. The cells were incubated for 3 days. On day 4 the growth medium was changed to enterocyte differentiation medium from BD Biosciences (CAT. 62405-446). This medium was changed to normal growth medium on day 6 of plating. The formation of monolayers was monitored through measurement of the Trans Epithelial Electrical Resistance (TEER).

2.5. Long term CaCO2 monolayers

Caco2 cells were seeded in transwell hanging inserts at the density of $3x10^5$ cells/cm^2 ($1x10^5$ cells/well), and incubated in DMEM growth medium supplemented with 10 % FBS, 1% NEAA and 1mM Sodium Pyruvate. The formation of the monolayer was monitored by measuring the TEER over a 21 day period. The growth medium was changed every alternate day.

2.6. Measurement of the Trans Epithelial Electrical Resistance (TEER)

The TEER of Caco2 monolayers was monitored using a Millicell ERS-2 Volt-Ohm Meter connected to chopstick electrodes. Prior to each measurement, the electrodes were dipped in 70% EtOH/H_2O and wiped. The TEER reading of an insert without cells was obtained as the blank reading and subtracted from the TEER reading of inserts with Caco2 cells. The growth medium was changed following TEER measurements.

2.7. Stimulation of CaCO2 monolayers with *E. coli* and *Salmonella typhimurium* LPS

Frozen stocks of E. Coli LPS O055:B5 (Sigma - Aldrich) and Salmonella Typhimurium were used for experiments. The concentrated stocks (1mg/ml) were diluted in DMEM growth medium to get the desired concentrations (1ng/ml, 10ng/ml, 100ng/ml, 1µg/ml and 10µg/ml). The differentiated CaCO2 monolayers were washed once carefully and gently with 1x PBS and growth medium containing growth medium was added to the apical and basal chambers of the transwell inserts through the sides so as to not disturb the monolayer.

2.8. Stimulation of short term and long term CaCO2 monolayers with cytokines

Frozen stocks of TNF-α (human recombinant, 50 µg/ml) and IL1-β (human recombinant 10 µg/ml) were used for this experiment. Concentrated stocks were thawed and diluted in growth medium to get the desired concentrations (10ng/ml, 25ng/ml and 50ng/ml). Prior to experiments, the TEER of differentiated short term CaCO2 monolayers was

measured and were washed 1x with PBS. The growth medium containing the cytokines was added to apical and basolateral chamber of the transwell inserts.

2.9. Immuno-staining of Claudin1

Caco2 monolayers were grown to confluency in CaCO2 in the depression of the Mattek slides. Confluent cells were treated with the enterocyte differentiation medium for 48 hours, following which they were treated with LPS and Cytokines for 48 hours. After 48 hours the supernatant was removed and the cells were washed with ice cold 1x PBS. Cells were fixed with ice cold methanol for 20 min at -20^{o}C. Cells were permeabilized with PBS containing 0.2% triton X. Primary antibody staining was carried out with Claudin1 antibody and secondary anti-rabbit antibody tagged with alexa flour 488. Images were captured using 100x oil immersion of Olympus BX40 epifluorescence microscope equipped with ImagePro Express 6.3.

2.10. CaCO2-PBMC co-culture model

2.10.1 Isolation of Peripheral Blood Mononucleated cells (PBMCs)

Blood samples from healthy blood donors were collected in vacutainers containing heparin as an anticoagulant. Fresh blood samples were diluted 1:1 with culture medium. 5 ml of diluted blood was overlaid on 4 ml of Histopaque 1077 in 15 ml centrifuge tubes and centrifuged at 800g for 20 minutes. Following centrifugation, the white viscous layer at the interface containing PBMCs was pipetted out and washed 3x with 1x PBS. Following PBS washes, the PBMCs were suspended in DMEM and counted using a haemocytometer under an inverted light microscope.

2.10.2 Co-culture of short term differentiated CaCO2 monolayers with PBMCs

PBMC isolated from blood were cultured in the basolateral chamber of the transwell inserts containing differentiated CaCO2 monolayers. The apical and the basal chamber of the transwell inserts contained DMEM growth medium. The following figure shows a schematic of the CaCO2 – PBMC co-culture model.

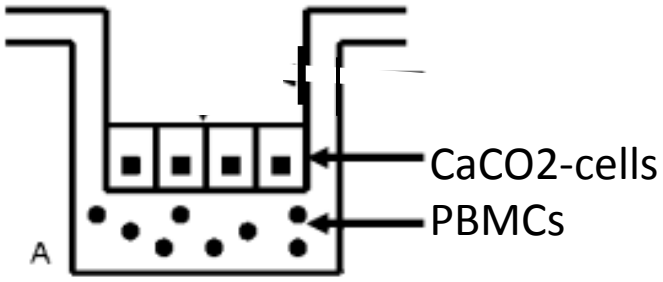


Figure 4: The CaCO2 – PBMC co-culture model

2.11. Formation of CaCO2 monolayers in Mattek® slides.

The short term CaCO2 monolayers were grown on mattek culture plates containing a coverslip covered depression at the bottom. Hundred micro-liters of media containing 1.5 x 10^6 cells/ml cells were seeded in this depression. The media was changed with 2 ml DMEM after sufficient cells were attached to the matrix. The cells were allowed to grow into a confluent monolayer. Post confluency the monolayers were differentiated by incubating with Enterocyte Differentiation Medium for 48 hours.

2.12. Effect of probiotic culture supernatant on compromised barrier

The *in vitro* 21-day CaCO2 monolayers compromised by inflammatory treatment were investigated for restorative effects of probiotics by treating with their culture supernatant. The probiotic conditioned media was diluted (1:1) in DMEM growth medium and added to apical and basal chambers of compromised monolayers.

2.13. Growth curve of bacteria

2.13.1. Bacteria grown in Brain Heart Infusion Media

The bacteria cultures (*Lactobacillus* delbruki, *Bacillus* coagulans, *Bifidobacterium* lactis Bb12, *Lactobacillus* rhamnosus GG, *Lactobacillus* casei shirota, were gown in Brain Heart Infusion Medium (Neogen, 7116). Actively growing bacteria were diluted into BHI medium to get an optical density reading of 0.1 at 600 nm in an iEMS 96 well plate

reader. Following this, the bacterial growth was monitored over a period of 48 hours, to plot the growth curve. Bulk cultures were grown at 37˚C in 5% CO_2 environment. The cultures were then harvested at the mid log phase and centrifuged to collect the supernatant. Filter purified supernatant was collected in aliquots and stored at -20˚C.

2.13.2. Bacteria in Slow digesting Starch(SDS)

Slow digesting starch containing 25% and 75% amylose was suspended in water at 10mg/ml and 20 mg/ml concentrations and autoclaved to get a gelly and viscous liquid media. The undisolved particulate matter was dissolved by stirring for 20 min at room temperature. Bacteria growing actively in BHI medium were diluted 1:10 in 200 µl of SDS medium in a 96 well plate. These plates were sealed with a transparent plastic film and incubated at 37˚C in the iEMS spectrophotometer for 48 hours. The optical density of growing cultures was measured at 600 nm after every hour and the growth curve was plotted to estimate the mid log phase.

Bulk cultures were grown and harvested in the mid log phase to extract the supernatant. The cultures were centrifuged at 4000 rpm in a temperature controlled centrifuge. The supernatant was harvested and stored in aliquots at -20˚C.

2.14. Alpha-Glucosidase activity assay.

2.14.1. Enzyme activity assay

A-Glucosidase inhibitory activities were evaluated according to the method described by Cheng et al. 2005 with some modifications. The enzyme solution contained 10µl α-glucosidase (0.066 g/ml) in 0.1 M phosphate buffer (pH 6.9). 100 µl of 8mM p-Nitro phenyl-α-D-glucopyranoside in the same buffer (pH 6.9) was used as a substrate solution. Ninety microliters of test samples was added to these wells. Ten micro-liters of enzyme solution were added and incubated at 37˚C for 30 min. The reaction kinetics was monitored by measuring the absorbance with an iEMS microplate reader 405 nm, while the reaction system without test samples was used as a control. The system

without α-Glucosidase was used as a blank and 90 µM Miglitol solution was used as a positive control. Each experiment was conducted in triplicates.

2.14.2. Calculating the enzyme activity

The remaining activity of the samples was calculated as follows:

$$Remaining\ Activity(\%) = \frac{[SlopeE{+}I{-}SlopeI]}{[SlopeE{-}SlopeBlank]} * 100$$

Where,

- Slope E+I is the slope of the mixture comprised of the inhibitor, enzyme and substrate
- Slope I is the slope of the mixture without the enzyme, which is used to control the background

Slope E is the slope of the mixture without the inhibitor, which is considered as the maximal activity under these conditions.

