Evaluation of potential antioxidant probiotics in \textit{in vitro} models of the gut epithelium

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\textbf{Abstract}

Inflammatory bowel disease (IBD) is a heterogeneous group of inflammatory disorders of the gut, which includes Crohn’s disease (CD) and ulcerative colitis (UC). IBD is characterized by chronic intestinal inflammation and increased permeability of the gut epithelial barrier. Reactive oxygen species (ROS), generated by the immune system and epithelium as part of the inflammatory response, increase epithelial barrier permeability through lipid peroxidation, apoptosis, DNA damage, and tight junction damage. Dysbiosis of the gut microbiota plays a critical role in exacerbating inflammation; therefore, there is significant interest in the use of beneficial microbes (probiotics) in the treatment of IBD to restore a healthy microbial community. Many \textit{Lactobacillus} species can reduce oxygen in culture, and are potential candidates for probiotic-based interventions to counter oxidative stress.

Herein, primary epithelial cell monolayers derived from murine colonic crypts were grown in a culture system allowing for physiological polarization with apical and basolateral access. In a pilot experiment to model the intestinal barrier damage caused by ROS in IBD, monolayers were apically treated with hydrogen peroxide. \textit{Lactobacillus} strains exhibiting oxygen reduction capabilities were co-cultured with epithelial monolayers. Effects to barrier permeability were assessed by measuring changes in trans-epithelial electrical resistance (TEER) and expression patterns of the tight junction marker ZO-1. Monolayers of Caco-2 BbE cells (human epithelial colorectal adenocarcinoma), often used in \textit{in vitro} studies of the epithelial barrier, were similarly treated. Further, candidate strains were evaluated for potential antioxidant activity using RT-qPCR analysis of target genes in respiratory pathways.
Damage to tight junctions and the cytoskeleton of Caco-2 BbE cells were observed after hydrogen peroxide treatment, with corresponding reductions in TEER. A similar treatment resulted in broad cell death of primary colonic epithelial cells, demonstrating a response distinct from typically used cell culture methods. The primary epithelial monolayer system used here to model inflammation-induced barrier damage will allow for further in vitro study of epithelial permeability; furthermore, the identified strains may be promising candidates for probiotic therapies for IBD.
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CHAPTER 1: BACKGROUND

Inflammatory bowel disease (IBD) is a group of gastrointestinal illnesses involving chronic inflammation of the gut epithelium. IBD has been grouped into two main categories: Crohn’s disease (CD) and ulcerative colitis (UC). CD is typically characterized by chronic inflammation throughout the tissue of the bowel wall at any point in the GI tract, whereas UC occurs only in the colonic mucosa. IBD is relapsing, and environmental triggers can cause activation of the disease followed by periods of remission.\(^1\) IBD is also associated with colorectal carcinogenesis, likely resulting from chronic gut inflammation.

The etiology of IBD is multifactorial with diverse genetic and environmental contributors to its pathophysiology. Both CD and UC are associated with genes involved in the maintenance of the gut epithelial barrier.\(^2\) However, significant evidence exists suggesting environmental factors, such as diet and stress.\(^3\) Higher intestinal permeability (a key marker of IBD) is observed in spouses of CD patients.\(^4\) Additionally, concordance rates in identical twins are low for both CD and UC.\(^5\) It is likely that genetic factors create disease susceptibility, while environmental triggers drive dysfunctional immune responses to gut microbes resulting in a chronic inflammatory state.

THE MICROBIOME AND IBD PATHOGENESIS

It is unclear whether dysbiosis in the gut microbiome is a primary cause of IBD, or a secondary effect from an inflammatory response. Regardless, the gut microbiome of IBD patients has distinct differences from healthy individuals.\(^6\) Loss of bacterial diversity is seen overall,\(^7\) as well as a general decrease in bacterial load.\(^8\)
In mucosal biopsies from CD and UC patients, the overall abundance of *Firmicutes* and *Bacteroidetes* was reduced as assessed by 16S rRNA gene amplicon sequencing, and there was an increased relative abundance of *Enterobacteriaceae*. Other populations of *Proteobacteria* were increased as well as members of the family *Actinobacteria*. Further studies have established reduced abundance of *Firmicutes* as characteristic of IBD; however, abundance of *Bacteroidetes* is inconsistently shown to be reduced, and is even elevated in some studies.

