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Effect of increased ratio of butyrate to physiological concentrations of acetate and propionate on intestinal integrity and IL-8 secretion in Caco-2 cells

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Effect of increased ratio of butyrate to physiological concentrations of acetate and propionate on intestinal integrity and IL-8 secretion in Caco-2 cells

by

Nidhi Shah

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
Suzanne Hendrich, Major Professor
Matthew Rowling
Nancy Cornick

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

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ABSTRACT

Background: Inflammatory bowel diseases (IBD) are a growing global health problem. Current pharmacological therapies are not effective in all IBD patients, tend to cause side effects and in some cases lose effectiveness when used for a prolonged period of time. Hence, there is a need to develop dietary interventions that are safe and be applicable to all. Chronic inflammation of the gut could be combated by increasing the consumption of dietary fiber which would lead to an increased butyrate production in the colon. Butyrate has been shown to improve intestinal integrity and suppress the secretion of pro-inflammatory cytokine, IL-8 – which is commonly found at high concentrations in IBD patients.

Hypothesis: When inflamed human intestinal cells (Caco-2) are exposed to an increased ratio of butyrate to physiological concentrations of acetate and propionate, the intestinal integrity would be improved and the secretion of IL-8 would be suppressed.

Experimental Design: Caco-2 cells were grown to 90-100% confluence on Transwell plate inserts for 4 days. Then, inflammation was induced for 24h. The inflamed and non-inflamed Caco-2 cells were then exposed to: (i) 60 mM acetate (Ac): 10mM propionate (Pr): 10mM butyrate(Bu) (lower butyrate ratio) or (ii) 60 mM Ac: 10mM Pr: 20mM Bu (higher butyrate ratio) for 0h, 12h or 24h. Intestinal integrity was measured by amount of Lucifer yellow transported from the apical to basolateral chamber of Transwell plates. IL-8 secretion was measured by ELISA and cell damage was measured by the lactate dehydrogenase cytotoxicity assay.

Results: Cells that were inflamed significantly decreased the intestinal integrity ($p=0.0094$), increased the secretion of IL-8 ($p<0.0001$) and induced higher cell damage ($p<0.0001$) than non-inflamed Caco-2 cells, $n=54$. SCFA mix containing higher butyrate ratio (20mM)

significantly reduced the intestinal integrity of inflamed cells ($p=0.0238$, $n=27$). Higher butyrate ratio did not affect the secretion of IL-8; but it led to greater cell-damage ($p=0.0161$, $n=54$). Prolonged exposure to SCFA improved intestinal integrity ($p<0.0001$), increased the secretion of IL-8 ($p<0.0001$) and led to greater cell damage ($p<0.0001$), $n=36$.

Conclusion: Our hypothesis was disproved. But the important lesson to be learned is that the effect of butyrate in colonocyte models should be studied in the presence of other SCFA, to assure physiological relevance.

CHAPTER 1: GENERAL INTRODUCTION

INFLAMMATORY BOWEL DISEASES (IBD)

What is IBD and its burden on society?

Chronic inflammation of the large intestine leads to development of inflammatory bowel disease (IBD). There are two kinds of IBD, namely, Ulcerative Colitis (UC) and Crohn's Disease (CD). According to Centers for Disease Control and Prevention (CDC), 1 – 1.3 million people suffer from IBD in USA. Although the prevalence of IBD is higher in the developed nations, the incidence and prevalence of IBD is growing in the developing nations as well (Molodecky *et al.*, 2012). USA spends more than \$6 billion in direct medical costs associated with IBD (Kaplan, 2015a). With the growing incidence rate in developing and developed nations, the direct and indirect medical cost is only going to increase, making IBD a global health problem that needs attention.

Which factors contribute towards development of IBD?

The pathogenesis of IBD is attributed to the complex interaction between genetics, environment, gut microbes and the immune system. With the advent of inexpensive ways of DNA sequencing, it has become easier to find the genes that might be associated with IBD or other diseases. Henderson and Satsangi (Henderson & Satsangi, 2011) have neatly summarized the genes and pathways that have been implicated in the etiology of IBD, as shown in Fig. 1.

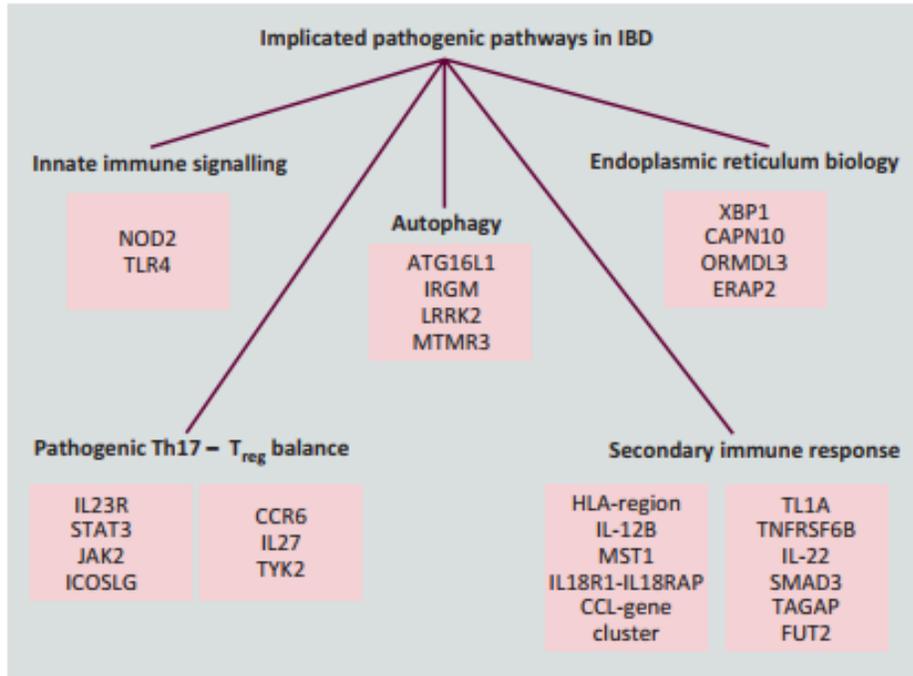


Fig.1. Broad overview of the genetic architecture of inflammatory bowel disease giving examples of confirmed and candidate genes implicated in each pathway. Treg - regulatory T cell. (Adapted from Henderson & Satsangi, 2011).

Several environmental factors have been shown to be associated with the risk of IBD. For example, consuming fruits and vegetables that is rich in dietary fiber is associated with decreased risk of developing both UC and CD (Molodecky & Kaplan, 2010; Ananthakrishnan, 2013). Similarly, cigarette smoking, appendectomy, psychosocial stress, depression, use of non-steroidal anti-inflammatory drugs (NSAID), dietary animal protein and usage of contraceptive pills have been associated with increasing the risk of developing CD; and on the other hand, consumption of vitamin D and dietary fiber have been associated with decreasing the risk of developing CD (Molodecky & Kaplan, 2010; Ananthakrishnan, 2013). The factors that increase the risk of developing UC are dietary arachidonic acid and linoleic acid, usage of NSAID, psychosocial stress, depression and post-menopausal hormonal use; and the factors that decrease the risk of developing UC are cigarette smoking,

appendectomy and dietary n-3 fatty acids (Molodecky & Kaplan, 2010; Ananthakrishnan, 2013).

The role of the immune system in developing IBD has been studied extensively (De Souza & Fiocchi, 2016). The gut microbiota plays an important role in the development of host immune system. Due to this connection between the immune system and the gut microbiota, it is safe to presume that the gut microbiota may play a role in pathogenesis of IBD.

Joossens *et al.* determined that when the fecal microbial composition of patients with CD was compared with that of their unaffected relatives, there was a significant decrease in the commensal bacteria like Firmicutes and increase in pathogenic ones like *Escherichia coli* (*E.coli*) (Joossens *et al.*, 2011). Similar such studies (Andoh *et al.*, 2011; Gong *et al.*, 2016) have found reduced microbial diversity and microbial composition are involved in the development of IBD – suggesting that the microbial composition could be manipulated to improve the effects of IBD.

In a nutshell, IBD is a growing global health problem. Genetic, environmental, immunological and gut microbial factors contribute towards the development of IBD. These factors could be targeted and modulated to develop effective strategies to prevent and cure IBD.

What can be done to prevent or treat IBD?

Disruption of the delicate equilibrium between the luminal microbial flora, intestinal epithelium and the immune system leads to inflammation of the gut. If the immune system becomes over-active, chronic inflammation persists that result into development of IBD. Hence, altering the microbial flora, improving the intestinal epithelial barrier function or targeting the immune system may be effective in treating or preventing IBD.

Caballero-Franco *et al.* determined the effect of a probiotic mixture, VSL#3, containing species from *Lactobacillus*, *Bifidobacteria* and *Streptococcus* genera on the mucus layer protein (mucin) secretion and expression of mucin secretion genes in 4-6 weeks old Wistar rats. The rats were fed 0.15 mg/kg (3×10^9 bacteria) of live VSL#3 probiotic formula dissolved phosphate-buffered saline (PBS) or PBS alone as a negative control, intragastrically for 7 days. VSL#3 treatment led to a 5-fold increase in the expression of mucin secretion gene, MUC2 and concomitantly, 60% increase in mucin secretion in the rat colons, in comparison with the control rat colons (Caballero-Franco *et al.*, 2007). This indicates how addition of beneficial microbes to the gut led to an improvement in development of the mucus layer which is the first line of defense for the gut.

In a randomized, placebo-controlled, cross-over study, Karczewski *et al.* determined the effect of duodenal administration of 10^{12} cells of *Lactobacillus plantarum* (*L.plantarum*) strain WCFS1 or placebo for 6h on secretion of tight junction proteins – zonula occludens(ZO)-1 and occluding (OC), in healthy volunteers (n =7). Tissue biopsies were obtained after each treatment by gastroduodenoscopy and then stained to be analyzed by using the confocal laser scanning microscopy. They found that the tissues obtained from the volunteers when *L. plantarum* was administered, were significantly highly stained for OC and ZO-1 when compared with tissues obtained from the volunteers during the placebo treatment. The researchers also determined the effect of 100 colony forming units (cfu) *L.plantarum* on tight junction proteins and permeability of Caco-2 BBe cell monolayers. They found that Caco-2 cells treated with *L.plantarum* for 6h stained significantly high for ZO-1 in comparison with those that were not treated with *L.plantarum*. They did not find the same result for OC. They also found that pre-treatment of Caco-2 cells with *L.plantarum* for 6h led

to significant protection against barrier dysfunction caused by phorbol 12,13-dibutyrate (PDBu) (Karczewski *et al.*, 2010). These results indicate that *L.plantarum* improves the intestinal barrier function and may prevent the entry of antigens or pathogens present in the colonic lumen.

Kelly-Quagliana *et al.* determined and compared the effect of diets containing 10g/100g cellulose (C), oligofructose (OF) or inulin (I) food on the immune function of 32-35 days old B6C3F1 female mice. They found that mice that were fed OF and I diets had higher natural killer cell activity from splenocytes and greater phagocytic activity of peritoneal macrophages in comparison with mice fed C diet. Mice fed OF and I diets also up-regulated macrophage-dependent (T-helper 1 type) immune responses (Kelly-Quagliana *et al.*, 2003). In conclusion, these studies are examples of how IBD could be prevented or treated through various mechanisms – changing the microflora, improving the intestinal barrier function or improving the immune response. One of the best ways to influence these changes is by increasing the intake of dietary fiber.

Diet has a major influence on the gut microbiome and intestinal epithelium. The gut microbiome can be altered by consumption of fiber-rich diet. The fiber-rich diet provides the necessary source of energy for the microbiome to thrive and the short chain fatty acids (SCFA) that are produced through microbial fermentation are used by the intestinal epithelium in the form of energy. Butyrate is one of the SCFA produced by microbial fermentation. Butyrate plays an important role in the growth, proliferation and differentiation of gut epithelial cells (Guilloteau *et al.*, 2010) It helps in protection against colorectal cancer by either causing the neoplastic cells to differentiate or by inducing apoptosis in them (Brouns *et al.*, 2002; Guilloteau *et al.*, 2010). Butyrate has anti-inflammatory properties and

has been shown to suppress the pro-inflammatory marker, IL-8 and it also improves the intestinal barrier function (Gibson & Rosella, 1995; Gibson *et al.*, 1999; Guilloreau *et al.*, 2010). Hence, improving the dietary fiber consumption of IBD patients might be an effective strategy combating the disease.

DIETARY FIBER

What is dietary fiber (DF)?

The definition of DF has evolved over time. Numerous countries and organizations define DF in different ways. But the definition developed by CODEX Alimentarius Commission (CAC) is accepted by 180 countries, making it an international standard for food and international trade (Jones, 2014). The definition states that:

Dietary fibre means carbohydrate polymers¹ with 10 or more monomeric units², which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

- 1. Edible carbohydrate polymers naturally occurring in the food as consumed.*
- 2. Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.*
- 3. Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.*

Footnote 1 states, “when derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds associated with polysaccharides in the plant cell walls. These compounds also may be measured by certain analytical method(s) for dietary fibre.

Footnote 2 states that, “Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.”

Most governments accept this definition, with slight modifications in the inclusion criteria for carbohydrates with more than two monomeric units, non-starch polysaccharides, resistant starch, resistant oligosaccharides and resistant maltodextrins.