Chapter 3: Results

3.1 Establishing a short term *in vitro* intestinal epithelial barrier using CaCO2 cells

3.1.1 Selection of Trans well Insert material

To mimic the intestinal epithelial barrier *in vitro*, it was desirable to have cells growing on a membrane and differentiate to form apical and basolateral sides and these sides are accessible to the investigator. Two types of materials for Trans well hanging insert were chosen for formation of differentiated CaCO2 monolayers. Trans well inserts with PTFE membrane is usable for live cell imaging and immuno fluorescence applications, and PET membrane is optimal for growth of attachment dependent cells. Varying densities of cells were seeded in the PTFE and PET membrane transwells and incubated in growth medium supplemented with FCS. A visual inspection of the monolayers 24 hours post seeding revealed lack of adhesion of CaCO2 cells on PTFE membrane. The cells were all clustered in bunches and were not spread to form a uniform layer. The cell-cell and cell-matrix adhesion required for formation of differentiated CaCO2 monolayers was absent. The cells in transwells with PET membrane were adherent. The CaCO2 monolayers grow and differentiate on PET membrane and not on PTFE membrane

3.1.2 Lower cell seeding densities produce compact monolayers

For an intact monolayer to form, the cells need to grow to an optimum density and then differentiate. Differentiation is enhanced post contact inhibition by stressors. To aid this, a confluent CaCO2 cell layer was incubated with Low serum `medium. This was tested by incubating varying cell densities ((0.5, 1, 2, 5) * 10^5) in low serum medium. The graph below reveals that low serum medium hastens the formation of the CaCO2 monolayers, and the optimum density of cells to form a monolayer is $1*10^5$ cells per insert with a surface area of 0.33 cm^2. It was also observed that the formation of monolayer is triggered with the right cell densities. Lower than optimum cell density takes longer time to grow and the cells grow in clusters and higher cell density promotes the clustering of cells on plating itself.

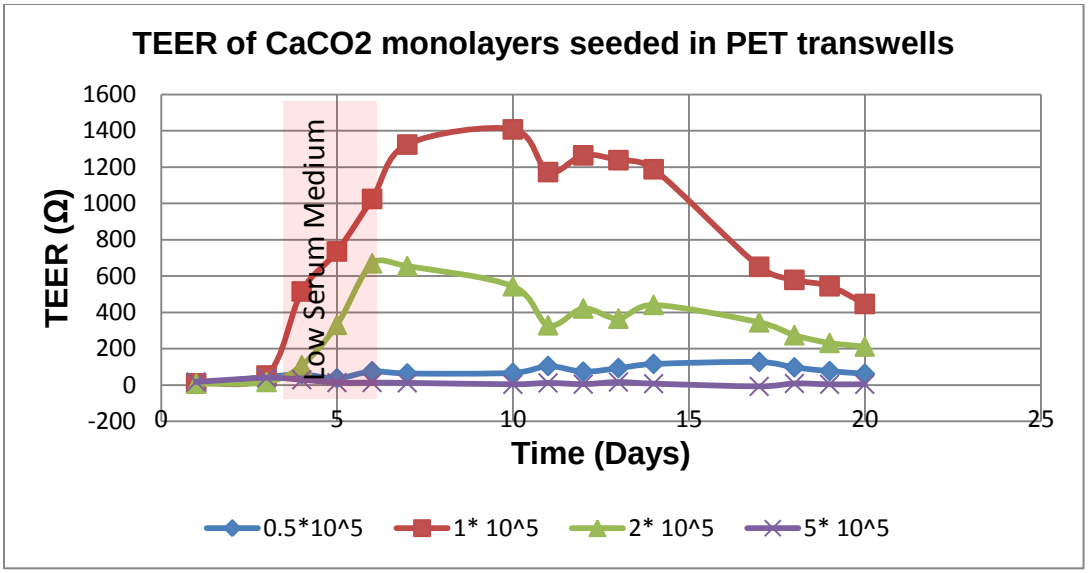


Figure 5: TEER of CaCO2 cells in PET inserts treated with Low Serum medium. CaCO2 cells were treated with Low Serum Medium three days post seeding in the Trans wells.

3.1.3 Enterocyte differentiation Media hastens the formation of CaCO2 monolayers

Enterocyte differentiation medium (EDM) is commercially available which claims better and faster differentiation. This medium is supplemented with butyrate. Cell densities $1*10^5$ and $2*10^5$ were seeded in transwell hanging inserts and EDM was added 24 hours post seeding. Treating with the differentiation medium demonstrated that lower seeding densities formed more compact monolayers.

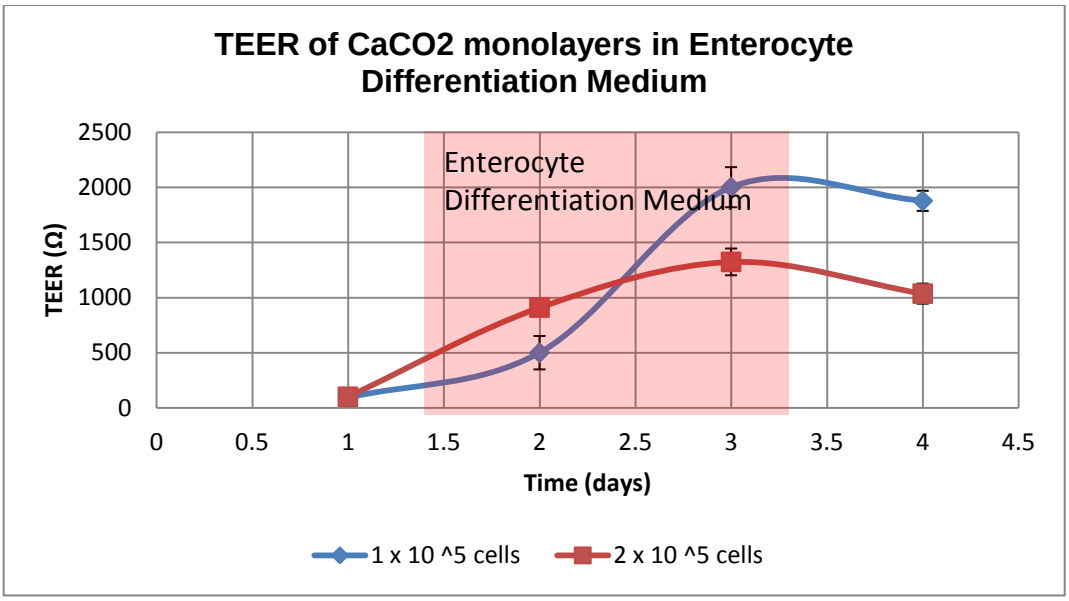
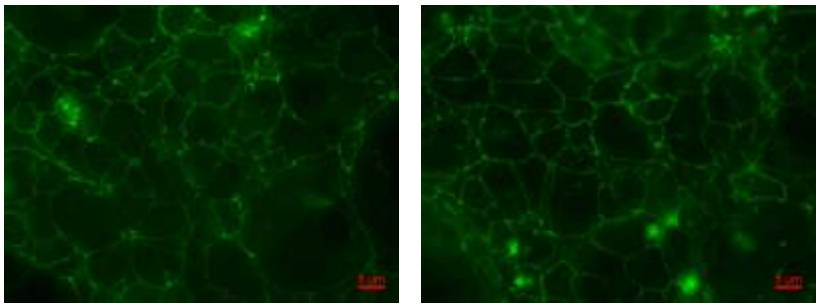


Figure 6: TEER of CaCO2 cells treated with Enterocyte Differentiation Medium. The TEER is multiplied with the area of the transwell inserts (0.33 cm^2). Values shown are mean ± SD, n=4

3.1.4 Claudin1 localizes in the Intestinal Tight Junctions

Claudin1 is an integral component of the intestinal Tight Junctions. The short term CaCO2 monolayers developed on mattek slides were probed for Claudin1 on the cell membrane.



Claudin1 Claudin1

Figure 7: Claudi1 protein localization in intestinal tight junctions. Scale bar = 5 µm

The 5-day protocol employed in the lab resulted in the formation of CaCO2 monolayers with assembly of tight junction components such as Claudin1 in the intercellular space.

3.1.5 EDTA mediated calcium chelation increases the CaCO2 monolayer permeability.

The Semi permeable nature of the CaCO2 monolayer is attributed to the tight Junction complexes in the intercellular space. Here, the tight junction proteins claudins and Occludins interact in Calcium dependent homophilic and heterophilic manner to form a semi permeable barrier. The assembly of tight junction requires calcium ions and a calcium chelator disassembles the cell to cell contact. Hence to validate the formation of TJ, the monolayers were treated with Calcium chelating agent EDTA. A follow up measurement of TEER of these monolayers showed a time and EDTA dose dependent decrease in the TEER. This observation validates that the semi permeable nature of short term layers formed in the inserts is attributed to tight junction complexes formed in the intercellular space.