The increases seen in *Proteobacteria*, a phylum containing many bacterial strains considered pathogenic, suggest that the gut community shifts towards microbes that promote inflammatory responses. Further, ileal biopsies of CD patients have shown an increase in pathogenic, adherent-invasive strains of *E. coli*. Other pathogens identified in driving inflammatory responses include *Helicobacter, Campylobacter*, and specific *Mycobacterium* subspecies. Further research has shown higher levels of pathogenic *Yersinia* and *Clostridium difficile* in CD patients.

The role of a pro-inflammatory gut microbial community is demonstrated in mice deficient in the transcription factor T-bet (crucial in inflammatory responses) and Rag2 (necessary for T and B cell generation) as well as in mice deficient in IL-10. For mice deficient in T-bet and Rag2, UC develops spontaneously. Transfer of their microbiota into wild-type mice results in intestinal inflammation, which histologically resembles a mild form of UC. Given that colitis in mice is dependent on the gut microbiota, studying the composition of gut microbial communities is likely critical to understanding IBD pathogenesis.
THE “VICIOUS CYCLE” OF IBD

Microbial dysbiosis is a key factor in perpetuating a “vicious cycle” of gut inflammation. Shifts towards pathogenic strains of microbes elicit inflammatory responses from the host, causing chronic inflammation. Intestinal epithelial cells express toll-like receptors (TLRs) on the basolateral surfaces (Figure 1). In particular, TLR2, TLR4, and TLR5 are responsible for recognizing microbial pattern recognition receptors (PRRs), causing immune responses via secretion of antimicrobial peptides or inflammatory cytokines. In the healthy gut, inflammatory pathways are suppressed to maintain tolerance towards resident gut microbes and a mucosal barrier, composed of both mucus and the epithelium, limits bacterial invasion of the epithelium.

In IBD, damage to the mucosal barrier increases epithelial permeability. This loss in barrier integrity leads to increased bacterial translocation through the lamina propria. TLRs located on the basolateral side of the epithelial barrier are then exposed to antigens such as bacterial flagellin or LPS, further amplifying the inflammatory response. Inflammation further damages the epithelium, trapping the host in a cycle of chronic inflammation. Thus, understanding how barrier damage occurs is critical to developing effective therapies for IBD.

THE EPITHELIAL BARRIER IN IBD

The gut epithelium consists of a single layer of cells, primarily absorptive enterocytes and secretory cells such as enteroendocrine cells, Paneth cells, and goblet cells. Tight junctions are crucial to maintaining a cohesive barrier resistant to mechanical stress, microbial invasion, and toxins. Composed of multiple transmembrane and cytosolic proteins, tight junctions allow for strong bonds between epithelial cells, preventing paracellular invasion of microbes and allowing...
for selective permeability of water and solutes. Tight junctions also maintain polarity of epithelial cells, and can act as “anchoring” proteins within a cell membrane.\textsuperscript{13,14}

To maintain immune tolerance towards the gut microbial community, contact with the epithelial surface is minimized to limit inflammatory responses and bacterial invasion. Goblet cells in the epithelial wall produce a mucus layer coating the epithelium, maintaining a physical barrier between the host and microbial community. The mucus layer is further fortified by antimicrobial peptides secreted by epithelial cells as well as IgA produced by B and T cells. Antimicrobial peptides may be constitutively expressed by Paneth cells (such as $\alpha$-defensins) or expressed upon stimulation of TLRs (such as RegIII$\gamma$). This physical and chemical “demilitarized zone” between the epithelial layer and gut microbes is crucial for maintaining intestinal homeostasis.\textsuperscript{11}

Failures in the gut epithelial barrier are key to IBD pathogenesis. In biopsies of the gut mucosa from IBD patients, higher levels of epithelial cell-associated bacteria are seen, indicating a failure to maintain separation between gut microbes and the epithelium.\textsuperscript{15} The integrity of the epithelial barrier can be assessed via the lactulose/mannitol (L/M) test, wherein lactulose and mannitol sugars are used as probes for intestinal permeability. In CD, intestinal permeability is elevated, decreasing in disease remission; increased permeability for patients in remission is predictive of relapse.\textsuperscript{16}

Defects in tight junction components, including claudins, occludins, and junctional adhesion molecules (JAM) are observed in IBD. In CD, claudin 2, 5, and 8 are abnormally distributed and expressed.\textsuperscript{17,18} Tanaka et al. (2015) studied conditional knockouts of claudin-7 mice, observing that claudin-7 deficiency triggered broad colonic inflammation and increased colonic epithelial permeability.\textsuperscript{19} JAM-A expression is reduced in IBD patients, as well as
occludin. Given the role of tight junctions in maintaining a cohesive connection between epithelial cells in mechanically stressed tissues, defects in tight junction expression and distribution may lead to increased barrier permeability and bacterial invasion.