Non-starch polysaccharides are defined as “the skeletal remains of plant cells that are resistant to digestion by enzymes of man measured as non α -glucan polymers measured by the Englyst (Type 2 Method). It includes NSP, which is comprised of cellulose, hemicelluloses, pectin, arabinoxylans, beta-glucan, glucomannans, plant gums and mucilages and hydrocolloids, all of which are principally found in the plant cell wall. It does not include oligosaccharides, resistant starch and resistant maltodextrins” (Jones, 2014).

Codex Alimentarius Commission issued a new definition in 2009, for dietary fiber to include resistant starch under carbohydrate polymers obtained from food raw material by physical, enzymatic or chemical means.

How is dietary fiber characterized?

Dietary fiber can be categorized based on various characteristics like: (a) chemical structure (sugar identity, linkage type, size) (b) food properties (biological origin, food processing) (c) gastrointestinal fate (rate of digestion and absorption) (d) metabolic properties (Glycemic index, substrate metabolism, fermentability and fermentation products, prebiotic effect) and (e) health effects (Englyst *et al.*, 2007).

There are different types of dietary fibers with varying chemical structures that would alter its properties like water-solubility, viscosity, digestibility and fermentability. For example, cellulose found in foods like whole grains, fruits and vegetables is a polysaccharide consisting of β -(1 \rightarrow 4)-linked glucose molecules. Cellulose polymers linked this way forms a rigid, insoluble and poorly fermentable structure. In the gastrointestinal tract cellulose has little impact, because as per an *in vitro* study by Barry et al., only 6-7% of cellulose was found to be fermentable. On the other hand, β -glucan found in cereals like oats and barley are made up of β -(1 \rightarrow 4)-linked glucose molecules, with intermittent β -(1 \rightarrow 3)-linkages every 2-4 glucose units. The β -glucan produced by yeast and mushrooms consists of β -(1 \rightarrow 3)-linkages as the backbone and β -(1 \rightarrow 6)-branches. This chemical structure makes β -glucan a soluble and highly fermentable dietary fiber. In the gastrointestinal tract, β -glucan increases the viscosity of the digesta which in turn reduces the rate of gastric emptying from the stomach to the small intestine; lowers absorption of nutrients and motility of small intestine; increases satiety; and increases fermentability in the large intestine (Mälkki & Virtanen, 2001).

Other types of dietary fibers that are insoluble include resistant starch, chitin, inulin and arabinoxylan and those that are soluble include chitosan, arabinogalactan, pectins, guar gum, xanthan gum, gellan, alginate, carrageenan, polydextrose, fructo-oligosaccharides, galacto-oligosaccharides and lactulose. These dietary fibers also vary in viscosity, fermentability and digestibility. Thus they have different effects in the gastrointestinal tract. For example, pectins and gums are soluble, viscous and highly fermentable fibers. The viscous nature of pectins and guar gum may enhance the mucous layer that protects the gastric lining from ulceration. The soluble nature of these dietary fibers also delays gastric emptying because of their water-holding capacity – leading to increased satiety, slower absorption of glucose and

fecal bulking. On the other hand, prebiotics like inulin and fructooligosaccharide are indigestible in the small intestine but highly fermentable in the colon. These dietary fibers selectively stimulate the growth of beneficial bacteria in the colon and thus improve the colon health (Rose & Hamaker, 2011).

To summarize, dietary fibers can be characterized by its physico-chemical properties, gastrointestinal fate, or the physiological or health effect they impart.

How is dietary fiber digested in the gut?

In the process of digestion, carbohydrates are first broken down by the salivary α -amylase in the mouth. Digestion of the carbohydrates in the stomach is limited. Carbohydrates are mainly digested in the small intestine. The pancreas secretes α -amylase which is delivered to the small intestine. The pancreatic α -amylase breaks down carbohydrates to monosaccharides, disaccharides like maltose and sucrose and oligosaccharides. The brush border enzymes of the small intestine break down the disaccharides and oligosaccharides into monosaccharides like glucose, galactose and fructose. However, not all carbohydrates present in the food is digestible in the small intestine. So the indigestible carbohydrates reach the large intestine, which is then fermented by the gut microbiota present there. The gut microbes break down the indigestible carbohydrates to short chain fatty acids (SCFA) like acetate, propionate, butyrate, iso-butyrate and more. 85% - 95% of the SCFA thus produced consists of acetate, propionate and butyrate. These short chain fatty acids have been known to have positive effect on the health of the host (Cummings & Macfarlane, 1991; Guilloteau *et al.*, 2010).

What is the physiological significance of dietary fiber?

Dietary fiber has been shown to exert beneficial effects on body weight, food intake, glucose homeostasis, and insulin sensitivity (Den Besten *et al.*, 2013). In a review by Galisteo *et al.*, several human and animal studies were summarized to indicate a correlation between higher fiber intake and a reduced risk of metabolic syndromes - obesity, cardiovascular disease and diabetes (Galisteo *et al.*, 2008). An inverse relation between fiber intake and the risk of developing IBD has also been established (Ananthakrishnan *et al.*, 2013). Ananthakrishnan *et al.* collected and analyzed data from 170,766 women who had participated in the Nurse's Health Study. Dietary information was collected by administration of semi-quantitative food frequency questionnaire every 4 years, for up to 26 years. Additionally, medical records were checked to confirm self-reported CD or UC. Using the Cox proportional hazards models, they concluded that the cohort of women who consumed the highest amounts of dietary fiber had 40% lower risk of developing CD in comparison with women who consumed lowest amount of dietary fiber. No such association was observed for UC (Ananthakrishnan *et al.*, 2013). For the purpose of this study, we will focus on the physiological effect of dietary fiber only on IBD.

Several cell culture, animal and human studies have been conducted to determine the role of dietary fiber in IBD:

Human Studies: Hallert *et al.* found that UC patients in remission who were fed 60g oat bran every day for 12 weeks as compared to UC patients in remission who did not receive the oat bran treatment, adapted to the diet leading to reduction of butyrate concentration. They observed 1.3 fold increase at 4wk and 8wk, but the concentration of butyrate normalized by 12 weeks and returned to its original concentration (Hallert *et al.*, 2003).

In an 8-week, randomized, single blind, cross-over study, James *et al.* compared the effect of increased dietary fiber in ulcerative colitis (UC) patients in remission with healthy subjects on food intake, bowel symptoms and tolerability, fecal indices and whole gut transit time (WGTT). During the dietary intervention period designated as 'low RS/WB', the participants consumed breads, cereals and muffins containing 2-5 g resistant starch (RS) and 2-5 g wheat bran (WB) per day, for 17 days. During the period of dietary intervention designated as 'high RS/WB', participants consumed breads, cereals and muffins containing 15g RS and 12g WB per day, for 17 days. There was a wash-out period of 14 days between each dietary intervention period, in this cross-over study.

At baseline, there were no significant differences between the UC patients and healthy ones in terms of fecal weight, pH, SCFA and phenolic compounds or WGTT. But at baseline, UC patients consumed significantly less iron and zinc. UC patients also consumed 1.5 times lesser amount of dietary fiber, which was not statistically significant. In spite of lower consumption of dietary fiber, output of fecal starch and NSP was three-fold higher in UC patients (James *et al.*, 2015).

Post-intervention, the researchers observed that during the 'high RS/WB' intake period, both subject groups consumed significantly higher amount of fiber as compared to the baseline, as expected. Both groups tolerated the 'low RS/WB' and 'high RS/WB' diets well. During the 'high RS/WB' intervention period, healthy controls excreted more than double amount of fecal starch and NSP as compared with baseline, but no differences were found in UC patients. Post-intervention, there were no significant differences between the UC patients and healthy ones in terms of fecal weight, pH, SCFA and phenolic compounds or WGTT. But usage of NSP was significantly reduced in UC patients as compared with healthy ones,

during either dietary intervention periods. Abundance of microbiota was similar in UC patients and healthy ones, but the composition was different, with UC patients having more diverse microbiota in their *Clostridium* cluster XIVa (James *et al.*, 2015).

Both groups tolerated both diets well. The only changes that were observed in this study were – two-fold increase in excretion of fecal starch and NSP in healthy patients, as compared with baseline; reduction in the usage of NSP in UC patients compared with the healthy ones; and differences in the diversity of gut microbiota. The researchers attributed these differences to lower fermentative capacity of NSP in UC patients as compared with healthy ones (James *et al.*, 2015).

In conclusion, increasing the dietary fiber intake of UC patients increased the butyrate concentration in the colon, which was normalized during long-term usage. High consumption of resistant starch also alters the microbial numbers and diversity in both – healthy and UC patients in remission. However, James *et al.* did not observe any significant changes in the fecal indices of UC patients when they were on high RS/WB diet. This could be because of dosage and type of fiber was not effective. The type and dosage of fiber may also have an effect on IBD markers.

Animal Studies: Zhou *et al.* found that when 21 days old, weaned Huanjiang piglets were fed standard diet supplemented with 0.5% soybean oligosaccharide (n=5, experimental group) as opposed to 0.5% corn starch (n=5, control group), the concentration of butyrate increased by 1.5 fold and the mRNA expression of IL-8 decreased by 26%, in the colonic contents obtained from the piglets after 14 days (Zhou *et al.*, 2014b).

Le Blay *et al.*, found that when Wistar rats were fed a standard diet supplemented with 9g/100g diet of fructooligosaccharide (FOS), there was 1.3 fold increase in the cecal SCFA

concentration after 2 wk, 8wk and 27wk; and, 3 fold increase in the butyrate concentration after 2 wk, and 27 wk, but 5 fold increase after 8 wk. Reduction in the butyrate concentration at 27 wk suggests that the rats adapted to the FOS supplemented diet and may have an increased number of FOS degrading gut bacteria (Le Blay *et al.*, 1999). Similar results of adaptation leading to decreased butyrate concentration were observed in the human study by Hallert *et al.*, 2003.

Nishitani *et al.* determined the anti-inflammatory effect of Lentinan - *Lentinula edodes* derived β -1,3; 1,6-glucan on 7-week old C57BL/6CrSlc mice that were induced to develop colitis when treated with 2% DSS w/v in drinking water for 7 days. Through intragastric administration, different doses of lentinan - 50, 100 or 200 μ g/mouse, were administered daily for 7 days prior to colitis induction via DSS and continued until sacrifice - 3 days after stopping the DSS-treatment. DSS- treated mice that received 100 μ g lentinan through oral administration, had significantly reduced weight loss (by 1.1-fold), colon shortening (by 1.2-fold) and histological score (by 60%), as compared with DSS-treated mice that did not receive lentinan. Additionally, lentinan treated DSS-mice significantly reduced the mRNA expression of pro-inflammatory cytokines IL-1 β by 50% in 200 μ g/mouse lentinan group and INF- γ by 40% in 100 and 200 μ g/mouse lentinan group as compared with untreated mice. This study suggests that the dietary fiber, β -glucan has anti-inflammatory properties that may help in ameliorating IBD (Nishitani *et al.*, 2013).

Majumder *et al.* determined the effect of isomaltodextrin (IMD) on the inflammatory markers found in DSS-induced colitis mouse model. Mice were provided with one of the doses - 0.5,1.0,2.5,5.0 % w/v IMD in drinking water for 23 days. On day 15, 5% DSS was added to the water until end of the experiment (day 23) to induce acute colitis. In contrast

with Nishitani *et al.*, IMD was not effective in improving the weight loss or colon shortening that occurs due to colitis. But, all doses of IMD was effective in reducing the concentration of pro-inflammatory cytokines TNF- α and IL-6 in DSS-treated mice as compared with those who did not receive IMD treatment. They also observed a tendency but not significant difference in reduction of other pro-inflammatory markers IL-1 β , MCP-1, and IL-17 and an increase in anti-inflammatory marker IL-10 – suggesting that IMD may be protective against colitis-induced gut inflammation (Majumder *et al.*, 2017).

Le Leu *et al.*, determined the effect of consumption of red meat with and without resistant starch on severity of DSS-induced colitis induced in male Balb/c mice. Colitis was induced in mice through 3% DSS in drinking water for the first 5 days of the dietary treatment. For the remaining 7 days of the treatment, they received plain drinking water. The daily disease activity index (DAI) increased in all dietary groups: control, red meat (RM), resistant starch (RS) and RM+RS. DAI was higher in the red meat group as compared with control and RS groups on most of the experimental days. But by 11th and 12th day of the experiment, there were no significant differences in any dietary groups. Histology scores were also significantly higher in the red meat group as compared with control and RS groups. Resistant starch appeared to be protective against the deteriorating effect of red meat, as indicated by the lower DAI scores than the RM group, but there was no statistical significance (Le Leu *et al.*, 2013). Although the researchers concluded that red meat aggravates DSS-induced colitis in mice, it should be noted that this was a short term study and the differences in DAI scores were not significant on last two days of the experiment. Hence, this study cannot be extrapolated to long term effects of red meat.

Scarminio *et al.* determined the protective effect of feeding green dwarf banana flour that is rich in type 2 resistant starch to male Wistar rats for 21 days. Colitis was induced by administration of trinitrobenzenesulfonic acid (TNBS)- for last 7 days of the experiment. Biochemically, the colonic damage caused by colitis was characterized by a reduction in colonic glutathione (GSH) levels and an increase in alkaline phosphatase (AP) and myeloperoxidase (MPO) activity. GSH is a marker of anti-oxidation and MPO and AP are markers of inflammation. The researchers found that feeding 20% banana flour diet significantly lowered the macroscopic and microscopic colonic damage score, maintained GSH levels and decreased AP and MPO levels in diseased mice as compared with non-diseased ones. They concluded that green dwarf banana flour helps in prevention and treatment of IBD mediated by its anti-oxidant and anti-inflammatory properties (Scarminio *et al.*, 2012).