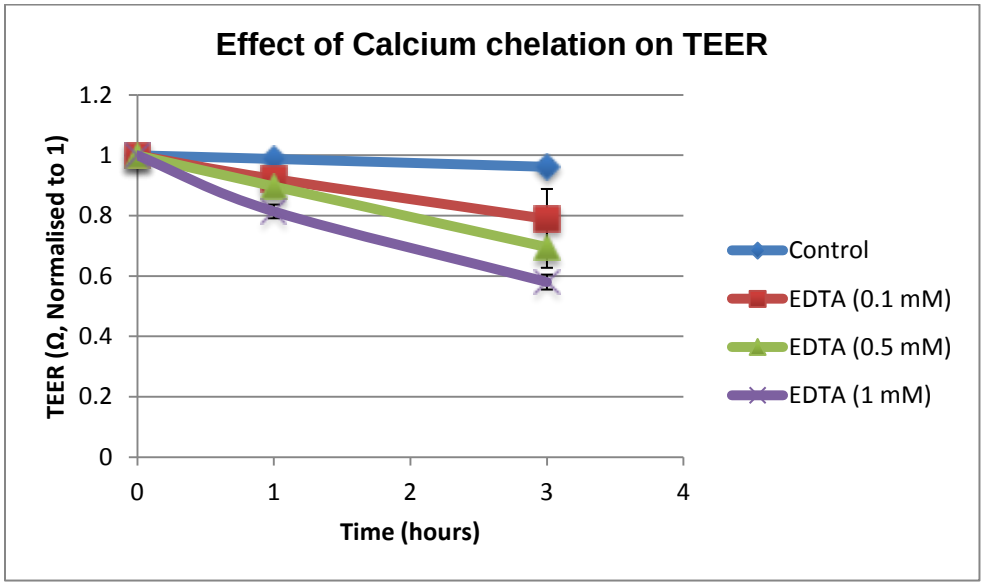
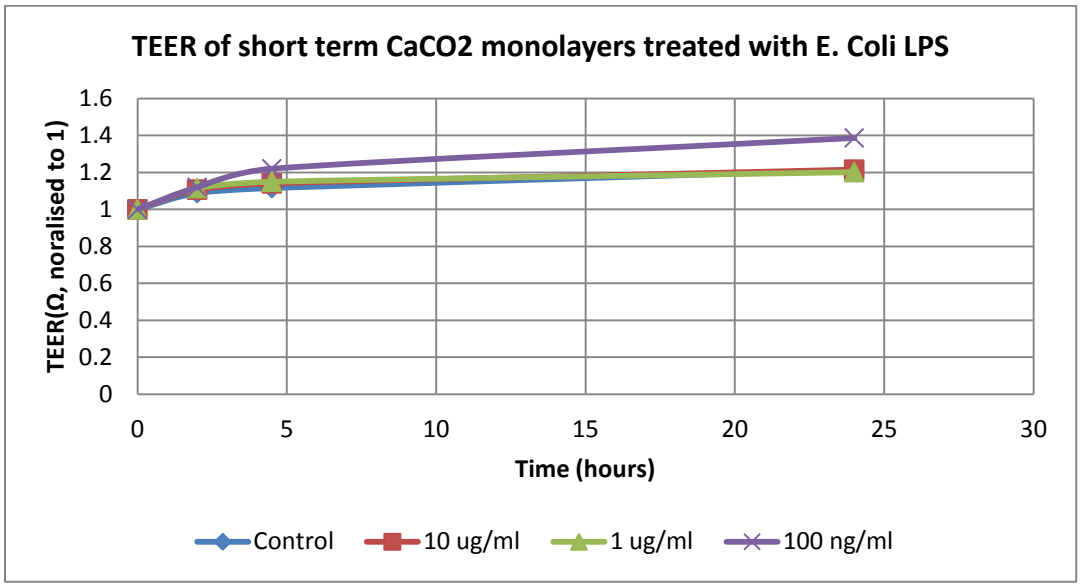


Figure 8: Effect of calcium chelation on TEER. Validation of the Short term CaCO2 monolayers. The short term CaCO2 monolayers formed in the Trans well inserts were treated with calcium Chelating agent EDTA. This showed a time and dose dependent decrease in the TEER of the short term CaCO2 monolayers. Data has been shown as mean ± SD of three replicates.

3.2 Evaluation of effect of endotoxins and cytokines on the short term CaCO2 monolayers.

3.2.1 Short term CaCO2 monolayers are not responsive to stimulation by LPS

a) *E Coli* LPS



b) *Salmonella* LPS

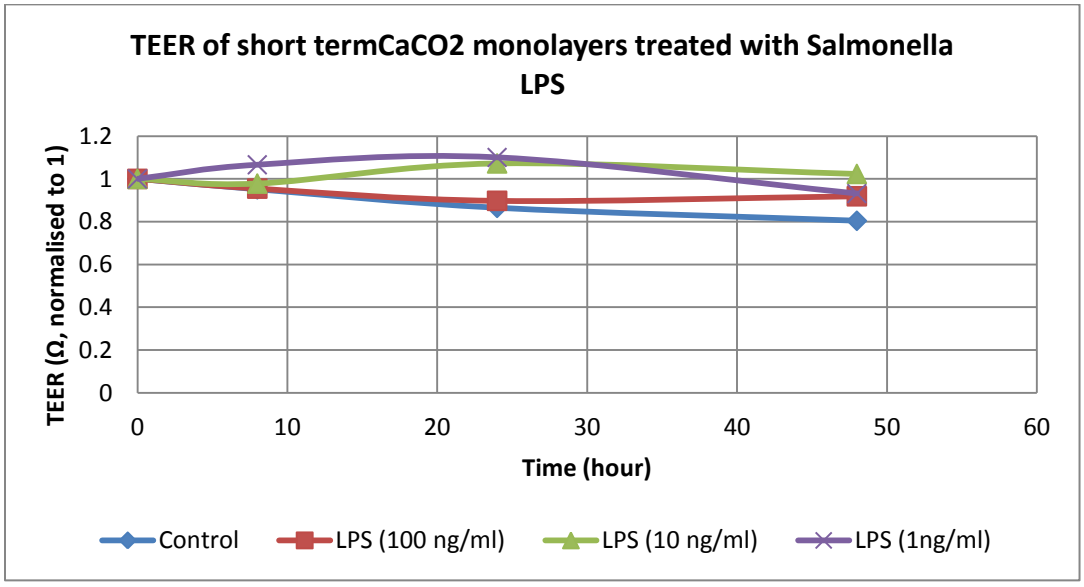


Figure 9: TEER of short term CaCO2 monolayers treated with a) E Coli LPS and b) *Salmonella Typhimurium* LPS. The TEER of each set was normalized to its initial value.

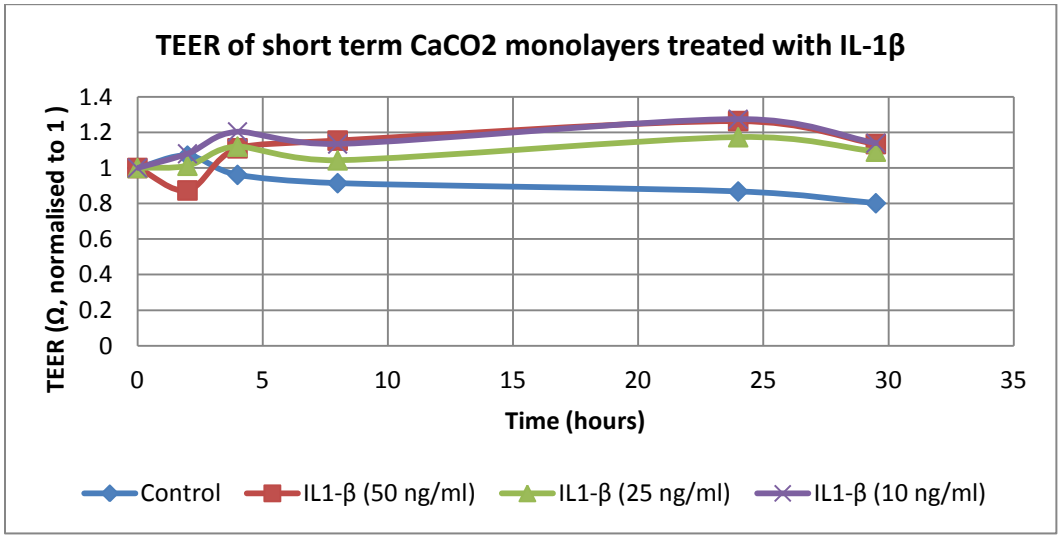
Short term CaCO2 monolayers differentiated using Enterocyte Differentiation medium were treated with varying concentrations of *E Coli* and *Salmonella* LPS. Following the treatment the TEER as a function of barrier function was measured over a 24 hour period. Any inflammation caused by LPS should show a decrease in the TEER.

There was no decrease in the TEER of short term monolayers treated with LPS either from *E coli* or from *Salmonella*. This raises the question whether short term CaCO2 model is suitable for studying inflammation caused by gram negative bacteria derived endotoxins such as LPS.