A well-documented cause of tight junction dysfunction in IBD is tumor necrosis factor-α (TNFα). TNFα is known to drive inflammation in IBD, and is shown to impact tight junction transcription. In cell culture models of the intestinal epithelial barrier, TNFα causes increases in intestinal permeability through expression of claudins and tight junction regulators. Further, monoclonal antibody therapy against TNFα (infliximab) therapy has proven effective in the treatment of both UC and CD through restoration of gut barrier integrity. Interestingly, pathogenic residents of the gut microbiota have been shown to dysregulate intestinal tight junctions; in particular, enteropathogenic *E. coli*, *Vibrio cholerae*, and *Clostridium perfringens* have been shown to disrupt tight junction structure.

Irregularities in the gut mucus layer are also seen in IBD, potentially contributing to disruption of immune tolerance to gut microbes by interfering with dendritic cell sampling of intestinal antigens. In CD patients, a thicker mucus layer is observed. An increase in abnormal mucin-2 (MUC2) expression is seen with a lack of typical viscosity, ultimately resulting in increased permeability. In UC, a decrease in overall goblet cell numbers results in a thin mucus layer, causing increased epithelial contact with gut microbes and leading to bacterial invasion of the epithelial layer.
OXIDATIVE STRESS IN BARRIER DAMAGE

Oxidative stress resulting from the presence of reactive oxygen species (ROS) in excessive levels is considered a major etiologic factor in IBD pathogenesis. The failure of native antioxidant mechanisms to detoxify ROS can result in excessive tissue damage and inflammatory responses. In the intestinal epithelium, reactive oxygen species may be generated by an influx of infiltrating immune cells, as well as dysfunctional mitochondria in intestinal epithelial cells.

Neutrophils and macrophages, upon stimulation by inflammatory cytokines, undergo “respiratory burst” wherein NADPH oxidase is suddenly released, causing the production of $\text{H}_2\text{O}_2$. Hydrogen peroxide can cause oxidative damage through the creation of hydroxyl radicals.\textsuperscript{29} Given the overstimulation of intestinal epithelial cell TLRs in the “vicious cycle” of chronic inflammation, a significant source of ROS in IBD is likely from infiltrating immune cells. In macrophages isolated from inflamed colonic tissue, a higher proportion were able to undergo respiratory burst in comparison to normal colonic mucosa.\textsuperscript{30} Additionally, for mice deficient in NOX2 (an enzyme crucial to respiratory burst), a greater resistance to experimentally induced colitis is seen.\textsuperscript{31}

The role of infiltrating immune cells and ROS may vary between CD and UC. In UC patients, a significant influx of neutrophils into the lamina propria is observed, and protein damage is associated with neutrophilic myeloperoxidase.\textsuperscript{32} However, in CD, neutrophils have impaired infiltration abilities, and data demonstrating their relative ability to generate ROS is inconsistent.\textsuperscript{29}

Structural abnormalities in gut epithelial mitochondria have been observed in both mouse models of experimental colitis as well as IBD patients, suggesting that mitochondrial ROS may
contribute to barrier damage. In healthy cells, mitochondria produce ROS during oxidative phosphorylation. Damage is kept in check through superoxide dismutase (SOD) neutralization of reactive superoxide in the mitochondrial membrane. However, in dysfunctional mitochondria, ROS may become excessive resulting in prolonged oxidative stress to the cell.

ROS may impact the gut epithelial barrier at multiple points, though specific targets are not yet clarified in clinical IBD. Firstly, membrane lipids and lipoproteins, high in polyunsaturated fatty acids, are targets of ROS damage in vitro. Lipid peroxidation causes structural disturbances to lipoproteins through the introduction of hydroperoxy groups into unsaturated fatty acids, disrupting membranes and damaging the integrity of cell and organelle barriers. Lipid peroxidation end products such as malondialdehyde cause further protein damage, and are linked to carcinogenesis. In active CD, higher levels of lipid peroxidation products are seen in blood plasma. Oxidative stress causes broad damage to other cellular proteins; in particular, hydrogen peroxide as well as IFN-γ and TNF-α treatment of epithelial cells causes disassembly of tight junction proteins.