Bassaganya-Riera *et al.* compared the protective effect of various soluble fibers and resistant starch against gut inflammation in wild type (WT) and IL-10 (IL-10^{-/-}) deficient C57BL/6J mice. IL-10^{-/-} mice tend to develop IBD, due to the lack of anti-inflammatory cytokine, IL-10. The researchers tested how diet supplemented with 4g fiber/100g diet affected the development of IBD in WT and IL-10^{-/-} mice. The fibers tested were PROMITOR soluble corn fiber (SCF), STA-LITE III polydextrose (PDX), Biogum (BG), Pullulan (PI-20), PROMITOR resistant starch-75 (RS-75), SCF&BG, RS-75&BG and inulin. They used dietary disease index (DAI), histology scores and cytokine production as biomarkers of gut inflammation. In IL10^{-/-} mice RS-75, SCF, RS-75&BG, and inulin suppressed IBD development by decreasing DAI. RS-75 and inulin were the only fibers that decreased inflammatory lesions in ileum and the colon of IL10^{-/-} mice. RS-75, SCF, and inulin

suppressed the production of pro-inflammatory cytokine interferon- γ (INF γ) and RS-75 increased the production of IL-10-expressing cells in WT mice. These results suggest that RS-75, SCF and inulin are protective against gut inflammation (Bassaganya-Riera *et al.*, 2011).

Moreau *et al.* compared the effect of time and type of dietary fiber supplementation on the efficiency of improving intestinal mucosa through SCFA production, in DSS-induced colitis model of male Sprague-Dawley rats. The diets compared were basal diet (BD), basal diet supplemented with type-3 resistant starch (BD+RS) and basal diet supplemented with fructooligosaccharide (BD+FOS). Colitis was induced by adding 50g DSS/L to the water of the DSS group for 7 days and all of them were fed the basal diet during this time. The control group received only water. Then, the DSS concentration was reduced to 30g DSS/L for rest of the experiment, and during that time, the mice received the experimental or basal diets. The effect of different diets was observed at 7 days and 14 days after starting the dietary intervention. Therefore, at each time point 6 groups were compared: BD-control, FOS-control, RS-control, BD-DSS, FOS-DSS and RS-DSS. In the control group, there were no significant changes due to diet on any parameters - food intake, body weight, disease activity index (DAI) or histological score. Between day 7 and day 14, there was a 1.2-fold reduction in food intake only in the FOS-DSS group, when compared with RS-DSS group ($p = 0.047$). At day 7, the body weight gain was 4-times and 6-times lower in the FOS-DSS compared with BD-DSS and RS-DSS groups, respectively ($p < 0.05$). At day 14, the body weight gain was 4-times and 9-times lower in the FOS-DSS when compared with BD-DSS and RS-DSS groups, respectively ($p < 0.05$). There was a 2-fold decrease in the DAI score in the RS-DSS group as compared with BD-DSS and FOS-DSS groups. At day 14, the histological score of

the RS-DSS group was 3 times lower than BD-DSS ($p=0.0082$) and FOS-DSS ($p=0.0306$) groups. The total histological score was significantly lower for RS-DSS when compared with BD-DSS ($p=0.0008$ at day 7, $p=0.0124$ at day 14) and FOS-DSS ($p=0.0195$ at day 7, $p=0.0406$ at day 14). DSS treatment decreased the SCFA concentration in the cecal-colonic contents of RS-DSS and FOS-DSS groups when compared with healthy controls; and, the butyrate concentration was higher in the cecum of rats in the RS-DSS group as compared with BD-DSS and FOS-DSS groups. In conclusion, RS diet seemed to improve the condition of inflamed colons, but FOS did not (Moreau *et al.*, 2003).

In a double-blind, placebo-controlled, randomized trial in canines, Segarra *et al.* determined the effect of a dietary supplement that contained chondroitin sulfate (CS) and prebiotics (resistant starch, β -glucans and mannanoligosaccharides (MOS)) on clinical disease activity, intestinal histology, gut microbiota, and selected serum biomarkers in dogs suffering from IBD, over 180 days. There were no significant differences in clinical disease activity and fecal gut microbiota between the placebo group and the supplemented group at any time point. However, there was a significant decrease (1.53-fold; $p < 0.01$) in the median overall histology score of the supplemented group, after treatment. Although the median overall histology score decreased by 1.07-fold in the placebo group after treatment, the difference was not significant. Higher concentration of serum cholesterol has been associated with better histologic score and they found significantly higher serum cholesterol ($p < 0.05$) after 60 days of treatment in the supplement group. Increased serum concentration of paraoxonase-1 (PON1), an antioxidant enzyme is associated with decreased oxidative stress and inflammation. The researchers found that after 60 days of dietary supplementation, there was a significant increase in the serum concentration of PON1 as compared with the placebo

group. The placebo group also showed significantly reduced serum total antioxidant capacity (TAC) levels after 120 days ($p < 0.05$), which could also be associated with increased oxidative stress. These results suggest that dietary supplements made of natural glycosaminoglycan – chondroitin sulfate and prebiotics helped in reduction of oxidative stress caused by IBD (Segarra *et al.*, 2016).

To summarize, SCFA production is increased with increased intake of DF. DF intake leads to an increased concentration of butyrate in the colon, probably because of the presence of high numbers of butyrate-producing microbes (Le Blay *et al.*, 1999; Zhou *et al.*, 2014a). DF has anti-inflammatory effect *in vivo* (Nishitani *et al.*, 2013; Majumder *et al.*, 2017) against colitis induced gut inflammation. DF may also impart its protective effect against IBD through its anti-oxidant properties (Scarminio *et al.*, 2012); and, resistant starch may be protective against the aggravation of IBD due to red meat consumption (Le Leu *et al.*, 2013).

Cell culture: The study done by Chen *et al.* is very similar to our experimental design. Chen *et al.* subjected four dietary fibers – raw potato starch (RS2), fructooligosaccharides (FOSs), sorghum arabinoxylan (SAX) and corn arabinoxylan (CAX) to *in vitro* digestion by sequentially exposing them to human salivary α -amylase, pepsin and pancreatin. The digesta (except for FOS) was then dialyzed against purified water for 24h and then freeze-dried.

Fecal samples were obtained from three healthy individuals and mixed equally in phosphate-buffered saline to prepare a fecal inoculum. 50mg of each fiber was fermented with 5% fecal inoculum for 12h. The fermenta products were collected and centrifuged. The supernatants were filter-sterilized and subjected to the cell monolayers. SAX fermentation produced highest amount of total SCFA followed by FOS then CAX and then RS2. In terms of butyrate concentration, FOSs produced highest amount of butyrate, followed by SAX, then

CAX and then RS2. Chen *et al.* claim that RS2 did not ferment well in this experiment.

Among the dietary fibers, they found that the intestinal integrity of the cells was improved by FOS, SAX, CAX when compared with RS2.

They then grew Caco-2 cells to confluence on Transwell plates for 7 days and subjected them to either pure SCFA mixture or sterilized fiber-fermented supernatants for 48h and measured the *trans*-epithelium resistance using a volt-ohm meter and paracellular permeability using fluorescent spectrophotometry. Caco-2 cells were exposed to one of the SCFA mixes (combination of acetate, propionate and butyrate) containing 10, 20 or 50% butyrate while maintaining the total SCFA concentration at 40mM for all mixes. They found that SCFA mixes containing 20% (8mM) and 50% (20 mM) butyrate had increased the TEER values by 1.3-fold and 1.5-fold, respectively, as compared to control (no SCFA added). Only butyrate showed a positive correlation with TEER values, suggesting that butyrate improves the intestinal barrier function (Chen *et al.*, 2017).

To determine the reparative effects of SCFA, Caco-2 cells were inflamed by addition of 10µg/ml LPS on apical side and 50ng/ml TNF α on the basolateral side for 48h. Inflamed Caco-2 cells were then exposed to one of the SCFA mixes (combination of acetate, propionate and butyrate) containing 5, 20 or 50% butyrate while maintaining the total SCFA concentration at 40mM for all mixes. To determine the protective effects, Caco-2 cells were exposed to the inflammatory stimulus and SCFA mixes simultaneously. Increasing proportion of butyrate improved barrier function in both –inflamed and non-inflamed Caco-2 cells (Chen *et al.*, 2017).

In order to determine the molecular mechanism through which IMD imparts its anti-inflammatory effect, Majumder *et al.* subjected ~80% confluent intestinal epithelial cells,

HT-29 to 0.01, 0.05, 0.1, 0.5 or 1 mg/ml IMD for 2h, followed by 10 ng/ml TNF- α induced inflammation for 4h and measured the secretion of pro-inflammatory marker IL-8 in the supernatant. To determine the role of Toll-like receptor (TLR)-4 in the anti-inflammatory effect of IMD, HT-29 cells were pre-treated with TLR-4 inhibitor – CL-095 for 6h before being exposed to different concentrations of IMD for 2h and then TNF α for 4h, as mentioned before. They found that 0.05 mg/ml – 1mg/ml IMD inhibited the expression of IL-8 in inflamed cells, as compared to inflamed cells that were not treated with IMD. They also determined that the presence of TLR-4 inhibitor along with IMD, further reduced the expression of IL-8 in inflamed HT-29 cells. After this observation, they tested for expression of TLR-4 receptors in the mouse colonic tissue and found that there was a significant reduction in it in the DSS-induced colitis mice that were treated with 5% w/v IMD. These results indicate that IMD imparts its anti-inflammatory effect by suppressing TLR-4 receptors (Majumder *et al.*, 2017).

To summarize, dietary fibers have been shown to be protective against inflammation, *in vitro*. This protective effect of dietary fiber was attributed to the increased production of butyrate due to microbial fermentation. To prove this Chen *et al.* tested the effect of pure SCFA on the intestinal integrity of Caco-2 cells and found that SCFA mixes containing 20% and 50% butyrate improves the intestinal integrity of inflamed Caco-2 cells.

General conclusions regarding dietary fiber and IBD

SCFA production is increased with increased intake of DF. DF intake leads to an increased concentration of butyrate in the colon, probably because of the presence of high numbers of butyrate-producing microbes (Le Blay *et al.*, 1999; Zhou *et al.*, 2014a). DF has anti-inflammatory effect *in vivo* (Nishitani *et al.*, 2013; Majumder *et al.*, 2017) against colitis

induced gut inflammation. DF may also impart its protective effect against IBD through its anti-oxidant properties (Scarminio *et al.*, 2012). Resistant starch may also be protective against the aggravation of IBD due to red meat consumption (Le Leu *et al.*, 2013). These beneficial effects of dietary fibers have been attributed to increased concentration of SCFA, specifically butyrate (Chen *et al.*, 2017). The studies that looked at the effect of SCFA on symptoms or markers of IBD are reviewed below.

SHORT CHAIN FATTY ACIDS (SCFA)

What are SCFA?

The gut microbiota in the large intestine ferment indigestible dietary fiber and resistant starches into SCFA. SCFA are one to six carbon organic acids; and the most abundant SCFA in the large intestine are acetate (C2), propionate (C3) and butyrate (C4). They are found in an approximate molar ratio of 60:10:10 in the gut (Cummings, 1981).

What is the role of butyrate in IBD?

Several studies have been conducted to determine the role of butyrate in IBD:

Human studies: The butyrate enema studies conducted in humans are summarized in Table 1. Most of the studies listed here indicate either an improvement or no effect of butyrate enema on disease activity, histology score, endoscopic score and alleviation of symptoms related to IBD.

Table 1: Butyrate enema studies conducted in humans.