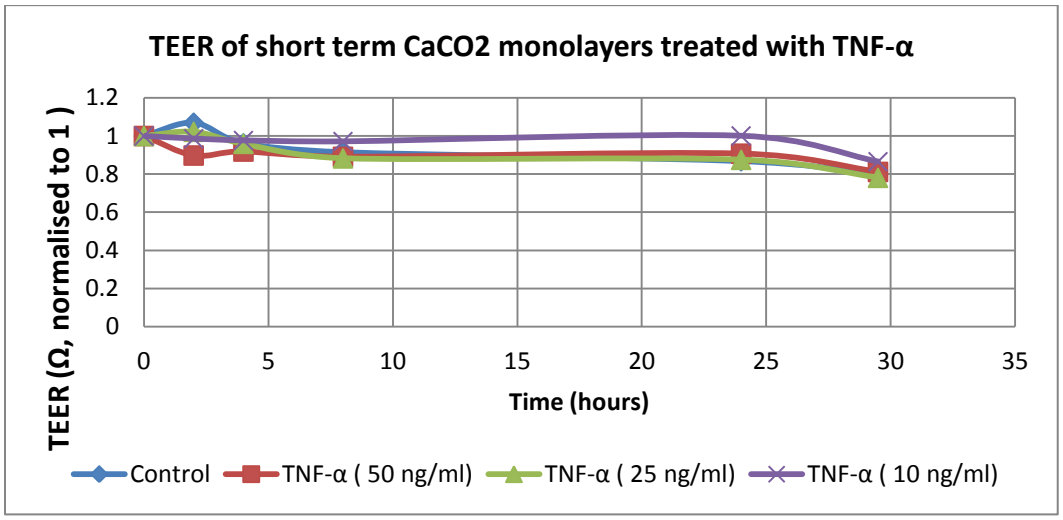
3.2.2 Inflammatory cytokines TNF-α and IL-1β don't affect the intactness of short term CaCO2 monolayers

One of the reasons that could be responsible for no effect observed with the LPS could be because of diminished response towards LPS that is not enough to disassemble tight junctions by the epithelial cells. In vivo, the intestinal epithelium is also interspersed with immune cells and the immune cells bring about greater responses and that is likely to bring about changes in assembly of tight junctions. To replicate that in an *in vitro* scenario, the short term CaCO2 monolayers were exposed to pro inflammatory cytokines TNF-α, IL1-β and co incubated with LPS and IL1-β.

a) IL1-β



b) TNF-α



c) LPS + IL1-β

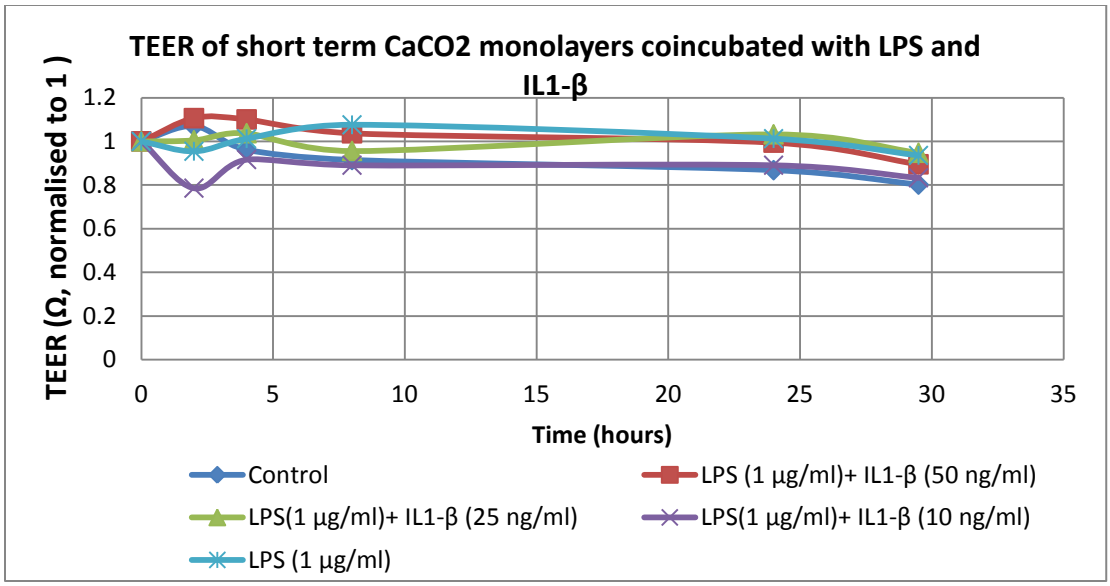


Figure 10: Effect of inflammatory cytokines a) IL1-β b) TNF-α and c) LPS + IL1-β on the short term CaCO2 monolayers

Exposure to pro inflammatory cytokines or a combination of cytokine with LPS as reported earlier (Wang et al., 2005) did not cause any change in the barrier function measured by TEER. This led to the thought process that is it just a single cytokine or a combination of cytokines that is responsible for the decrease of barrier function.

3.3 The short term CaCO2 – PBMC co-culture model to study inflammation

A co culture model was developed to mimic the systemic inflammation caused by activation of the intestinal epithelial lymphocytes by pathogenic bacteria. Here Peripheral Blood Monocytes were cultured in the basal chamber of short term CaCO2 monolayers and treated with LPS.

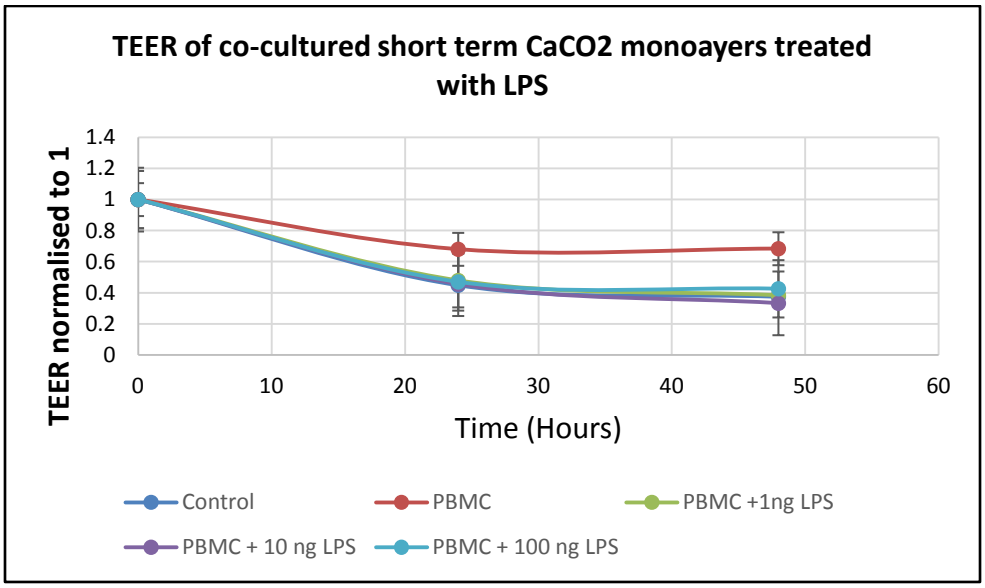


Figure 11: TEER of CaCO2 monolayers co cultured with isolated PBMCs and treated with E Coli LPS.

The idea behind this approach was that the inflammation caused by activated PBMCs would cause a decrease in the TEER of short term CaCO2 monolayers. However it was observed that the TEER of untreated control Trans wells, decreased too.

Exposure of the intestine epithelium to pro inflammatory cytokines causes an increased permeability, measured by TEER and Mannitol permeability. Incubation of CaCO2 monolayers with inflammatory cytokines compromises the semi permeable barrier of 21 day CaCO2 monolayers (Capaldo & Nusrat, 2010b). However, incubation with pro inflammatory cytokines did not induce any compromise in the barrier function of short term CaCO2 monolayers. Though the short term cultures have been reported for transfer studies, its suitability to use as an *in vitro* model to study inflammation in the intestine is uncertain.

3.4 Establishing the long term (21 days) CaCO2 monolayers

3.4.1 The TEER of long term (21-day) CaCO2 monolayers is stable for 21 days

Given that the short term CaCO2 monolayers were unstable in a co-culture model and unresponsive to the stimulation by LPS and inflammatory cytokines TNF-α and IL1-β, a long term CaCO2 culture was envisaged.