Mitochondrial as well as nuclear DNA can be damaged by ROS, resulting in mutagenesis or cell death. H₂O₂, upon reaching the nucleus, reacts with iron bound within chromatin to produce OH•, which then reacts with DNA residues. Cell death may be induced by ROS damage from DNA damage and lipid peroxidation; further cell death could occur through direct modulation by ROS of apoptotic processes.
PROBIOTIC THERAPIES FOR IBD

Restoration of intestinal epithelial integrity has been a target for IBD therapies. Anti-TNF-α drugs have proven effective in treatment of CD, and supplementation of tight-junction boosting additives such as short-chain fatty acids (SCFAs) have been investigated in mice. However, given the central role gut dysbiosis plays in perpetuating an inflammatory “vicious cycle”, restoring the epithelial barrier via probiotic therapies is of interest. Limited success in inducing remission has been found in clinical trials of probiotic treatment of UC, and treatment of CD has only been investigated in a handful of human studies. Animal studies have focused on several potential mechanisms. Administration of probiotic bacteria could help prevent the outgrowth of pathogenic bacteria via nutrient competition or production of antibiotics, restoring a healthy microbial community. Additionally, probiotics may produce SCFAs to restore epithelial integrity by promoting tight junction assembly or inducing mucus secretion.

Probiotics targeting ROS-mediated damage have also been investigated in animal models. Strains of lactic acid bacteria (including Bifidobacterium and Lactobacillus species) exhibiting high antioxidant activity were administered to rats, effectively reducing experimentally induced oxidative stress. Lactobacillus casei strains engineered to produce superoxide dismutase or catalase were shown to induce remission in a murine model of CD involving intrarectal administration of trinitrobenzenesulfonic acid (TNBS). In colitis induced by dextran sodium sulfate (DSS) in mice, a strain of Lactobacillus casei engineered to express superoxide dismutase reduced inflammation. Ballal et. al. 2015 identifies a strain of Lactococcus lactis that ameliorates colitis in three mouse models through bacterial production of superoxide dismutase A (SodA). Interestingly, lysis of L. lactis was required to release SodA and
attenuate colitis, suggesting a model wherein SodA delivery is targeted through lysis of *L. lactis* at inflamed sites.

*In vitro* studies have begun to isolate specific mechanisms of antioxidant probiotics. In a study of Caco-2 BbE (a human epithelial colorectal adenocarcinoma cell line) monolayers, damaged induced by hydrogen peroxide could be prevented by treatment with *Lactobacillus rhamnosus GG*-produced soluble proteins. Redistribution of epithelial tight junctions and restoration of monolayer integrity resulted. Live probiotics have also been co-cultured with epithelial cell cultures; *Boswellia serrata* was shown to counteract damage induced by hydrogen peroxide and a IFN-γ/TNF-α treatment in Caco-2 BbE cells. Though no clear mechanism has been isolated, *Lactobacillus plantarum* has also been shown to prevent tight junction disassembly induced by enteroinvasive *Escherichia coli*. *Lactobacillus* is a genus of lactic acid bacteria, often studied as a potential probiotic therapy due to their relatively long persistence in the host cecum and colon after administration. Herein, Caco-2 monolayers as well as a Transwell™ model of differentiated primary epithelial cells were used to assess the potential antioxidant capabilities of two *Lactobacillus* strains. *Lactobacillus plantarum* WCFS1 has previously been identified for notable oxygen respiration capabilities and alongside *Lactobacillus* sp. 121247, and was co-cultured with cell monolayers subjected to oxidative stress via addition of hydrogen peroxide. Changes in monolayer integrity were assessed using transepithelial electrical resistance (TEER) as well as fluorescence microscopy.
Figure 1. The gut epithelial barrier. Schematic diagram of the components of the mucosal epithelial barrier.
CHAPTER 2: DATA AND METHODS

INTRODUCTION

To assess the effects of *Lactobacillus plantarum* WCSF1 and *Lactobacillus* sp. 121247 in countering damage from oxidative stress, primary colonic epithelial cells were grown in a polarized monolayer in a Transwell™ system. Alongside Caco-2 BbE cells, primary epithelial monolayers were treated with H$_2$O$_2$ to model oxidative stress conditions in IBD. *Lactobacillus plantarum* WCFS1 and *Lactobacillus* sp. 121247 were co-cultured with treated monolayers to assess for potential antioxidant capabilities. Primary epithelial cells were found to be more susceptible to H$_2$O$_2$ treatment, demonstrating that traditional cell culture models of the epithelial barrier may not be effective representations of barrier function. H$_2$O$_2$ treatment disrupted cytoskeletal structure and tight junction expression.