Reference	Patients	Methods	Results
(Guillemot <i>et al.</i> , 1991)	Diversion colitis	Randomized, placebo-controlled, double blind study Placebo: saline enema, n = 6 Trt: Mix of 60 mM Ac, 30 mM Pr and 40 mM Bu enema, n=7 Dose and frequency: Twice a day for 14 days	↔ Endoscopic score at the end of treatment period ↔ Histology score at the end of treatment period
(Steinhart <i>et al.</i> , 1996)	Distal Ulcerative colitis	Randomized, placebo-controlled, double blind study Placebo: saline enema, n = 19 Trt: 60 ml of 80 mM butyrate enema, n = 19 Dose and frequency: Once a day for 6 weeks	↔ Disease Activity Index (UCDAI) score at the end of treatment period ↔ Endoscopic score at the end of treatment period ↔ Histological score at the end of treatment period

(Table 1 continued)

(Talley <i>et al.</i> , 1997)	Radiation proctitis	<p>Double-blind, placebo-controlled, crossover study</p> <p>Placebo: 60 ml saline enema, n=12</p> <p>Trt = 60 ml of 40 mM butyrate enema, n =12</p> <p>Dose and frequency: Twice per day for 2 weeks; Washout period: 1 week</p>	<p>↔ Endoscopic score at the end of treatment period</p> <p>↔ Histological score at the end of treatment period</p> <p>↔ Symptom score at the end of treatment period</p>
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(Table 1 continued)

(Pinto <i>et al.</i> , 1999)	Radiation proctitis	<p>Randomized, placebo-controlled, double blind study</p> <p>Placebo (C): saline, n = 5</p> <p>Trt (T): 60 mL mix of 60 mM Ac +30 mM Pr + 40 mM Bu enema, n= 9</p> <p>Dose and frequency: Twice daily for 5 weeks (therapeutic phase - TP)</p> <p>Follow up for up to 6 months after treatment</p>	<p>TP: Compared with baseline, ↓ number of days/week for rectal bleeding (p<0.01) in treatment group compared to placebo group</p> <p>Follow up: Compared with baseline, ↓ number of days/week for rectal bleeding but not significant (p =0.13)</p> <p>TP: ↓ Endoscopic score (p < 0.001) for SCFA group, ↓ Endoscopic score (p<0.01) for placebo</p> <p>Follow up: ↔</p> <p>Endoscopic score , ↔</p> <p>Histological score at the end of the treatment period</p>
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(Table 1 continued)

(Lührs <i>et al.</i> , 2002)	Ulcerative colitis	<p>Randomized, placebo-controlled</p> <p>Placebo: Saline enema, n = 5</p> <p>Trt: 60ml of 100 mM butyrate enema, n =6</p> <p>Dose and frequency: Twice daily for 8 weeks</p>	<p>↓ Disease activity index (DAI) after 4 wks (p=0.011) and 8 wks (p=0.001) of treatment in the butyrate-treated patients</p> <p>↓ Endoscopic score only after 8 wks of treatment (p=0.007) in the butyrate-treated patients.</p> <p>↔ Overall histological score between groups at any time point</p>
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(Table 1 continued)

(Vogt & Wolever, 2003)	Healthy adults	<p>Crossover study, placebo controlled</p> <p>10 trts: 3 saline infusions (at the start, middle and end of the study) and 7 SCFA infusion in random order: Ac, Pr, Bu, Ac+Pr, Ac+Bu, Pr+Bu, Ac+Pr+Bu</p> <p>Concentration used for trts Ac: 60 mM, Pr: 20 mM, Bu: 20 mM</p> <p>Volume: 300 ml of test solution;</p> <p>Washout period: 3 days</p>	<p>% absorption of butyrate > % absorption of acetate</p> <p>% absorption of butyrate not greater than % absorption of propionate</p> <p>Total SCFA absorbed was positively correlated to the total SCFA concentration infused, except for APB</p> <p>Amount of Ac absorbed from APB solution was negatively correlated with fecal molar % of Ac</p>
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(Table 1 continued)

(Hamer <i>et al.</i> , 2009)	Healthy	<p>Double blind, placebo-controlled, crossover study</p> <p>Placebo: 60 mL saline, n =16</p> <p>Trt: 60 mL of 100 mM butyrate enema, n = 16</p> <p>Dose and frequency: Once a day for two weeks</p>	<p>↔ Histological score at the end of the treatment period</p> <p>↓ Oxidative stress at the end of the treatment period</p>
(Hamer <i>et al.</i> , 2010)	Ulcerative colitis patients in remission	<p>Randomized, placebo-controlled, double-blind study</p> <p>Placebo: 60 mL saline, n = 18</p> <p>Trt: 60 mL 100 mM butyrate enema, n = 17</p> <p>Dose and frequency: Once a day for 20 days</p>	<p>↑ Colonic IL-10/IL-12 ratio in butyrate-treated group compared with baseline (p=0.022)</p> <p>↔ Histology score between the treatment and placebo groups</p> <p>Minor effects on inflammatory and oxidative stress parameters</p>

(Table 1 continued)

(Luceri <i>et al.</i> , 2016)	Enterostomy patients	Randomized, placebo-controlled, double blind study Placebo: 30 ml saline enema, n = 7 Trt: 30 ml of 600 mM butyrate enema, n = 10 Dose and frequency: Twice daily for 30 days	↓ Endoscopic score in butyrate-treated patients, as compared with baseline (p = 0.0083) ↔ Histological score in either groups ↓ Atrophy score in trt group as compared with baseline, but not significant
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Animal studies: Argenio *et al.* tested the effect of 80 mM butyrate enema in trinitrobenzene-sulphonic acid (TNBS) colitis induced model, in Wistar rats. 2 weeks of butyrate treatment led to a 2.5-fold reduction in the macroscopic score and nearly 3-fold reduction in the histological scores when compared with rats that received only saline enema. They also tested for the effect of 80 mM butyrate on development of colitis associated colorectal cancer. One week after induction of colitis with TNBS in Wistar rats, the researchers administered azoxymethane (AOM) for 5 weeks and continued treatment until 27 weeks. They found that butyrate treatment led to a 2-fold reduction in the number of tumors formed and >3-fold reduction in the size of the tumors when compared with saline treatment (D' Argenio *et al.*, 2007).

Vieira *et al.* determined the effect of oral administration of 0.5% butyrate for 14 days on the histological score and cytokine profile and DSS-induced colitis C57BL/6 mice. The crypt length was reduced by 50% in the UC-mice that were fed the standard diet, when compared with control mice that were on standard diet. But the UC-mice that were fed the diet containing butyrate, the crypt length was 1.5-fold longer than UC-mice on standard diet. They also found that UC-mice that were fed butyrate-supplemented diet had half the concentration of neutrophils and eosinophils when compared with UC-mice on standard diet. They also observed an improved inflammatory cytokine profile in the butyrate-fed UC-mice (Vieira *et al.*, 2012).

General conclusions from human and animal butyrate enema studies

The human studies suggest that for the treatment to be effective the butyrate concentration should be ≥ 100 mM and the treatment should be given for at least 6-8 weeks to observe an improvement in the severity and symptoms of colitis. The concentration and number of days for treatment is reduced in the rodent studies, probably because of lower body weight and size. But it is important to note that dietary fiber fermentation also produces other SCFA like acetate, propionate. Additionally, other branched chain-fatty acids like iso-butyrate, valerate etc., which are fermentation products of amino acids; organic acids like lactate and succinate and products like indoles, phenols, amines and ammonia are also present in the colonic lumen (Cummings & Macfarlane, 1991). Although, 85% - 95% of the products contain acetate, propionate and butyrate – indicating that they are present in highest amounts in the colon (Cummings & Macfarlane, 1991).

Most of the enema studies reviewed above have looked at the effect of only butyrate on the mucosal damage caused by IBD. Only 3 groups of researchers reviewed above tested for the

effect of enemas containing acetate, propionate and butyrate (Guillemot *et al.*, 1991; Pinto *et al.*, 1999; Vogt & Wolever, 2003). Hence, to determine the protective effect of dietary fibers more realistically, it is important to study the effect of its fermentation byproducts in the presence of other colonic lumen particles. However, only the concentrations of acetate, propionate and butyrate are well characterized in the colon. Hence, the focus of our research is to determine the effect of increased butyrate in the presence of acetate and propionate on the intestinal integrity and secretion of pro-inflammatory chemokine, IL-8 in Caco-2 cells.

INTERLEUKIN-8 (IL-8)

What is IL-8?

IL-8 is an pro-inflammatory marker belonging to IL-1 family (Guan & Zhang, 2017). IL-8 belongs to the class of chemokines that have the cysteine – amino acid – cysteine (CXC) structure and binds to CXC receptors (CXCR) on the cells. IL-8 binds to CXCR1 and CXCR2 (Kobayashi, 2006). The primary role of IL-8 is the activation and migration of neutrophils from the peripheral blood into the tissues (Múzes *et al.*, 2012). When acute inflammation occurs, IL-8 is secreted from the epithelial cells that bind to the CXCR2 on neutrophils. IL-8 thus acts as a chemo-attractant for neutrophils, which then migrate and infiltrate the injured tissue (Kucharzik & Williams, 2003; Kobayashi, 2006).

What is the role of IL-8 in IBD?

One of the inflammatory markers used to characterize IBD is IL-8. Gibson and Rosella isolated colonic crypt cells from the resected mucosa of patients with varied colon diseases: normal (n=16), colorectal cancer (CRC) (n = 20), Ulcerative colitis (UC) (n=17) and Crohn's Diseases (CD) (n=14). The isolated cells were then cultured for 16-24 hours and IL-8 secretion was measured in the supernatant by ELISA. They found that IL-8 secretion was 2.5

times higher from the uninflamed mucosa of colonic crypt cells that were isolated from UC and CD patients, as compared with that of normal patients (Gibson & Rosella, 1995).

In a recent study by Rodriguez-Peralvarez *et al.* the serum cytokine profile of UC patients (n=67), was characterized and compared with that of healthy ones (n=21), with the goal of determining the serum biomarkers that could be used to diagnose UC. Using the univariate analysis they determined that IL-6 was 1.8-times higher (p=0.007), IL-8 was 1.3 times higher (p=0.002) and IL-10 was 1.3 times higher (p=0.008) in UC patients when compared with healthy ones. Using the multiple logistic regression model they showed that UC patients were characterized by higher concentration of IL-8 (odds ratio [OR] = 1.37, p=0.002) and IL-10 (OR = 3.88, p=0.012) and lower concentration of IFN- γ (OR = 0.95, p=0.002). This serum profile had 74.6% sensitivity, 85.7% specificity, and 77.3% accuracy in discriminating between UC patients and controls. When the data from only newly diagnosed patients were used, the accuracy of the model increased to 96.4% accuracy. Higher serum concentration of IL-8 was also related to moderate to severe disease clinical activity (OR=1.16, p=0.012); endoscopic (OR=1.10, p=0.026) and histology (OR = 1.33, p=0.017) severity (Rodríguez-Perálvarez *et al.*, 2012)

In conclusion, IL-8 concentration is high in the serum and colonic crypt cells of UC patients and could possibly be used as a biomarker for diagnosis.

How is IL-8 affected by butyrate?

Several studies indicate that butyrate suppresses the secretion of IL-8 from colonic cells:

Gibson and Rosella isolated uninflamed colonic crypt cells from the resected mucosa of patients with varied colon diseases: normal (n=16), colorectal cancer (CRC) (n = 17),

Ulcerative colitis (UC) (n=13) and Crohn's Diseases (CD) (n=10). The isolated cells were

then cultured for 24 hours with 1 mM acetate, propionate or butyrate. While acetate and propionate had no significant effect on the secretion of IL-8 from the cells, butyrate significantly reduced the secretion of IL-8 in from the cells isolated from normal patients by 20%, from CRC patients by 25%, from UC patients by 30% and from CD patients by 22% , when compared with cells that were not treated with butyrate. There was no significant difference across disease groups (Gibson & Rosella, 1995).

In a different study by Gibson *et al.* they tested the effect of butyrate and TNF α alone and effect of butyrate after pre-incubating the cells with TNF α for 24h, on IL-8 secretion. They found that when the cells were incubated with 2mM butyrate alone or 10^{-7} M TNF α for 24h, IL-8 secretion more than doubled. But, when Caco-2 cells were pre-incubated with TNF α for 24h, washed and then subsequently exposed to 2 mM butyrate for 24h, IL-8 secretion was suppressed by 34% from the basal levels ($p = 0.03$).

The Caco-2 cell-line is an *in vitro* model that is used to determine the mechanisms and effects of various compounds on enterocytes or colonocytes. Caco-2 cells are a good model for IBD because they secrete high amounts of IL-8 when stimulated by inflammatory stimuli. The secretion of IL-8 is also dependent on the differentiation phase of the cells. Mariadason *et al.* determined the effect of butyrate treatment on secretion of IL-8 from Caco-2 cells at various phases of differentiation. In a 21-day growth period, they found that 5-days old cells secreted highest amount of IL-8 ($1352\% \pm 166\%$ above control), after being treated with 2 mM butyrate for 72 hours. The secretion of IL-8 was high until the Caco-2 cells were 7 days old and this trend seemed to wear off by day 21. Huang *et al.* observed similar amount of secretion in 5-days old (pre-confluent) cells that were stimulated by IL-1 β for 4 hours. They also observed a 6-fold decrease in the secretion of IL-8 in 18-days old (post-confluent) cells

and in 5-days old (pre-confluent) cells that were treated with 2.5 mM sodium butyrate for 72 hours, in comparison with untreated 5-days old cells (Huang *et al.*, 1997).

Through these studies, it can be concluded that exposure of inflamed Caco-2 cells to 2mM butyrate leads to a significant suppression of IL-8 concentration when compared with untreated, inflamed Caco-2 cells.

OVERALL SUMMARY AND RATIONALE

Increasing the intake of dietary fiber has been proven to reduce the risk of developing CD (Ananthakrishnan *et al.*, 2013) and resistant starch has been shown to be protective against development of UC (Le Leu *et al.*, 2013). These effects could be attributed to the anti-inflammatory (Nishitani *et al.*, 2013) or anti-oxidant (Scarminio *et al.*, 2012) properties of dietary fiber.

It has been proposed that dietary fiber imparts its protective effect against IBD through its fermentation byproduct – butyrate (Guilloteau *et al.*, 2010). Several studies have been conducted to determine the mechanisms through which butyrate is protective against IBD (Hamer *et al.*, 2010). However, these studies have limited implications because only the effect of butyrate has been studied alone. But other major fermentation products like acetate and propionate are also present in the colon (Cummings & Macfarlane, 1991). It is unknown if the effect of butyrate is affected by the presence of other short chain fatty acids. Hence, to fully understand the effect of butyrate against IBD, it is important to study its effect in the presence of other short chain fatty acids – acetate and propionate.