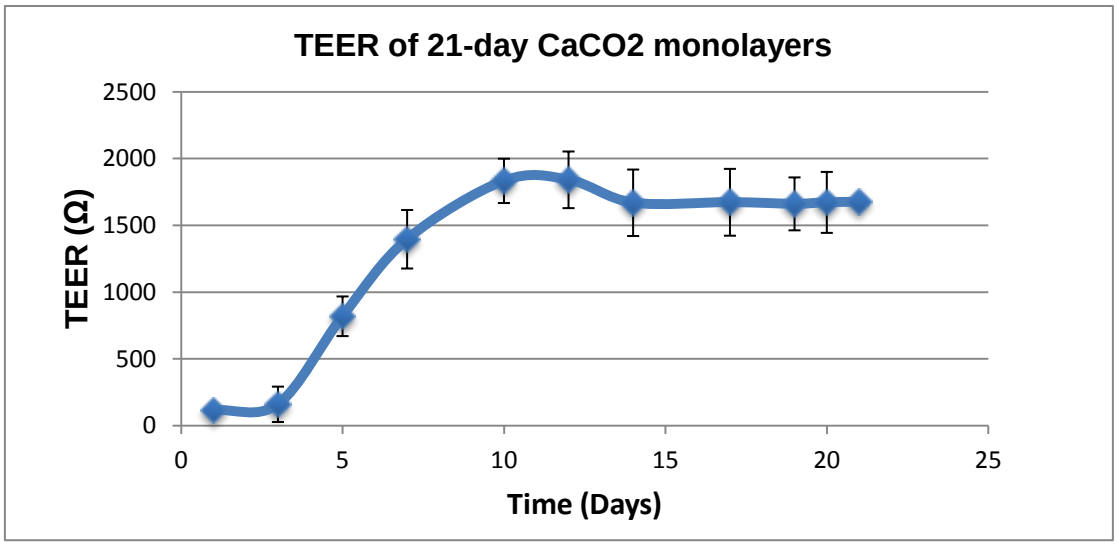


Figure 12: TEER of Long term CaCO2 monolayers cultured in DMEM growth medium for 21 days. Data has been reported as mean ± SD of n=4.

The TEER of long term CaCO2 monolayers increased over a period of 14 days and stabilized around 1600 Ω. Cell density of $1*10^5$ cells was used per transwell insert, as in published literature. It was observed that the peak TEER of stabilized monolayers in long term culture is lower than that of the TEER observed in the short term CaCO2 monolayers differentiated by using Enterocyte Differentiation Medium. However, the long term monolayers were stable for a longer duration, which was not the case in short term CaCO2 monolayers.

3.4.2 Inflammatory stimuli from LPS activated PBMC affect the integrity of the long term CaCO2 monolayers.

CaCo2 monolayers have been used to study the effects of inflammation in the intestinal epithelium, and inflammatory cytokines TNF-α and IL1-β affect the integrity of the barrier in a non-apoptotic manner. The long term CaCO2 monolayers developed in the

lab were treated with culture supernatant of PBMCs (isolated form healthy volunteers) treated with 10ng/ml LPS, culture supernatant of untreated PBMCs and the inflammatory cytokine TNF-α. The graph below depicts their effect on integrity of the long term CaCO2 monolayers.

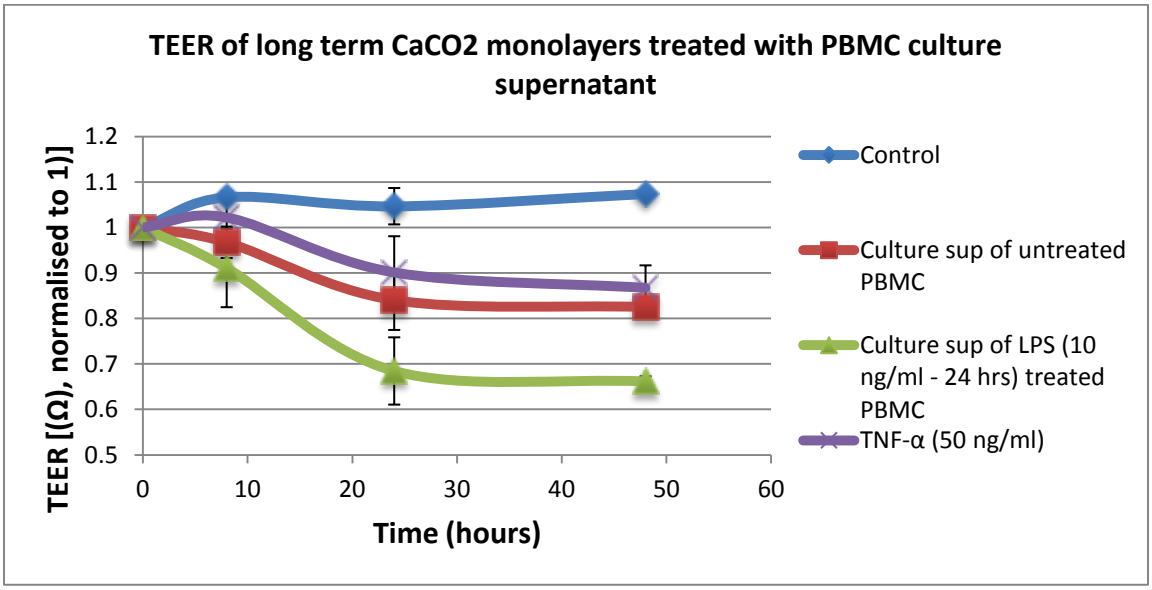


Figure 13: Effect of Inflammatory stimuli on 21 day CaCO2 cultures. 21 day CaCO2 monolayers treated with PBMC culture supernatant (diluted 1:1 in growth media) and TNF-α. Data has been reported as mean ± SD of three replicates.

The TEER of the long term CaCO2 monolayers decreased after treating with culture supernatant of PBMCs treated with LPS. Moreover TEER of CaCO2 monolayers treated with culture supernatant of untreated PBMCs decreased too. This was not surprising as there would be a basal level of cytokine secretion that will invariably affect the barrier function. TEER of monolayer treated with TNF-α decreased though this decrease was very small, it was significant ($p<0.05$).

3.5 *Bifidobacterium lactis* Probiotic conditioned media alleviates PBMC induced TEER compromise in long term CaCO2 monolayers.

Long term (21days) CaCO2 monolayers were treated with *E coli* LPS treated PBMC (24hrs) culture supernatant. The effect of inflammatory mediators on the barrier formed in the CaCO2 monolayer was followed by measuring the TEER. Supernatant of both

untreated PBMC and PBMC treated with LPS caused a decrease in the TEER of long term CaCO2 monolayers after 48 hours of incubation. The relative TEER decrease in monolayers PBMC-LPS culture supernatant was higher than those treated with PBMC only culture supernatant.

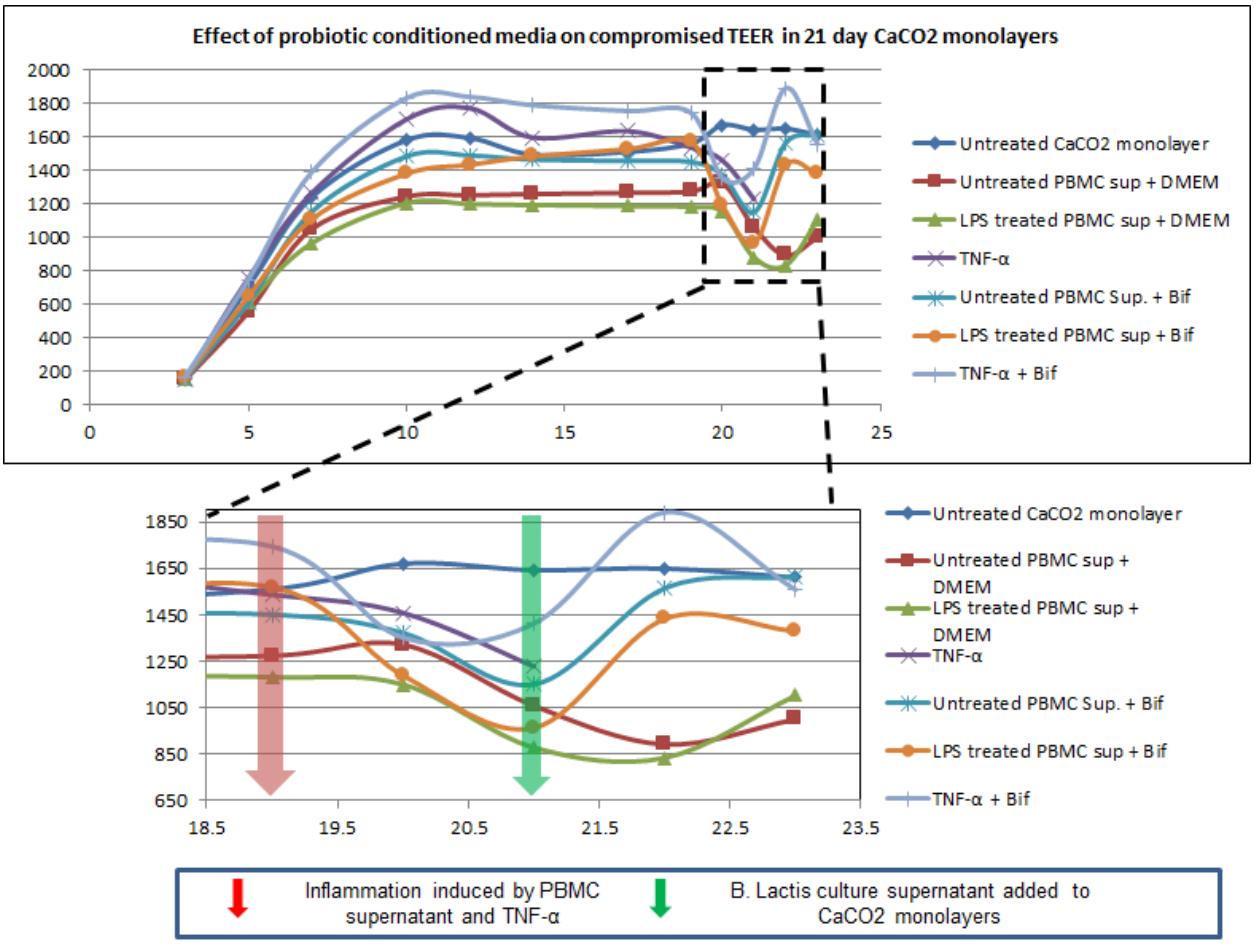
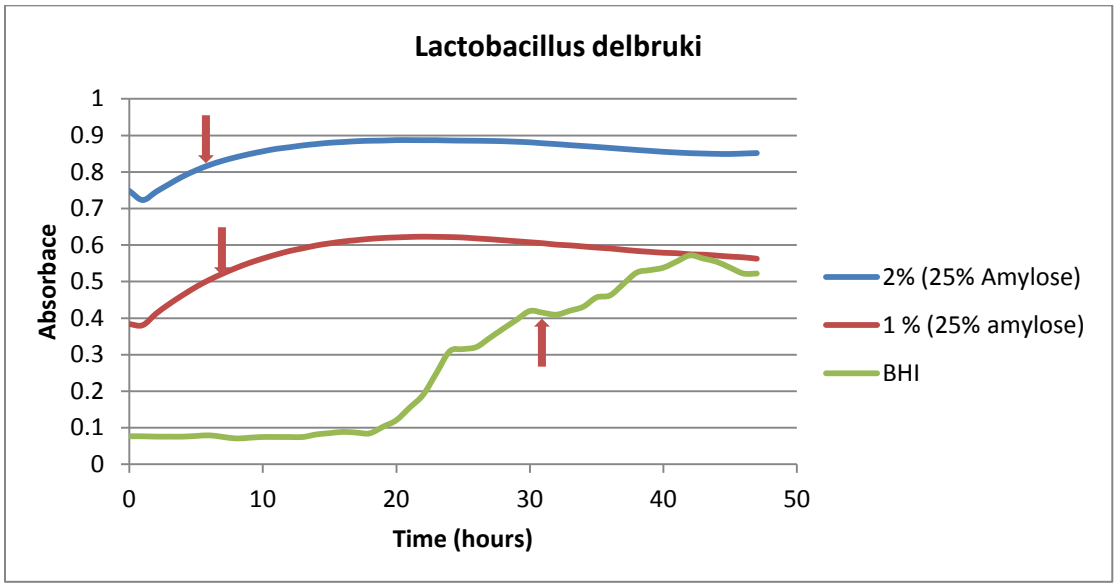