MATERIALS AND METHODS

Isolation of primary colonic epithelial cells and organoid culture:

Primary epithelial cells were isolated from mouse colonic crypts, as described in Miyoshi and Stappenbeck 2013 with modifications.$^{50}$ Female C57Bl/6J CEABAC10 mice expressing human CEACAM6 (used to allow for the possibility of experiments with adherent-invasive *E. coli* LF82) were sacrificed at 8-10 weeks of age. The mouse colon was dissected and cleaned before being gently scraped to remove villi. The colon was washed in washing media (DMEM/F12 with L-glutamine, HEPES, and 15% fetal bovine serum (FBS); Westnet) and cold phosphate-buffered saline (PBS). In order to loosen crypts, the washed colon was treated with PBS 2% FBS 5mM EDTA (VWR) and 5mM HEPES at 4°C in a fixed rocker.
The colon was then shaken in a series of 50mL conical tubes containing PBS to release the crypts. Tubes were centrifuged to form a pellet and filtered through 70µm cell strainers to remove debris. After further washes with washing media, isolated crypts were counted under a light microscope, and suspended in 100µl Matrigel (BD Biosciences) per 3000-5000 crypts.

10-15 µL of the Matrigel suspension was plated in 24 well culture plates. Plates were inverted and incubated at 37°C for 10 minutes, and 500 µL of 50% L-WRN with 10µM Y-2763 (ROCK inhibitor, R&D Systems); and 10µM SB-431542 (TGF-β type I receptor inhibitor, R&D Systems) to prevent differentiation were added. Penicillin/streptomycin and gentamycin (VWR) were also added. Plates were incubated in a CO₂ incubator at 37°C.

Organoid and spheroid development is seen after 24 hours, at which point culture medium is replaced without inhibitors. Cultures were split 2:1 at 48 hours (with the addition of 10µM Y2763 to culture media for 24 hours after expansion). After 4 passages without bacterial contamination, Matrigel was plated in 10mL culture dishes with 10mL of culture media.

Development of a primary colonic epithelial monolayer:

Organoid cultures were plated to Transwells™ in order to form a polarized monolayer, as described in Moon et al. 2014 with modifications. Polycarbonate 0.33cm² Transwells™ with 0.4 µm pores (Corning) were coated with 50µL of collagen type I (Sigma-Aldrich) in 60% ethanol. Transwells™ were allowed to dry before being sealed and stored at 4°C for 24 hours before use.

100 wells of organoid cultures generally yielded 4-8 0.33cm² Transwells™. Organoid cultures were suspended and washed with PBS before treatment with trypsin (Sigma-Aldrich) for 5 minutes at 37°C. Washing media (DMEM/F12 with Hepes, 15% FBS, penicillin, and streptomycin) was added 1:1, and 1.5mL aliquots were individually disaggregated by pipetting.
100 times with a 1000µL pipet. Cells were washed with media and strained with a 40µm cell strainer. After subsequent washes, cells were counted and plated in the apical chamber at a concentration of 500,000 cells/Transwell™ in L-WRN (with 10µM Y2763, penicillin, streptomycin, and gentamicin). 600 µL of media was added to the basolateral compartment. Transwells™ were washed with media 24 hours after plating, and media was replaced (without Y2763) daily.

After primary epithelial cell cultures reached confluence, differentiation was induced with 10uM DAPT (Millipore) and 1µg/mL LPS (Sigma) in DMEM/F12 with 20% FBS. DAPT and LPS treatment lasted 48 hours; thereafter, DMEM/F12 with 20% FBS was used as the culture medium.

Development of a Caco-2 BbE monolayer:

Caco-2 BbE cells were cultured in DMEM (Corning) with 15% FBS and penicillin/streptomycin. Cells were passaged at 80-90% confluency using trypsin-EDTA, and were seeded on collagen-coated 0.4 µm polycarbonate Transwells™ at a density of 1x10⁵ cells/Transwell™. Transwells™ were maintained for 9-14 days with media changes in the basolateral chamber every 2 days to achieve confluence.