Brouns *et al.* suggested that increasing the consumption of resistant starch would lead to an increase in the concentration of butyrate in the gut. Butyrate plays an important role in growth, proliferation and differentiation of gut epithelial cells. Butyrate has have anti-

inflammatory and anti-oxidative properties (Guilloteau *et al.*, 2010). Butyrate enemas have been shown to be protective against the mucosal damage caused due to IBD (Hamer *et al.*, 2010; Luceri *et al.*, 2016). Gibson and Rosella have showed that butyrate suppresses the secretion of the pro-inflammatory chemokine, IL-8. Since high concentrations of IL-8 has been proven to be a characteristic of UC and CD patients (Gibson & Rosella, 1995), suppression of secretion of IL-8 by butyrate may help in attenuating the effects of IBD. Hence, we hypothesized that an increased ratio of butyrate in the presence of physiological concentrations of acetate and propionate (as produced after fermentation of dietary fiber), would improve the intestinal integrity and suppress IL-8 secretion in Caco-2 cells.

RATIONALE FOR METHODS USED

Caco-2 cells are human adeno-carcinoma cells that behave as colonocytes when grown *in vitro* for up to 7 days (Engle *et al.*, 1998). To understand the protective effect of dietary fiber, it is important to study its effect on colonocytes and not enterocytes, because dietary fibers are highly fermented to SCFA in the colon (Cummings, 1981; Cummings & Macfarlane, 1991). Caco-2 cells also secrete high concentrations of IL-8 when inflamed - similar to what occurs in IBD patients (Gibson & Rosella, 1995). Suppression of IL-8 by butyrate has also been observed in Caco-2 models (Huang *et al.*, 1997; Mariadason *et al.*, 2001). Due to all these characteristics, we selected to use Caco-2 cells as our model.

Caco-2 cells were grown on Transwell plates such that it separated each well into two chambers – apical and basolateral. The apical chamber represents the apical surface of the intestinal epithelial cells and the basolateral chamber represents the basal and lateral surfaces of the intestinal epithelium. The apical surface of the epithelial cells faces towards the

colonic lumen, where the gut microbes are present and the basolateral surface faces towards the blood vessels (Fig 2).

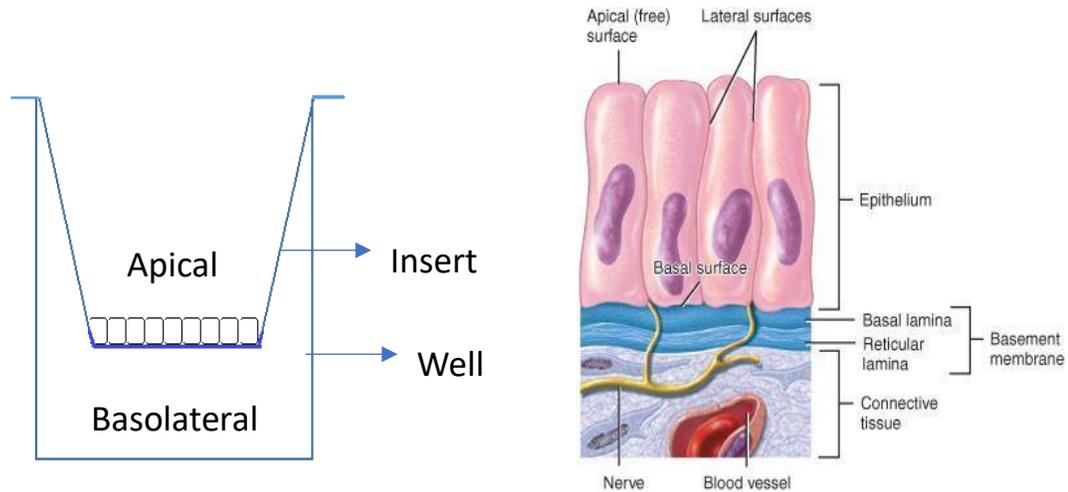


Fig 2: (Left) Cartoon representation of how an insert divides the well into two chambers – apical and basolateral. (Right) The apical and basolateral surfaces found in the intestinal epithelium as mimicked in the Transwell plates.

To mimic the chronic inflammation observed in IBD patients, we used lipopolysaccharide (LPS) as an inflammatory stimulus on the apical side. LPS represents the microbial community found on the apical side of the epithelium. On the basolateral side, we used a mixture of pro-inflammatory cytokines tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and interferon- γ (INF- γ) as used previously in our lab to inflame the cells (Qiang *et al.*, 2011).

The intestinal integrity of the cells was tested by the colorimetric method using Lucifer Yellow (LY). When the cells are highly permeable, the LY is easily transported from the apical chamber to the basolateral chamber. Hence, when high concentration of LY is measured in the basolateral chamber, it is concluded that the intestinal integrity of the cells is compromised. Several other researchers have used this method to test the intestinal integrity

of Caco-2 cells (Kadota *et al.*, 2013; Nemoto *et al.*, 2015). Cell integrity was also measured by the quantifying the damage caused to the cells, by measuring the lactate dehydrogenase (LDH) activity. LDH is a soluble enzyme located in the cytosol. When the cell is damaged or lysed, LDH is released in the surrounding culture medium. LDH activity in the culture medium can therefore be used as an indicator of cell integrity (Haslam *et al.*, 2000). Several other researchers have used this method to test for cell damage/cell permeability of Caco-2 cells (Carrasco-Pozo *et al.*, 2010; Chen *et al.*, 2017).

Hence, our objective was to determine the effect of increased ratio of butyrate in the presence of physiological concentrations of acetate and propionate on the intestinal integrity and IL-8 secretion in inflamed and non-inflamed Caco-2 cells.

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**CHAPTER 2: EFFECT OF INCREASED RATIO OF BUTYRATE TO
PHYSIOLOGICAL CONCENTRATIONS OF ACETATE AND PROPIONATE ON
INTESTINAL INTEGRITY AND IL-8 SECRETION IN CACO-2 CELLS**

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ABSTRACT

Background: Chronic inflammation of the gut could be combated by increasing the consumption of dietary fiber which would lead to an increased butyrate production in the colon. Butyrate has been shown to improve intestinal integrity and suppress the secretion of pro-inflammatory cytokine, IL-8.

Experimental Design: Caco-2 cells were grown to 90-100% confluence on Transwell plate inserts for 4 days, then inflammation was induced for 24h. The inflamed and non-inflamed Caco-2 cells were then exposed to: (i) 60 mM acetate (Ac): 10mM propionate (Pr): 10mM butyrate(Bu) (lower butyrate ratio) or (ii) 60 mM Ac: 10mM Pr: 20mM Bu (higher butyrate ratio) for 0h, 12h or 24h. Intestinal integrity was measured by amount of Lucifer yellow transported from the apical to basolateral chamber of Transwell plates. IL-8 secretion was measured by ELISA and cell damage was measured by the lactate dehydrogenase cytotoxicity assay.

Results: Cells that were inflamed significantly decreased the intestinal integrity ($p=0.0094$), increased the secretion of IL-8 ($p<0.0001$) and induced higher cell damage ($p<0.0001$) than non-inflamed Caco-2 cells, $n=54$. SCFA mix containing higher butyrate ratio (20mM) significantly reduced the intestinal integrity of inflamed cells ($p=0.0238$, $n=27$). Higher butyrate ratio did not affect the secretion of IL-8; but it led to greater cell-damage ($p=0.0161$,

n=54). Prolonged exposure to SCFA improved intestinal integrity ($p < 0.0001$), increased the secretion of IL-8 ($p < 0.0001$) and led to greater cell damage ($p < 0.0001$), n=36. **Conclusion:** Our hypothesis was disproved, but the important lesson to be learned is that the effect of butyrate in colonocyte models should be studied in the presence of other SCFA, to assure physiological relevance.

Key words: inflammatory bowel disease, IBD, butyrate, IL-8, Caco-2, inflammation

INTRODUCTION

Inflammatory bowel diseases (IBD) are a global health problem. According to Centers for Disease Control and Prevention (CDC)¹, 1 – 1.3 million people in USA, 2.5-3.3 people in Europe and nearly 200,000 people in Canada suffer from IBD (Kaplan, 2015b). The direct health care cost in these three countries is nearly \$13 billion (Kaplan, 2015b). Due to the influence of industrialization from Western civilizations, the incidence of IBD is on the rise in developing nations of Asia, South America and the Middle East. By 2025, the prevalence of IBD in the developing nations will match the current prevalence rate of the western civilizations (Molodecky *et al.*, 2012; Kaplan, 2015b).

There are two types of IBD. Chronic inflammation of the large intestine (colon and rectum) leads to development of Ulcerative Colitis (UC) and chronic inflammation of the digestive tract leads to development of Crohn's disease (CD). IBD has been treated with pharmacological drugs like non-steroidal anti-inflammatory drugs (NSAIDs), mesalazine, sulfasalazine, aminosalicylic acid, TNF-inhibitors or COX-inhibitors. While these drugs are effective in alleviating the symptoms of IBD in several individuals, the effects could be short-term; the sufferers of IBD may develop side-effects to these drugs; or these drugs are not equally effective in all individuals suffering from IBD (Meyer *et al.*, 2006; Paiotti *et al.*,

2012). Hence, there is a need to develop therapies that are effective in all individuals without causing the development of side-effects. This can be achieved through therapeutic dietary interventions.

Increasing the consumption of dietary fiber (DF) among IBD patients is one of the tested dietary intervention strategies. Dietary fiber has been proven to impart anti-inflammatory properties and alleviate some of the IBD symptoms in several animal studies (Bassaganya-Riera *et al.*, 2011; Le Leu *et al.*, 2013; Nishitani *et al.*, 2013; Majumder *et al.*, 2017). The hypothesis is that by increasing the consumption of DF, the gut microbiota would receive more of it and they would ferment the DF to produce butyrate and other short chain fatty acids (SCFA).

. Butyrate is the main energy source for colonocytes; promotes the expression of anti-inflammatory cytokines and suppresses the expression of pro-inflammatory cytokines (Guilloteau *et al.*, 2010). Moreover, butyrate enema studies in humans have shown that butyrate is anti-inflammatory and tends to reduce the symptoms related to UC (Hamer *et al.*, 2009; Hamer *et al.*, 2010; Luceri *et al.*, 2016). Similarly, treatment of induced-colitis with butyrate in mice (Vieira *et al.*, 2012) and rats (D' Argenio *et al.*, 2007) have shown similar alleviation properties of butyrate. Moreover, D' Argenio *et al.* also showed that butyrate treatment reduced the number and size of tumors against colitis induced colorectal cancer, in rats. The anti-inflammatory and anti-oxidative properties of butyrate make it an attractive form of treatment against UC.

Gibson *et al.* determined that the colonic crypt cells obtained from UC and CD patients have increased concentration of pro-inflammatory cytokine IL-8, which can be suppressed by butyrate. Other researchers have found similar suppressive effect of butyrate

against IL-8 (Huang *et al.*, 1997; Pedersen *et al.*, 2000; Lin *et al.*, 2015; Pedersen, 2015).

Hence, IL-8 is a good marker to test the effects of butyrate.

Caco-2 cells are human adenocarcinoma cells, which when grown in the lab for seven days, retain the characteristics of colonocytes (Huang *et al.*, 1997; Engle *et al.*, 1998; Mariadason *et al.*, 2001). This is important because the fermentation of dietary fiber occurs in the colon and the greatest amounts of SCFA are produced in the colon (Cummings, 1981; Cummings *et al.*, 1987). Additionally, Caco-2 cells can be inflamed by exposing them to pro-inflammatory cytokines like tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and interferon- γ (INF γ) (Qiang *et al.*, 2011). Hence, Caco-2 cells are a good model for ulcerative colitis and to determine the effect of SCFA on both – inflamed and non-inflamed cells.

Most of the studies conducted so far have determined the effect of only one short chain fatty acid - butyrate on IL-8 (Gibson & Rosella, 1995; Mariadason *et al.*, 1999; Mariadason *et al.*, 2001). But physiologically, acetate and propionate are also present in high amounts in the colonic lumen (Cummings, 1981; Cummings *et al.*, 1987). Hence, to make it physiologically relevant, we aimed to determine if an increased ratio of butyrate with respect to physiological concentrations of acetate and propionate would suppress the secretion of IL-8 from inflamed and non-inflamed Caco-2 cells.

At the time of designing this experiment, there were no published studies that determined the effect of butyrate on intestinal integrity in the presence of other SCFA. But recently, Chen *et al.* published a paper that determined the effect of exposing inflamed and non-inflamed cells to total SCFA concentration of 40 mM containing 5, 10, 20 or 50% butyrate for 48h on intestinal integrity. However, there are differences between the two studies. For this study, inflamed and non-inflamed Caco-2 cells were exposed to either 60

mM acetate: 10mM propionate: 10mM butyrate or 60 mM acetate: 10mM propionate: 20mM butyrate SCFA mix for 24h, which is more physiologically relevant. To inflame the Caco-2 cells Chen *et al.* exposed them to 10 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) on the apical side of the Transwell plates and 50 ng/mL TNF α on the the basolateral side of the Transwell plates for 48h. In this study, the Caco-2 cells were exposed to 1 $\mu\text{g}/\text{mL}$ LPS on apical side and a cocktail of 25 ng/mL IL-1 β , 50 ng/mL TNF- α and 50 ng/mL IFN- γ on the basolateral side of the Transwell plates for 24h. Due to these differences in the ratio of SCFA, time of exposure to SCFA, concentration and composition of the inflammatory stimulus and time of exposure to the inflammatory stimulus, the two studies are comparable but not same.