Figure 14: Effect of *B. lactis* supernatant on 21 day CaCO2 monolayers treated with supernatant of LPS treated PBMC.

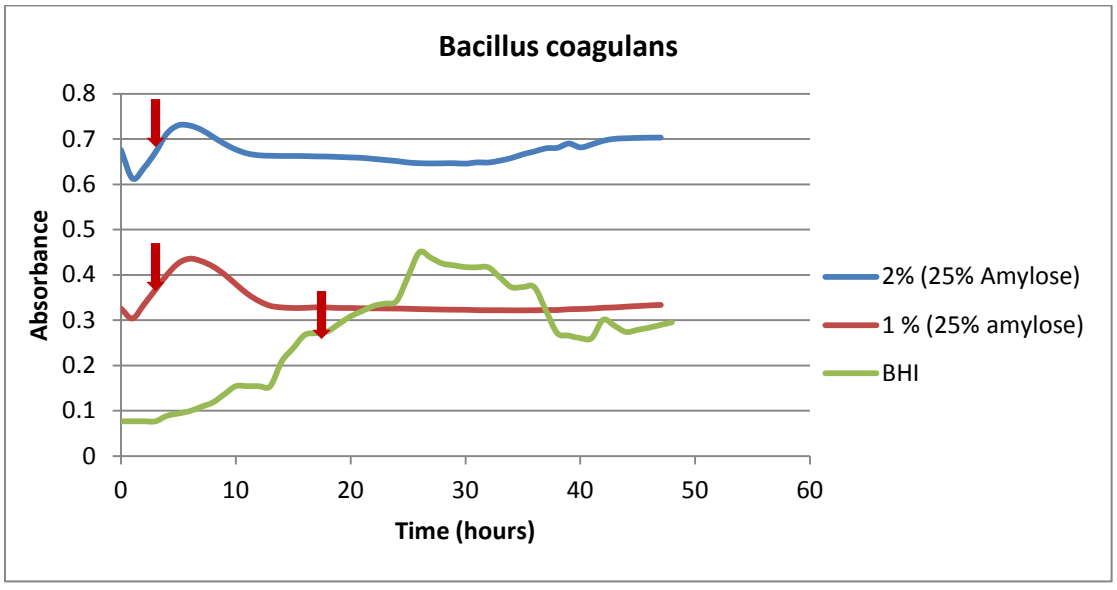
The monolayers compromised by PBMC supernatant were treated with culture supernatant from *B. lactis, L. casei* and normal growth medium (supernatant diluted 1:1 in DMEM growth medium). This showed that the compromised barrier function was being restored as understood by TEER measures (36 % increase in TEER). The TEER of monolayers treated with DMEM post PBMC supernatant treatment failed to increase.