Establishing treatment conditions via Annexin V/PI apoptosis detection

In order to determine the appropriate concentration of hydrogen peroxide to treat monolayers, Caco-2 BbE cells were seeded onto 96-well plates in DMEM with 15% FBS and penicillin/streptomycin. Upon reaching confluence (after 24 hours), wells were treated with hydrogen peroxide at concentrations ranging from 0.25 mM to 2 mM for 18 hours. To assess
relative levels of apoptosis, a Biolegend APC Annexin V Apoptosis Detection Kit with PI was utilized (Biolegend). Flow cytometry using a BD LSR II (BD Biosciences) was performed on the stained cells, and data were analyzed with FACSDiva software 6.1.3 (BD Biosciences) and FlowJo 10.0.5 software (TreeStar).

**Bacterial culture and monolayer treatment:**

*Lactobacillus plantarum* WCFS1 and *Lactobacillus* sp. 121247 were grown from a frozen stock in Difco Lactobacilli MRS Broth (BD Diagnostics) at 37°C. Overnight cultures were subcultured 1:100 into fresh media and grown for 4 hours, then centrifuged and resuspended in PBS, and adjusted to an OD600 = 0.1 (~10⁷ cells/mL). Bacteria was resuspended in cell culture media and 10⁶ cells were added to the apical chamber of experimental Transwells.

For Transwells™ treated with hydrogen peroxide, 3% hydrogen peroxide (Sigma-Aldrich) was diluted to 1mM in cell culture media in both apical and basolateral chambers.

**Indirect immunofluorescence microscopy staining:**

Transwell™ membranes were rinsed twice in PBS, and fixed in 4% paraformaldehyde for 1 hour (Sigma-Aldrich). After three ten minute washes in PBS, the membranes were blocked for 30 minutes with 3% donkey serum diluted in staining buffer (3% BSA, 0.1% TritonX 100, 0.1% saponin in PBS). ZO-1 Polyclonal Antibody (Invitrogen) was added at 5µg/mL in staining buffer and added to the membranes for overnight incubation at 4°C.

After three 10 minute washes in staining buffer, Alexa Fluor 488 donkey anti-rabbit IgG secondary antibody (Invitrogen) was added at 5µg/mL in staining buffer for an hour at room temperature, in the dark. Following another 10-minute wash in staining buffer, membranes were stained for 30 minutes in the dark with Alexa Fluor 594 Phalloidin (Invitrogen) at 5µg/mL in
staining buffer. Finally, membranes were stained with DAPI at 1µg/mL in staining buffer for 2 minutes and washed in PBS.

Membranes were cut out of their supports and mounted on glass slides using Aqua-Poly/Mount (Polysciences).

**Microscopy imaging:**

Transwell™ membranes were imaged using a Nikon Eclipse Ni microscope. Digital photographs were taken using a Nikon DS-Qi1Mc camera and a Nikon Digital Sight DS-U3. Images were processed using NIS-Elements BR 4.11 (Nikon Instruments).

**Evaluation of monolayer integrity:**

Confluence and epithelial damage was assessed via trans-epithelial electrical resistance (TEER), using an EVOM2 and chopstick electrode set (World Precision Instruments). Electrodes were placed in the apical and basolateral chambers of the Transwell™. A resistance lower than 100Ω indicated a bare membrane. Confluent Caco-2 monolayers TEER values ranged from 500Ω - 1000Ω. For confluent differentiated primary epithelial cell monolayers, TEER values ranged from 100Ω - 600Ω.

**Lactobacillus respiration assay**

*Lactobacillus plantarum* WCFS1 and *Lactobacillus* sp. 121247 were grown in WMB10 medium under aerobic conditions, and conditions driving respiratory growth (2.5 µg/mL hemin and 1 µg/mL menaquinone-4). WMB10 medium Cultures were grown for 24 hours at 37°C in a shaking incubator, and samples were collected at 16, 20, and 24 hours. The OD600 at each time
point was evaluated and real-time quantitative PCR was performed on samples at each time point, using primers targeting genes of interest in *Lactobacillus* respiratory pathways.

Quantitative real-time PCR was performed to assess for potential alterations in respiratory pathways using a Stratagene Mx3005P instrument (Agilent Technologies). The KAPA Biosystems SYBR Fast Universal qPCR Kit was used, with *fusA2* and *rpoB* housekeeping genes used to normalize samples. Primers used are listed in Table 1.

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<th>Table 1. RT-qPCR Primers</th>
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RESULTS

In order to establish optimal treatment conditions for the pilot study, Caco-2 BbE seeded onto 96-well were treated with varying concentrations of \( \text{H}_2\text{O}_2 \) for 18 hours, and evaluated for relative levels of damage and apoptosis via flow cytometry (Figure 2). Analysis indicated that 1 mM of hydrogen peroxide treatment for 18 hours resulted in the optimal balance of alive Caco-2 BbE cells and apoptotic cells; therefore, this concentration was used in future experiments.