Our hypothesis is that when inflamed Caco-2 cells are exposed to an increased ratio of butyrate to physiological concentrations of acetate and propionate (as produced after fermentation of dietary fiber), intestinal integrity is improved and IL-8 secretion is reduced. The aim of this study is to determine the effect of increased ratio of butyrate to physiological concentrations of acetate and propionate on intestinal integrity and suppression of IL-8 secretion.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), 0.5% trypsin - EDTA, antibiotic-antimycotic, L-glutamine, HEPES and Hank's Balanced Salt Solution (HBSS) were purchased from Life Technologies Corp. (Carlsbad, California). Interferon γ – (INF γ), Tumor Necrosis Factor α (TNF α), Interleukin – 1 β (IL-1 β) , lipopolysaccharide (LPS) and Lucifer yellow (LY), sodium acetate, sodium propionate and sodium butyrate were purchased from

Fisher Scientific (Hanover Park, Illinois). The ELISA kit to determine the concentration of IL-8 was purchased from Qiagen (Valencia, California).

Study design

Colorectal carcinoma (Caco-2) cells were used to determine the effect of an increased butyrate concentration in a mixture of butyrate, acetate and propionate, on secretion of IL-8. To assure that Caco-2 cells retained the characteristics of a colorectal cell (Engle *et al.*, 1998), Caco-2 cells were grown for 3 days on inserts of a Transwell plate such that the inserts divided the wells into two chambers: apical and basolateral (Fig. 1).

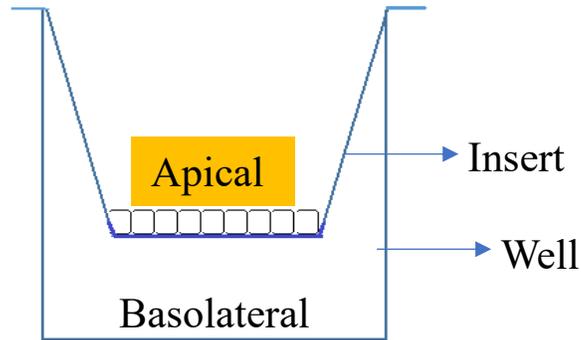


Fig 1: Cartoon representation of how an insert divides the well into two chambers – apical and basolateral.

On the fourth day, Lucifer Yellow transport experiment was conducted to determine the intestinal integrity of the Caco-2 cell monolayer formed on the inserts. On the fifth day, half of wells were inflamed for 24 hours, by exposing the Caco-2 cells to a cocktail of inflammatory stimuli. Following inflammation, the Caco-2 cells were exposed to two different ratios of acetate: propionate: butyrate (Ac : Pr : Bu) for 0h, 12h and 24h. The media was collected after each time point, to determine the concentration of IL-8 secreted by the Caco-2 cells. In this 2 X 2 factorial design, there were four experimental groups. For every

group, there were three replicates and the entire experiment was conducted three times. Hence, $n = 9$ for each of the four experimental groups.

Cell culture

Caco-2 cells were purchased from American Type Culture Collection (ATCC) at passage number 17. Once received, Caco-2 cells were revived and further grown to confluence on 25 cm² flasks. The cells were grown in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 2 mM L-glutamine and 1% antibiotic-antimycotic reagent. The cells were incubated at 37 °C in an atmosphere of 5% CO₂ (Hubatsch *et al.*, 2007).

For the experiments, cells were grown on 75 cm² flasks. Passage numbers 31-36 were used for the experiments. On day 1, the cells were seeded on 12mm polyester membrane inserts with pore size of 0.4 μm. These inserts were fitted in the chambers of a 12-well plate at 1.2×10^5 cells/cm². The fitted inserts would divide the wells into two chambers – apical (AP) and basolateral (BA) as shown in figure 1. By day 3, the cells were ready for the experiment as they were 80% – 90% confluent (Borthakur *et al.*, 2008; Lecona *et al.*, 2008). On day 4, Lucifer Yellow Transport Experiment was conducted to assess the permeability of Lucifer yellow from the AP chamber to the BA chamber. This experiment helped us determine the permeability of the cells. On day 5, DMEM containing an inflammatory stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL) and IFN-γ (50 ng/mL) was added to the BA chamber of half of all the wells, and LPS (1 μg/mL) was added to both – AP and BA chambers of those wells. The other half of wells received only DMEM. After 24 hours, the media was replaced with media containing one of the two ratios of freshly prepared short chain fatty acid (SCFA) mixes: (1) 60 mM acetate: 10 mM propionate: 10

mM butyrate (2) 60 mM acetate: 10 mM propionate: 20 mM butyrate. The medium was collected after 0, 12 or 24 hours of exposure to SCFA mixes from all wells. This medium was then analyzed using ELISA, to determine the concentration of secreted IL-8. In every experiment, three replicates were used for each of the experimental groups: Non-inflamed + SCFA Mix 1, Non-inflamed + SCFA Mix 2, Inflamed + SCFA Mix 1, Inflamed + SCFA Mix 2. The entire experiment was conducted three times. Hence, $n = 9$ for each experimental group.

Lucifer Yellow Transport Experiment

Lucifer Yellow transport experiment was conducted on the fourth day of every experiment to determine the integrity of the barrier function of Caco-2 cells. To conduct the experiment, DMEM in the AP chamber was replaced with 100 μ M Lucifer yellow (LY) in HBSS and only HBSS in the BA chamber. After incubating the plates for one hour at 37°C, the solution from each chamber was collected separately. Absorbance of each sample was measured in duplicates, at 450 nm using the microplate reader (Biotek, Synergy2). Using the standard curve for LY, the absorbance values were then converted to concentration of LY present in each sample to determine the amount of LY that was transported from the AP chamber to the BA chamber (Qiang *et al.*, 2011).

Determination of IL-8 concentration using ELISA

On the fifth day of the experiment, the cells were exposed to inflammatory stimuli for 24 hours, and then treated with SCFA for 0h, 12h or 24h. At the end of each time-point, the medium was collected from each of the AP and BA chambers and stored at -80°C until further analysis. Following the manufacturer's protocol, the medium collected from AP chamber was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit from

Qiagen (Valencia, California), in duplicates. The amount of IL-8 secreted from Caco-2 cells was then calculated using a standard curve.

Cell viability test

The lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Cayman Chemical, Michigan. The kit was used to measure the LDH activity as an indicator of cell death, as suggested in the protocol. Supernatant was collected twice, from the plates during the experiment- post-inflammation and post SCFA exposure. 100µl of the supernatant from each well was transferred to a 96-well plate and then 100µl of the LDH reaction solution (prepared as per the protocol) was added. The plates were gently shaken on an orbital shaker at 37°C for 30 minutes. Then absorbance was read at 490 nm using a microplate reader (Biotek, Synergy2). The absorbance values were compared between treatments.

Statistical Analysis

The data was analyzed using the ANOVA and MIXED models to determine the statistical significance of the differences between the treatments using SAS 9.4 software. P-values < 0.05 were considered to be significant.

RESULTS

Effect of inflammation, SCFA ratio and time on intestinal integrity

LY transport experiment was conducted twice – before inflaming the cells (before inflammation) and after SCFA-exposure. When the concentrations of basolateral LY of all the wells were combined (12 wells for each time point X 3 time points (0h,12h,24h) X 3 repetitions of the experiment), the average concentration of basolateral LY before inflammation was 3.08 ± 2.41 mM (n=108) and after SCFA exposure was 2.44 ± 1.94 mM (n=108). One way ANOVA showed that the average basolateral concentration of LY after

SCFA-exposure was significantly lower than the average basolateral concentration of LY before inflammation ($p=0.0327$, $n=108$), indicating an improvement in the intestinal integrity (Fig 2).

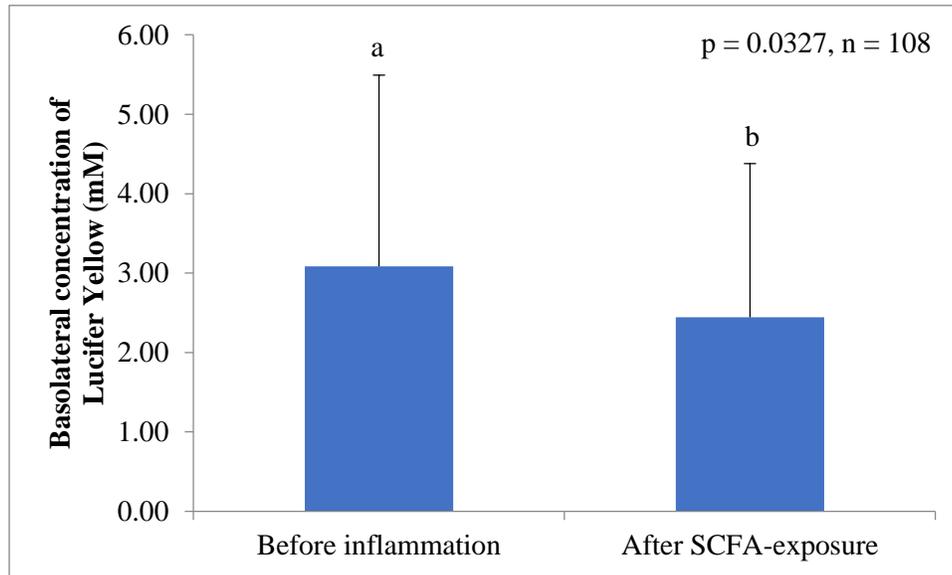


Fig 2: Basolateral concentration of Lucifer Yellow before inflammation and after SCFA-exposure for designated amount of times (0h, 12h, 24h). $p = 0.0327$, $n = 108$. Different letters indicate a significant difference at $p < 0.05$ (Tukey comparison).

In order to determine how the experimental treatments would have contributed towards improving the intestinal integrity, the basolateral concentrations of LY after SCFA-exposure were analyzed by 3-way ANOVA with respect to the three independent variables – inflammation, SCFA ratio and time and interactions between them. Statistical analysis showed that the average basolateral concentration of LY of inflamed cells was significantly greater than the average basolateral concentration of LY of non-inflamed cells ($p=0.0094$, $n=54$), indicating that the intestinal integrity of the cells was weakened (Fig 3).

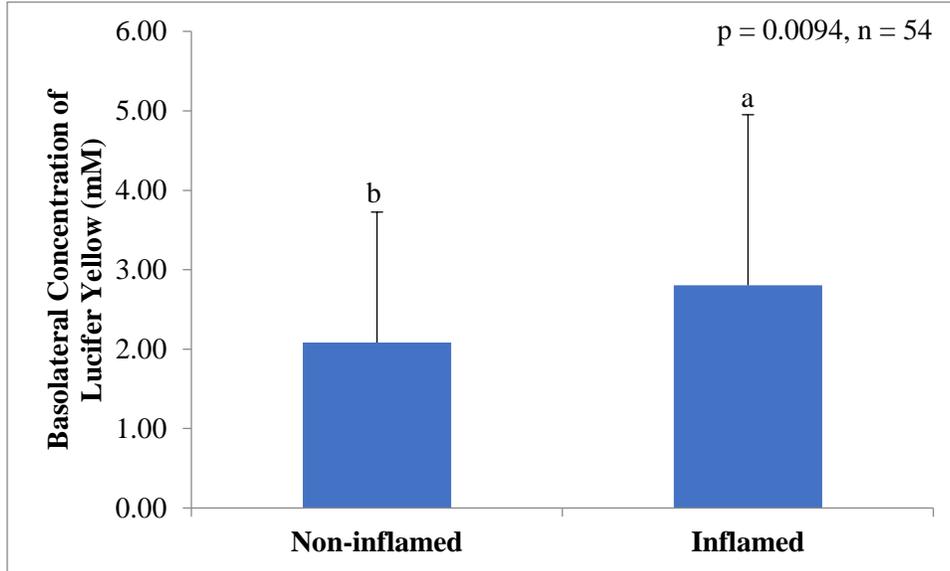


Fig 3: Basolateral concentration of Lucifer Yellow of all inflamed and non-inflamed cells, irrespective of the SCFA ratio and the time of exposure to SCFA mix. Different letters indicate that the treatments were significantly different from one another. $p = 0.0094$, $n = 54$.

Post-SCFA exposure, 3-way ANOVA analysis showed that the average basolateral concentration of LY exposed to the SCFA ratio containing higher amount of butyrate (20 mM) was greater than the average basolateral concentration of LY exposed to the SCFA ratio containing lower amount of butyrate (10 mM), but it was not statistically significant ($p=0.1020$, $n=54$). This indicates that the increased amount of butyrate did not have any significant effect on the intestinal integrity of the cells (Fig 4).