3.6 Growth of Probiotics in nutrient enriched and resistant starch culture Medium

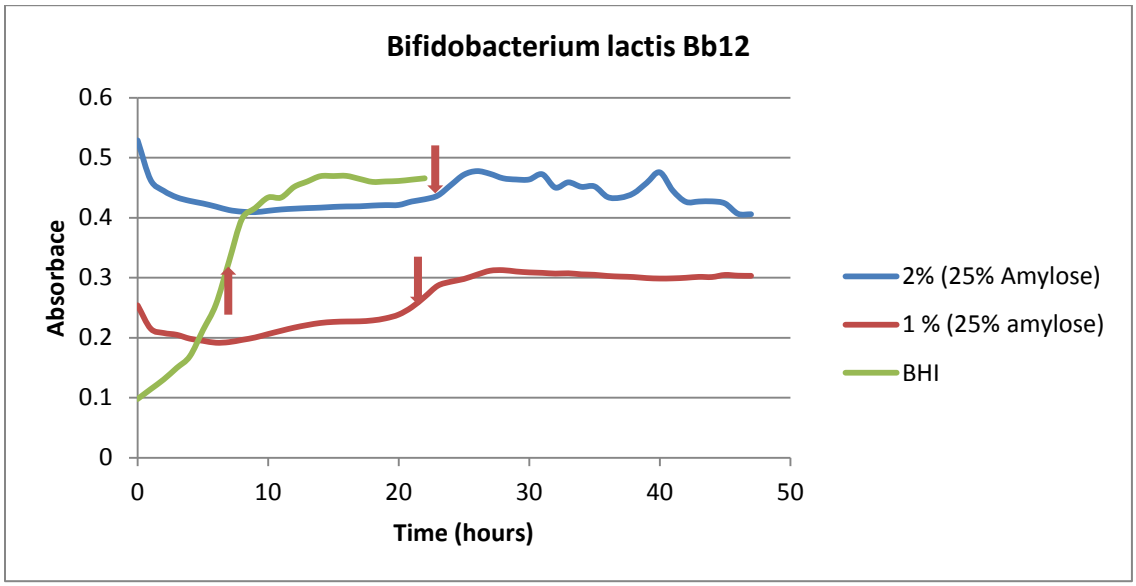
a) Lactobacillus delbruki



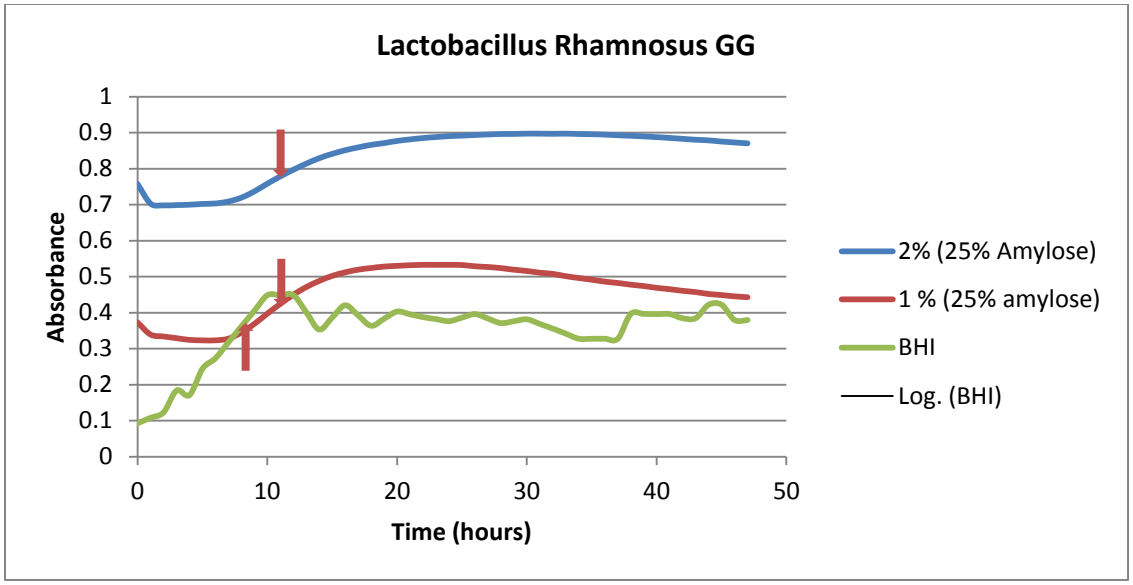
b) Bacillus coagulans



c) *Bifidobacterium lactis Bb12*



d) *Lactobacillus rhamnosus GG*



e) Lactobacillus casei shirota

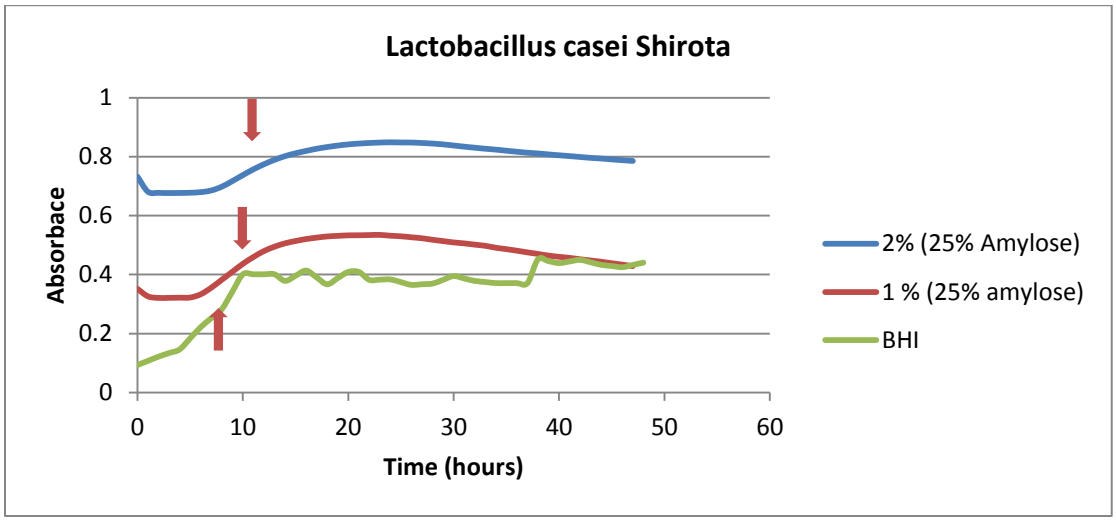


Figure 15: Growth curves of probiotic bacteria a) *Lactobacillus delbruki* b) *Bacillus coagulans* c) *Bifidobacterium lactis Bb12* d) *Lactobacillus rhamnosus GG* and e) *Lactobacillus casei shirota*, grown in different growth media i) Brain Heart Infusion (BHI) Broth ii) Slow digesting starch 25% amylose in 0.01 g/ml and 0.02 g/ml concentrations.

Time taken to achieve mid log phase			
	Brain Heart Infusion **BHI**	25% amylose 2%	25% amylose 1%
B. coagulans	17	6	6
L. delbruki	30	12	12
B. lactis Bb12	9	22	22
L. rhamnosus GG	9	15	15
L. casei shirota	10	10	10

Table 2: Time taken to achieve the mid log phase of the growth curve for the probiotic bacteria

Absorbance of probiotic cultures: The growth pattern of the bacteria was monitored by measuring the absorbance at 600 nm. The absorbance at the starting point of bacteria grown in BHI remained largely invariant at 0.1. However, it was also observed

that the initial absorbance of bacterial incubations varied depending upon the strain, though the same dilutions were used in each of the probiotics chosen. The resistant starch containing growth medium showed higher absorbance as compared to the nutrient enriched BHI medium. This absorbance of resistance starch varied depending on the concentration of amylose.

Growth pattern of probiotics: The pattern of growth curve varied for each of the probiotics. Growth curve of probiotics *L. casei, L Delbruki, B. lactis* and *L. Rhamnosus* followed a sigmoidal pattern, showing a lag phase followed by log phase and growth saturation. The pattern remained independent of the culture media conditions. However, the time taken to reach each phase varied across strains and culture conditions. *B. coagulans*, however did not follow this pattern. This bacteria started to grow immediately following incubation and there was little to no lag phase. Activiely growing cultures did not follow the sigmoidal growth pattern, rather the absorbance peaked after 8 hours in SDS and 30 hours in BHI. This was followed by a decrease in the absorbance of the cultures, which remains an unexplained observation.

Mid log phase: The growth profile of the probiotic bacteria varied depending upon the nutrients in the growth media. The time taken to attain the mid log phase in the two types of culture media varied for each of the probiotics. Probiotics *B. coagulans, L. delbruki,* attained the mid log phase earlier in 25% amylose (6 and 12 hours resp.) as compared to the nutrient enriched BHI media (17 and 30 hours resp.). While *L. rhamnosus* grew slower in resistant starch medium (15 hours) as compared to in enriched BHI media (9 hours). Growth of *L. casei* remained unaffected by the growth medium conditions (10 hours). The probiotic *Bifidobacterium* attained mid log phase in 8 hours in the nutrient enriched BHI medium, however this was delayed (22 hours) in growth medium containing resistant starch.

3.6.1 Probiotics conditioned media doesn't affect the activity of intestinal α-Glucosidase

The effect of probiotic culture supernatant on rat intestinal α-Glucosidase was analysed by an *in vitro* activity assay. The following graph shows the effect of probiotic culture supernatants on its activity.

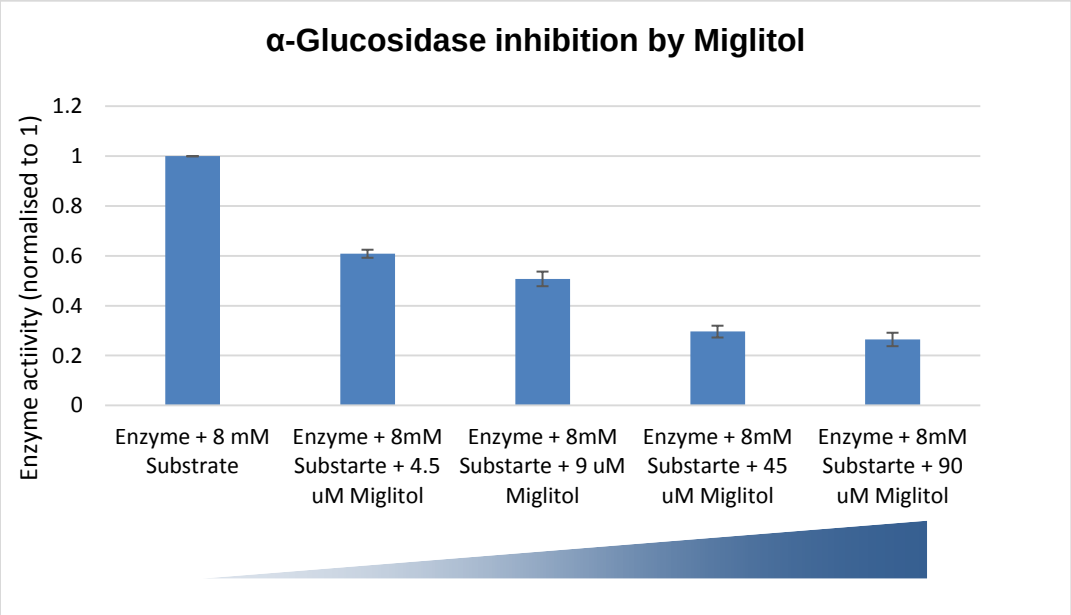


Figure 16: Effect of inhibitor Miglitol on the activity of rat-intestinal α-glucosidase. Varying concentration of Miglitol (4.5 µM, 9µM, 45µM, and 90µM) were assayed for its effect on α-glucosidase activity. The data is shown as mean ± SD of three replicates

The *in vitro* assay for inhibition of α-Glucosidase with its inhibitor Miglitol showed a dose dependent inhibition of its activity. The *in vitro* assay to study effect of probiotic metabolites on the activity of rat intestinal α-Glucosidase activity showed that the culture supernatant of the chosen probiotics did not have any inhibitory effect on the activity of rat α-Glucosidase.