**Figure 2: Determining optimal treatment conditions using Caco-2 BbE cultures.** Caco-2 BbE cells were seeded onto 96-well plates in DMEM with 15% FBS and penicillin/streptomycin. Upon reaching confluency, wells were treated with hydrogen peroxide at concentrations ranging from 0.25 mM to 2 mM for 18 hours. Cells were stained with Annexin V-FITC and propidium iodide (PI)-PE and analyzed via flow cytometry. A) illustrates the gating strategy utilized to isolate single cells. Cells positive for Annexin V-FITC and PI-PE were in a late apoptotic state; positive for PI-PE and negative for Annexin V-FITC necrotic, and Annexin V-FITC positive and PI-PE negative early apoptotic. Flow cytometry indicated that 1 mM of hydrogen peroxide treatment for 18 hours resulted in the optimal balance of apoptotic and alive Caco-2 BbE cells; therefore, this concentration was used in future experiments (B).
Caco-2 BbE cells were grown in polarized monolayers on Transwells™ to evaluate a co-culture assay with candidate *Lactobacillus* strains and hydrogen peroxide. After 18 hours of treatment with 1mM H$_2$O$_2$, TEER values decreased (Figure 3E, F). Post-treatment, confluency of monolayers was still apparent, though H$_2$O$_2$ caused damage in irregular patches to cytoskeletal structure and tight junction integrity (Figure 3B). For Caco-2 BbE monolayers treated with H$_2$O$_2$ and *Lactobacillus* strains, similarly damaged areas were not visible. ZO-1 localization appeared similar to untreated controls, co-expressing with actin (Figure 3C-D).

Addition of *Lactobacillus* strains had no significant effect on TEER or structural changes inflicted by H$_2$O$_2$ treatment (Figure 3E-F). This was not due to death of cultures during the treatment, as *Lactobacillus* strains remained viable after 18 hours of H$_2$O$_2$ exposure (Figure 5).
Figure 3. H$_2$O$_2$ treatment of Caco-2 BbE monolayers with *Lactobacillus* co-culture. Caco-2 BbE monolayers grown on 0.4 µm pore collagen-coated Transwell™ inserts were treated for 18 hours with 1 mM H$_2$O$_2$ (B), 1 mM H$_2$O$_2$ with *Lactobacillus* sp. 247 (C) and *Lactobacillus plantarum* WCFS1 (D) or left untreated (A). Monolayers were evaluated by immunofluorescence immediately after treatment, staining for DNA (DAPI), actin (Phalloidin), and ZO-1 at 20x. Monolayer integrity was evaluated by transepithelial electrical resistance (TEER) immediately before and after treatment period (E). The percent change in TEER ([posttreatment TEER-pretreatment TEER] x 100 / pretreatment TEER) after the treatment period was calculated (F). Error bars represent standard deviation.
The assay was repeated using the described methods for culturing primary colonic epithelial monolayers to evaluate the technique’s use in probiotic studies involving epithelial barrier function. Primary colonic epithelial monolayers treated with 1mM H$_2$O$_2$ were severely damaged, regardless of additional bacterial treatment. In most replicates, TEER values post-treatment decreased to 100 Ω, indicating total cell death on the membrane (Figure 4C). However, TEER decreased significantly for untreated controls (Figure 4C, D), indicating that TEER measurements were somewhat inconsistent in evaluating monolayer integrity. In any remaining cell clumps, disrupted cytoskeletal structure was seen in comparison to the control via Phalloidin staining against actin (Figure 4A, B). Staining for ZO-1 (a marker for tight junction expression) showed decreased localization of tight junctions to cell membranes when monolayers were treated with H$_2$O$_2$ (Figure 4A, B).