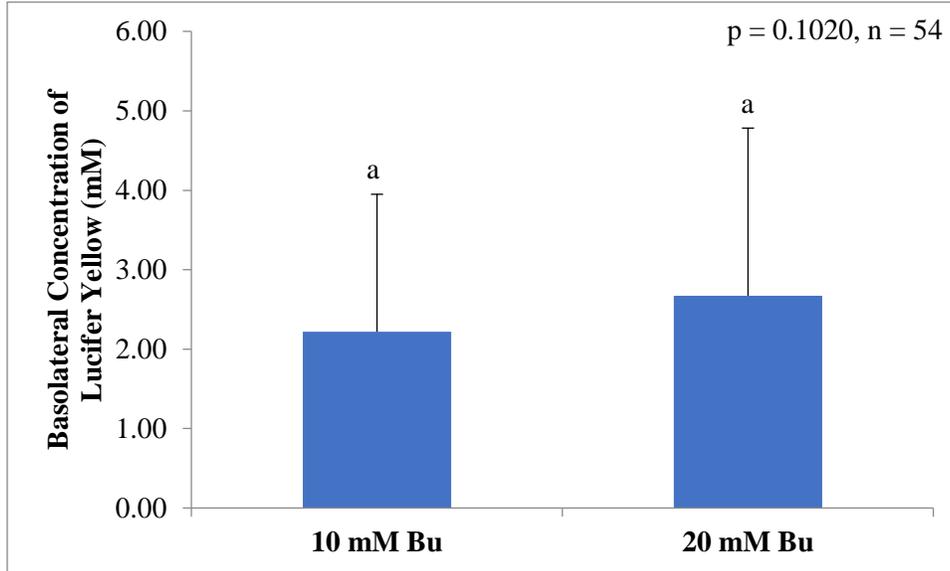


Fig 4: Basolateral concentration of Lucifer Yellow of all the cells exposed to SCFA ratio containing lower butyrate (10mM Bu) or higher butyrate (20mM Bu), irrespective of inflammation and the time of exposure to specific SCFA mix. Different letters indicate that the treatments were significantly different from one another. $p=0.1020$, $n = 54$.

Although there was no significant effect of SCFA ratios on the basolateral concentration of LY, there was an interaction effect between inflammation and the ratio of SCFA mix used, on the basolateral concentration of LY ($p = 0.0238$, $n=27$). The average basolateral concentration of LY of inflamed cells that were exposed to the SCFA ratio containing higher (20mM) concentration of butyrate was significantly greater than that of inflamed cells that were exposed to the SCFA ratio containing lower (10mM) concentration of butyrate ($p = 0.0387$, $n = 27$), indicating that the intestinal integrity of the cells was weakened (Fig 5). The basolateral concentration of LY of the inflamed cells exposed to the SCFA ratio containing higher (20mM) butyrate was significantly greater than that of non-inflamed cells exposed to the SCFA ratio containing higher (20mM) butyrate ($p=0.0103$, $n=27$), indicating that the intestinal integrity of the cells was weakened (Fig 5).

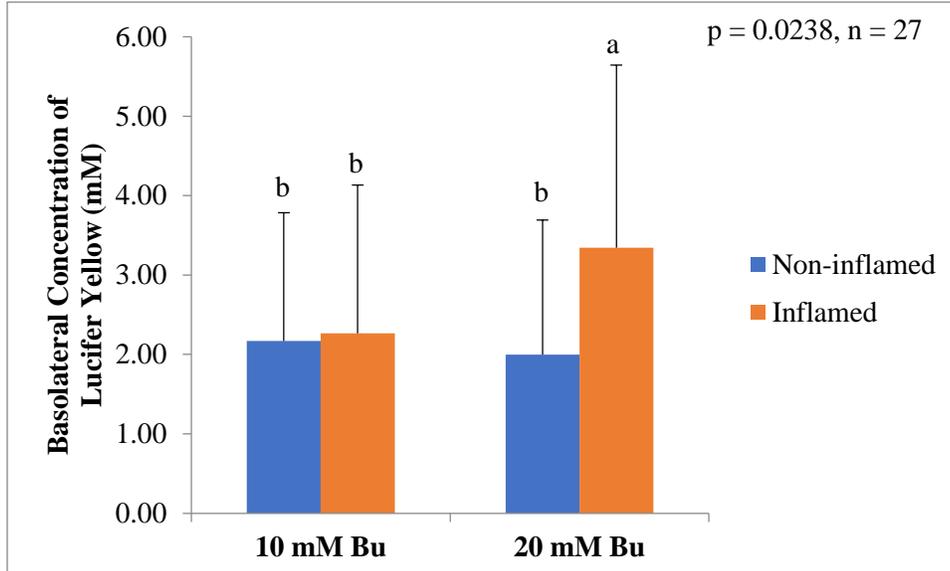


Fig 5: Basolateral concentration of Lucifer Yellow of all the inflamed and non-inflamed cells that were exposed to SCFA ratio containing lower amount of butyrate (10 mM Bu) or higher amount of butyrate (20 mM Bu), irrespective of the time of exposure to SCFA. This graph shows the interaction effect between inflammation and SCFA mix ratio on basolateral concentration of LY. Different letters indicate that the treatments were significantly different from one another. $p=0.0238$, $n = 27$.

The basolateral concentration of LY of inflamed and non-inflamed cells that were treated with SCFA for 12h or 24h, was significantly lesser ($p<0.0001$, $n=36$) than that of inflamed and non-inflamed cells that were treated with SCFA for 0h. However, there was no significant difference between the cells that were treated with SCFA for 12h and the cells that were treated with SCFA for 24h. These results indicate that the intestinal integrity of the cells improved when they were exposed to SCFA for a longer time (Fig 6). There was no significant interaction effect between time and inflammation or time and SCFA ratio on the basolateral concentration of LY. Also there was no significant interaction effect between inflammation, SCFA ratio and time on the basolateral concentration of LY.

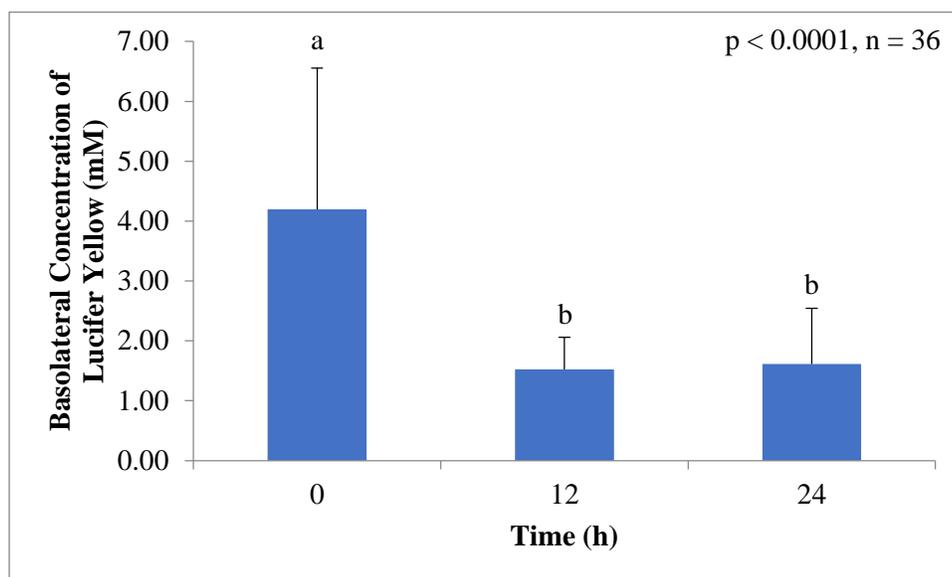


Fig 6: Basolateral concentration of Lucifer Yellow of all the cells exposed to SCFA for specific time-points, irrespective of inflammation and the SCFA ratio they were exposed to. Different letters indicate that the treatments were significantly different from one another. $p < 0.0001$, $n = 36$.

Effect of inflammation, SCFA ratio and time on IL-8 secretion

In order to determine the effect of the independent variables (inflammation, SCFA ratio and time) and interactions between them on the secretion of IL-8, the concentrations of IL-8 determined by ELISA were analyzed using 3-way ANOVA. Statistical analyses showed that cells that were inflamed, secreted significantly greater amount of IL-8 ($p < 0.0001$, $n = 54$) than the non-inflamed cells (Fig 7), which was expected because IL-8 is a pro-inflammatory chemokine.

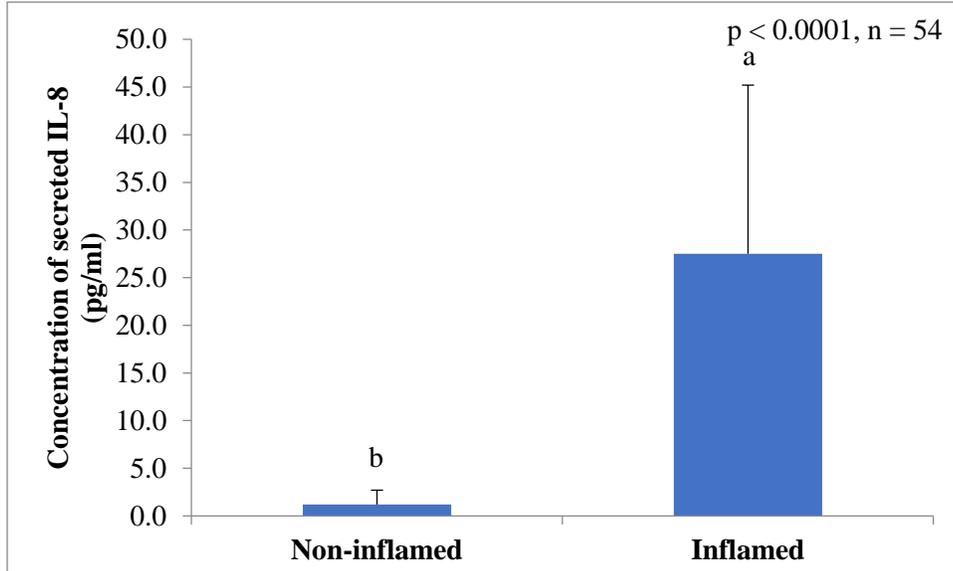


Fig 7: Concentration of IL-8 secreted from all inflamed and non-inflamed cells, irrespective of the SCFA ratio used and the time of exposure to SCFA mix. Different letters indicate that the treatments were significantly different from one another. $p < 0.0001$, $n = 54$.

Post-SCFA exposure, 3-way ANOVA analysis showed that the cells that were exposed to SCFA mix containing higher (20mM) amount of butyrate slightly increased the secretion of IL-8, but was not significantly different ($p=0.1162$, $n=54$) when compared with the cells that were exposed to SCFA mix containing lower (10mM) amount of butyrate. This indicates that the ratio of SCFA mixes used did not have any significant effect on secretion of IL-8 (Fig 8).

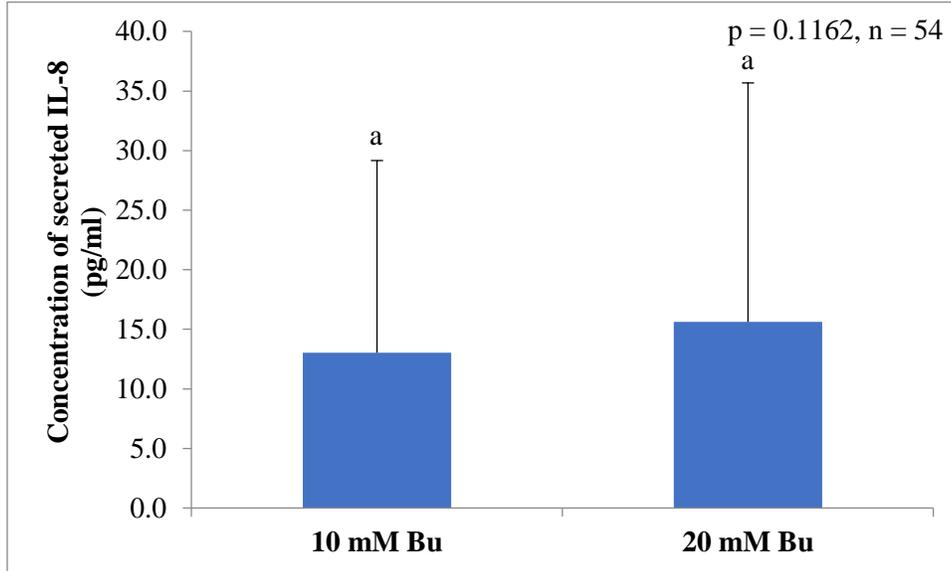


Fig 8: Concentration of IL-8 secreted from all the cells that were exposed to SCFA mix containing lower amount of butyrate (10mM Bu) or higher amount of butyrate (20mM Bu), irrespective of inflammation and the time of exposure to specific SCFA mixes. Different letters indicate that the treatments were significantly different from one another. $p=0.1162$, $n=54$.

Post-SCFA exposure, the time for which the cells were exposed to specific SCFA ratios had a significant effect ($p<0.0001$, $n=36$) on the secretion of IL-8; and, the secretion of IL-8 increased with an increase in the amount of time for which the cells were exposed to the SCFA mixes (Fig 9).