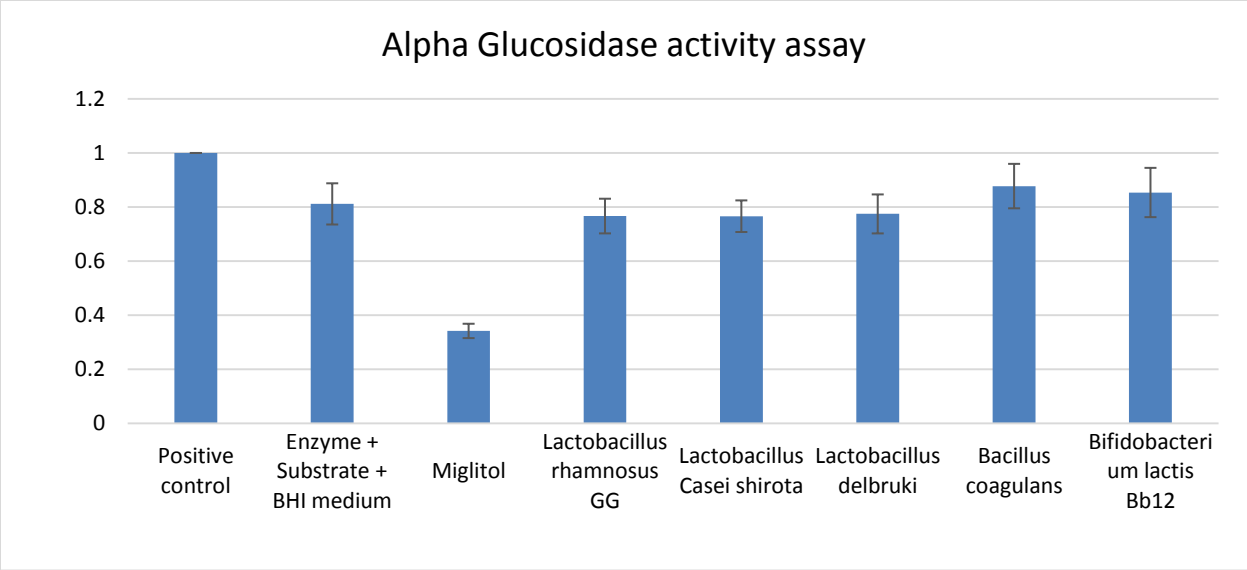


Figure 17: Effect of probiotics conditioned on α-Glucosidase activity. Culture medium harvested in the mid-log phase of growth curve of probiotics was tested for their effect on the activity of rat intestinal α-Glucosidase. The data is shown as mean ± SD of three independent replicates

The drug Miglitol was chosen as a control for the inhibition assay. At 90 µM concentration, it showed 70% inhibition in the activity of α-Glucosidase. The enzyme incubated with BHI culture media had an activity of 80% of that of the control reaction. Compared to this culture supernatant of probiotic bacteria did not demonstrate any effect on the enzyme activity.

Discussions

Various types of experimental models are used to study the intestinal epithelial barrier function. Of these in vivo animal models, *ex vivo* animal tissue extracts and *in vitro* cell culture models are used. The experimental and ethical restrictions associated with using an animal model makes *in vitro* assay systems a lucrative alternative to study the barrier properties. To this effect established cell lines such as MDCK, HT 29 and CaCO2 have seen widespread increase in their use. Having permeability characteristics closest to the intestinal epithelium, colorectal cancer cell line CaCO2 and its modified cell lines have seen tremendous growth in their applications in the past 10 – 15 years.

The traditional CaCO2 cell culture systems include a 21 days differentiation period for formation of polarized monolayers on semipermeable membranes. Labor intensive process, un-physiological growth conditions, and lack of reproducibility limit its usability for high throughput uses. However, shorter three day and five day alternatives to this system are developed (Yamashita et al., 2002). The more convenient and productive accelerated five day culture systems have been reported to have the permeability characteristics comparable to the 21-day systems. It has been reported that the permeability characteristics of drugs in 5 day system have a correlation with 21 day systems and is proposed to be suitable for high throughput permeability characteristics investigation(Caldwell et. al., 2014). Using this 5 day CaCO2 culture system we tried to evaluate its suitability for studying inflammation induced barrier compromise.

The TEER of low serum medium based CaCO2 monolayers developed in the lab depended inversely on the initial seeding density of the CaCO2 cells in the transwell inserts. Highest TEER of 1400 Ω was observed at day 7 post differentiation with the seeding density of $1*10^5$ per transwell. Similarly, accelerated CaCO2 monolayers differentiated by using the Enterocyte Differentiation Medium showed a peak TEER of 1600 Ω on day 3 post seeding with the cell densities of $1x10^5$ per transwell. However this TEER was not stable after a day7, as expected in accelerated CaCO2 monolayers (Yamashita et al., 2002). These monolayers differentiated by the EDM were used as a model to study the compromised barrier function on treatment with endotoxins and cytokines.

To setup a model to study inflammatory mediators induced barrier dysfunction, short term CaCO2 monolayers were treated with endotoxins, inflammatory cytokines and LPS activated PBMCs. The endotoxin LPS from *E Coli* and *S. typhimurium* did not have any detrimental effect on the integrity (TEER) of short term monolayers. Similarly inflammatory cytokines TNF- and IL-1β showed no effect on the TEER. These observations with short term cultures were contradictory to previous studies with 21-day CaCO2 cultures where *S. typhimurium* LPS, cytokines TNF-α and IL-1β induced barrier dysfunction has been reported previously (Yeung et al., 2013)(Bruewer et al., 2003). Similarly co-incubation of cytokine IL-1β with endotoxin LPS did not have any effect on the TEER of short term CaCO2 monolayers, an observation disagreeing with earlier reports in 21-day cultures (Al-sadi, Ma, Al-sadi, & Ma, 2014).

Similarly, the CaCO2-PBMC co-culture model system used to study systemic inflammations was used to study the effect of inflammation on short term CaCO2 monolayers. Stimulation of co-culture systems with LPS resulted in a TEER decrease of about 60% in 24 hours. This observation was not unique with this treatment alone but similar responses were observed in co-culture systems not stimulated by LPS and untreated cells. So this drop in TEER can be attributed to the stability of the monolayer rather than the effect of cytokines. Co culture systems of 21-day CaCO2 monolayers have previously been used to study Immuno-stimulatory effects of cytokines and immuno-modulatory effects of probiotics and commensal bacteria (Pozo-Rubio et al., 2011). However, the short term CaCO2 monolayers developed in the lab were unsteady and their use for studying effect of inflammation is rather questionable.

To test the effect of the same stimuli on traditional CaCO2 monolayers a 21-day CaCO2 culture was developed. This model had peak TEER at day 12 and stabilized at 1600Ω after day 15. This model was used to study the effects of supernatant from LPS activated PBMC cultures. Culture supernatant of LPS activated PBMCs induced a decrease in TEER of 21-day CaCO2 monolayers by 35%. Furthermore the TEER of CaCO2 monolayers treated with culture supernatant of untreated PBMC reduced by 20%. The cytokine TNF-α (50 ng/ml) caused a small decrease in TEER (20%, $p<0.05$). These results indicated a suitability of the given model to study the effects of probiotics

on inflammation induced barrier dysfunctions. The 21-day monolayers compromised by LPS activated PBMC culture supernatant was treated with probiotics (*B. lactis Bb12* and *L. casei shirota*) conditioned media. Supernatant of *B. lactis Bb12* restored the monolayers in 24 hours post incubation. Furthermore the compromised CaCO2 monolayers could not be restored with either culture supernatant of *L. casei shirota* or DMEM growth medium. This suggested that *B. lactis* conditioned media has metabolites with potential to enhance the integrity of the barrier function. Furthermore it indicates that this effects is a species specific phenomena.

The intestinal α glucosidase is a target to control the glucose absorption in the intestine. Drugs such as Miglitol, Acarbose, Voglibose are therapeutic tools to manage the intestinal glucose digestion and absorption in diabetic individuals. To investigate any possible role of probiotic metabolites in regulating the α-Glucosidase activity, we employed an *in vitro* assay. This assay revealed that the conditioned media (BHI) of the given probiotics has doesn't have any factors (metabolites) which affect the activity of rat intestinal α-Glucosidase.

In conclusion, it can be said that the short term CaCO2 monolayers developed in the lab are unsuitable to study effects of inflammation mediators in the intestine. The short term protocols are employed for high throughput analysis and screening of intestinal absorption rates. These short term CaCO2 monolayers are however not responsive to inflammatory mediators such as endotoxins and inflammatory cytokines. However when used in the co culture system for studying systemic inflammations these monolayers are unstable. Analyses of the same inflammatory cytokines on 21-day CaCO2 monolayers indicate it to be a more viable alternative to study the effects intestinal inflammations and their modulation. The inflammation induced by LPS activated PBMC in 21-day CaCO2 monolayers was found to be restored by *B. lactis Bb12* conditioned media. Moreover this restoration was found to be in addition to the butyrate produced by probiotics (data not shown). For evaluations of ingredients that induce intestinal barrier defects due to inflammation and a possible restoration by ingredients we will have to resort to the 21day long CaCO2 monolayer model and not the short term model.

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I would also like to thank other lab members at Unilever R&D, who extended constant help and support in running experiments. Discussions and positive inputs from the lab members have been invaluable.

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