Figure 4. H$_2$O$_2$ treatment of primary colonic epithelial cells with Lactobacillus co-culture. Monolayers of primary colonic epithelial cells grown on 0.4 µm pore collagen-coated Transwell inserts were treated for 18 hours with H$_2$O$_2$ (B), 1 mM H$_2$O$_2$ with Lactobacillus plantarum WCSF1 and Lactobacillus sp. 121247 (data not shown), or left untreated (A). Monolayers were evaluated by immunofluorescence immediately after treatment, staining for DNA (DAPI), actin (Phalloidin), and ZO-1 at 20x. Monolayer integrity was evaluated by transepithelial electrical resistance (TEER) immediately before and after treatment period (C). The percent change in TEER ([posttreatment TEER - pretreatment TEER] x 100 / pretreatment TEER) after the treatment period was calculated (D). Error bars represent standard deviation. Scale bars represent 100 µm.
Lactobacillus plantarum WCFS1 and Lactobacillus sp. 121247 were evaluated for potential antioxidant capabilities in preliminary experiments. Strains were grown in WMB medium containing 10g/L of glucose under aerobic conditions, and conditions driving respiratory growth (2.5 µg/mL hemin and 1 µg/mL menaquinone-4). Cultures were grown for 24 hours and samples were collected at 16, 20, and 24 hours. The OD600 at each time point was evaluated (Figure 6A) and real-time quantitative PCR was performed on samples at each time point, using primers targeting genes of interest in Lactobacillus respiratory pathways and evaluating the fold change in mRNA expression between aerobic (AE) and respiratory (RS) growth states (Figure 6B, C). Notably, in Lactobacillus plantarum WCFS1 under respiratory growth conditions,

Figure 5. Lactobacillus viability during H₂O₂ treatment. Primary colonic epithelial or Caco-2 BbE monolayers were treated with H₂O₂ with and without Lactobacillus plantarum WCFS1 and Lactobacillus sp. 121247 for 18 hours. After growth of Lactobacillus strains in MRS broth, cultures were added to the apical chamber of the Transwell™ culture system. Serial dilutions were plated on MRS agar before and after 18 hours of H₂O₂ treatment to calculate cfu/mL.
mRNA levels of NADH dehydrogenase (Ndh1) and cytochrome D subunit 1 (CydA) were elevated, both crucial to *Lactobacillus* respiratory pathways and indicating a possible increase in respiratory activity (Figure 6B). In *Lactobacillus* sp. 121247, increased CydA expression was also elevated, suggesting similar activity (Figure 6C). Other genes evaluated include *nox* (NADH oxidase), involved in detoxifying hydrogen peroxide and reducing NADH, as well as *poxB* (pyruvate oxidase), involved in lactate and acetate production pathways in *Lactobacillus*. *narG* (nitrate reductase) was also investigated as a potential marker for nitrate reduction activity in respiratory growth conditions.52–54

![Figure 6. Evaluation of respiratory pathways in *Lactobacillus* WCSF1 and *Lactobacillus* sp. 121247.](image)

*Lactobacillus plantarum* WCSF1 and *Lactobacillus* sp. 121247 were grown in WMB10 medium under aerobic conditions, and conditions driving respiratory growth (2.5 µg/mL hemin and 1 µg/mL menaquinone-4). Cultures were grown for 24 hours and samples were collected at 16, 20, and 24 hours. The OD600 at each time point was evaluated (A) and real-time quantitative PCR was performed on samples at each time point, using primers targeting genes of interest in *Lactobacillus* respiratory pathways (B, C).
CHAPTER 3: DISCUSSION AND PERSPECTIVES

Preliminary experiments demonstrate the potential use of primary colonic epithelial monolayers in probiotic studies of barrier integrity, though further optimization and modification of protocols are necessary to fully take advantage of the methodology.

LIMITATIONS:
A limited research period prevented further optimization of the primary epithelial monolayer protocol, as well as thorough investigation of further probiotic candidate strains.

While primary epithelial monolayers recapitulate the gut epithelium by allowing development of multiple cell types without relying on a cell line derived from tumors, generation is costly and inefficient. 100 wells of Matrigel-cultured organoids were required to plate 4-8 Transwells, involving multiple expansions of organoid cultures over the course of weeks. As a low-throughput method, experimental replicates are difficult to conduct.

FUTURE RESEARCH:
Future research may focus on increasing the output of the primary epithelial monolayer system, in order to refine its use in modeling gut epithelial function.

To fully assess the relative effectiveness of the primary epithelial monolayer model, other common cell lines used to study the colonic epithelium in vitro could be investigated in addition to the Caco-2 BbE line. Given that many cell lines used in in vitro study are derived from cancers, cells may respond abnormally to oxidative stress as compared to the mouse-derived primary epithelial monolayer used here. Additionally, gut organoids have previously been developed from human iPS cells, and could similarly be plated in the Transwell culture system used here. Human-derived primary epithelial monolayers could act as a personalized screening
method for IBD therapies, a potentially useful tool given the complex etiology of IBD and other gut epithelial barrier-related diseases.