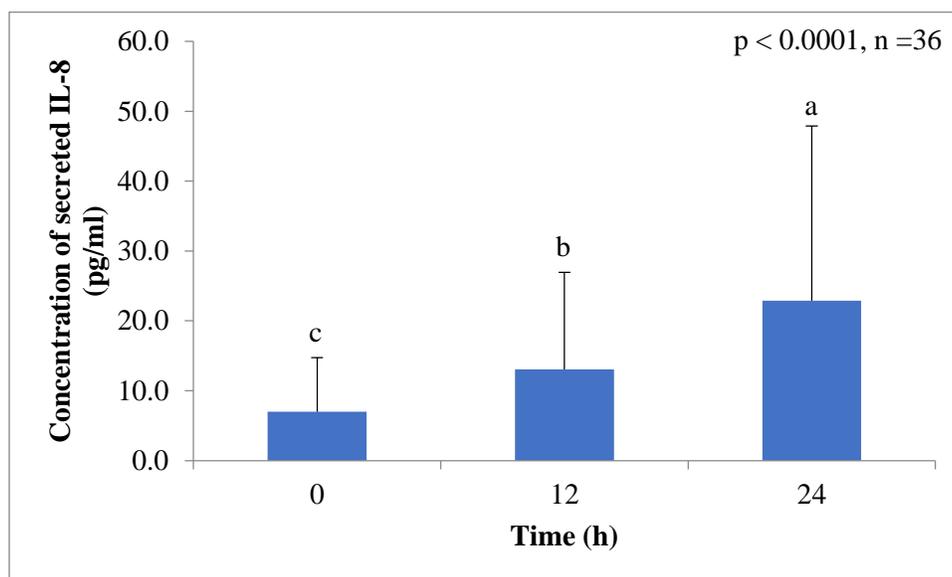


Fig 9: Concentration of IL-8 secreted from all the cells exposed to SCFA for specific time-points, irrespective of inflammation and the SCFA mix ratio they were exposed to. Different letters indicate that the treatments were significantly different from one another. $p < 0.0001$, $n = 36$.

Statistical analysis showed that there was a significant 2-way interaction effect between inflammation and time on the secretion of IL-8 (figure not shown). However, there were no significant 2-way interaction effects between inflammation and the SCFA ratio or time and SCFA ratio, on the secretion of IL-8. Also there was no significant 3-way interaction effect between inflammation, SCFA ratio and time, on the secretion of IL-8.

Effect of inflammation, SCFA ratio and time on LDH activity

LDH activity of the cells from each well was measured from the medium collected after inflammation and after SCFA exposure, using the LDH cytotoxicity assay. Post-inflammation, one-way ANOVA showed that the LDH activity of inflamed cells was significantly higher than the LDH activity of non-inflamed cells ($p < 0.0001$, $n = 54$), indicating that the inflamed cells were more damaged than the non-inflamed cells (Fig 10).

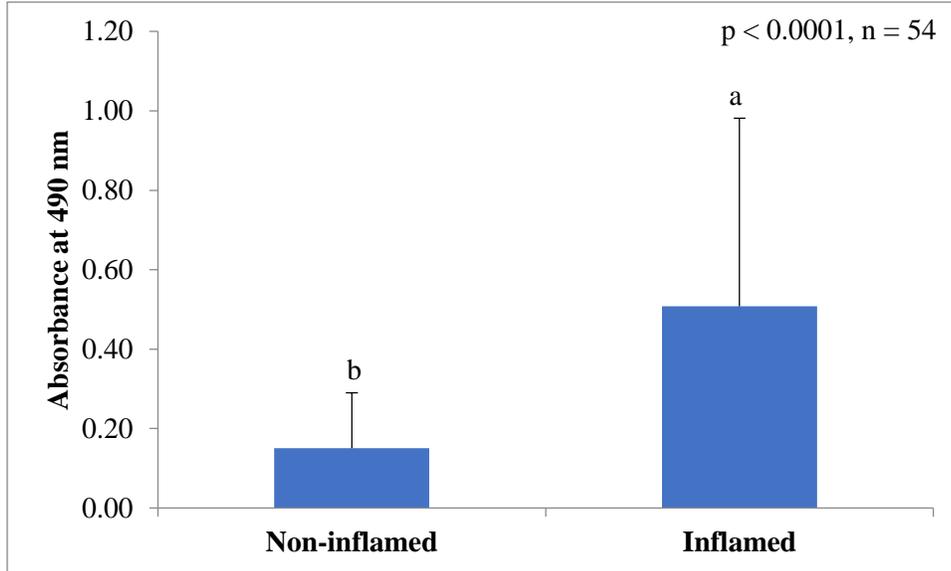


Fig 10: Post-inflammation, activity of lactate dehydrogenase of inflamed and non-inflamed cells. Different letters indicate that the treatments were significantly different from one another. $p < 0.0001$, $n = 54$.

Post-SCFA exposure, 3-way ANOVA analysis showed that the LDH activity of the cells that were exposed to SCFA mix containing higher (20mM) amount of butyrate was significantly greater ($p=0.0161$, $n=54$) than the LDH activity of the cells that were exposed to SCFA mix containing lower (10mM) butyrate, indicating that the cells that were exposed to higher butyrate containing SCFA mix were more damaged than the cells that were exposed to lower butyrate containing SCFA mix (Fig 11).

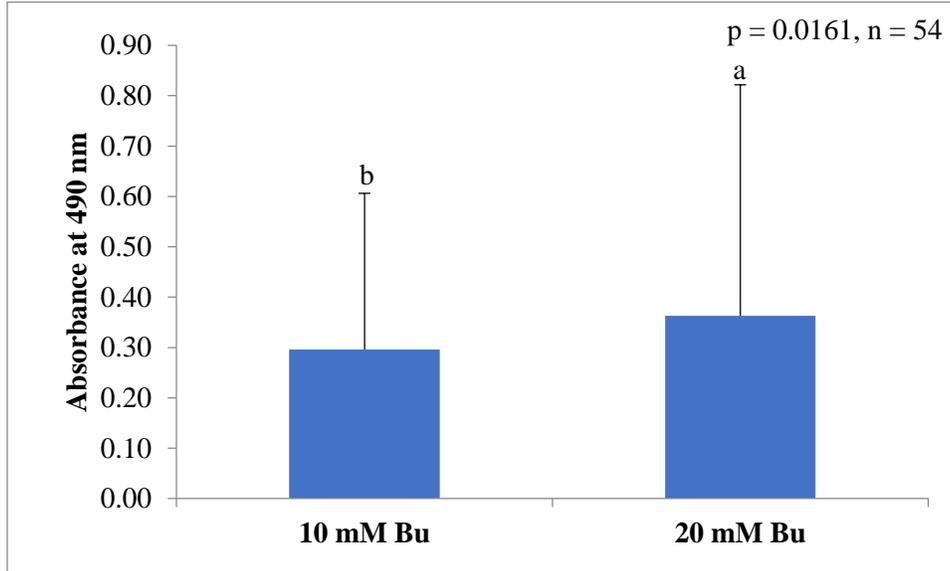


Fig 11: Post-SCFA exposure, activity of lactate dehydrogenase of the cells that were exposed to SCFA mix containing lower amount of butyrate (10 mM Bu) or higher amount of butyrate (20 mM Bu), irrespective of inflammation and the time of exposure to specific SCFA mixes. Different letters indicate that the treatments were significantly different from one another. $p=0.0161$, $n=54$.

Post-SCFA exposure, the LDH activity significantly increased with increased time of exposure to SCFA ($p<0.0001$, $n=36$). This indicates that the cell damage increased with an increase in the amount of time the cells were exposed to SCFA mixes (Fig 12). All the pairwise interactions between inflammation, SCFA ratio and time also had a significant effect on LDH activity.

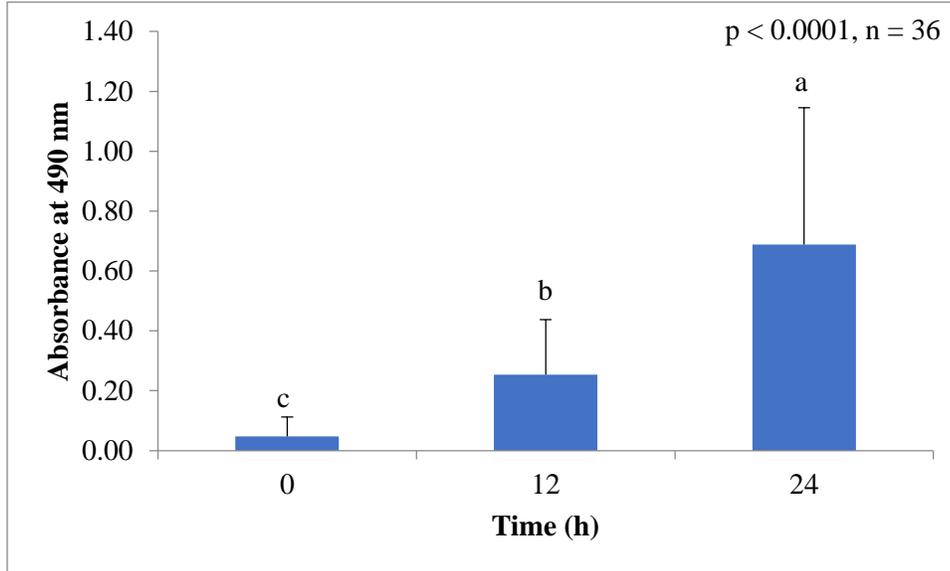


Fig 12: Post SCFA exposure, activity of lactate dehydrogenase of cells that were exposed to SCFA mixes for specific amount of times, irrespective of inflammation and SCFA ratio they were exposed to. Different letters indicate that the treatments were significantly different from one another. $p < 0.0001, n=36$.

DISCUSSION

Transfer of Lucifer Yellow from the apical chamber to the basolateral chamber has been measured as a marker to determine intestinal integrity in Caco-2 cells by several researchers (Kadota *et al.*, 2013; Nemoto *et al.*, 2015). According to this study, inflammation compromised the intestinal integrity of Caco-2 cells, as measured by the transfer of the permeability marker - Lucifer yellow, which was expected. Research has shown that TNF- α , IL-1 β and INF- γ decrease the intestinal integrity and make them more permeable (Ma *et al.*, 2004; Beaufepaire *et al.*, 2009; Pedersen, 2015). But in this study, the time of exposure to SCFA, tended to improve the intestinal integrity of the cells between 0h to 12h and 0h to 24h, but not between 12h to 24h (Fig 6). While there was no significant effect of the ratio of SCFA used, the interaction effect of inflammation and the SCFA ratio tended to affect the

intestinal integrity of the cells, in this study. Chen *et al.* saw a similar increase in the intestinal integrity of Caco-2 cells as measured by trans-epithelial electrical resistance (TEER), when they were exposed to SCFA over 48h. Moreover, they found that the TEER values were significantly correlated to total SCFA concentration ($r = 0.923$, $p = 0.009$), acetate ($r = 0.953$, $p = 0.003$), and butyrate ($r = 0.813$, $p = 0.049$). They found that when Caco-2 cells were exposed to the SCFA ratios: 24mM (60%) acetate: 8mM (20%) propionate: 8mM (20%) butyrate or 15mM (37.5%) acetate: 5mM (12.5%) propionate: 20mM (50%) butyrate, the TEER values at 48h were increased by 1.4-fold and 1.7-fold, respectively (Chen *et al.*, 2017). Contrary to our hypothesis, the results of our study indicated that inflamed cells that were exposed to the ratio of 60 mM Ac: 10mM Pr: 20mM Bu (higher butyrate) increased the transport of Lucifer Yellow from the apical side to the basolateral side. This would be possible only when the Caco-2 cell monolayer was more permeable due to compromised intestinal integrity. These results in contrast with Chen *et al.* suggest that the ratio and concentration of SCFA used may influence the intestinal integrity of the cells. The other thing to note is that butyrate may act differently in the presence or absence of SCFA. Peng *et al.* found that when Caco-2 cells were exposed to 8mM butyrate, the TEER values were not significantly different from that of Caco-2 cells not treated with butyrate for 24h. But by 48h the TEER values of Caco-2 cells exposed to 8 mM butyrate was 1.25-fold lower than the TEER values of untreated Caco-2 cells and it kept declining until it was lower by 8-fold at 96h. Whereas the TEER values of Caco-2 cells that were exposed to 2mM butyrate for 72 hours, were 1.5-fold significantly higher than that of untreated Caco-2 cells at 48h and they remained steady until 96h (Peng *et al.*, 2007). These results are in contrast with that of Chen *et al.* While Peng *et al.* reported that cells treated with 8 mM butyrate had lower TEER

values by 48h, Cheng *et al.* observed an improvement in the TEER values in cells treated with 8mM butyrate containing SCFA mix for 48h. These differences in the TEER values may be due to the presence of other SCFA. Hence, it is crucial that future projects that aim to study the effect of butyrate on intestinal integrity, be conducted in the presence of other SCFA.

Similar to the LY results, inflammation and the time of exposure to SCFA increased the secretion of IL-8 but as opposed to our hypothesis, the ratio of SCFA used did not have any effect on the secretion of IL-8. These results are different from that of previous researchers. Huang *et al.* found that when 5-days old Caco-2 cells that were inflamed by exposure to 5 ng/ml IL-1 β for 4hours, they secreted high concentrations of IL-8. When these inflamed cells were then treated with 2.5 mM sodium butyrate for 72 hours, the IL-8 secretion was reduced by 6-fold (Huang *et al.*, 1997). Gibson *et al.* observed that when Caco-2 cells were exposed to 10 μ M TNF- α for 24h, the secretion of IL-8 doubled in comparison with untreated Caco-2 cells. IL-8 secretion nearly doubled even when the Caco-2 cells were exposed to 2mM butyrate for 24h. However, when Caco-2 cells were pre-treated with 10 μ M TNF- α , washed and then exposed to 2 mM butyrate, IL-8 secretion decreased by 34% below basal levels ($p = 0.03$). These differences could probably be because these researchers used only butyrate as opposed to the mixture of acetate, propionate and butyrate used for this study. The difference in our results from that of previous researchers could also be attributed to the concentration of butyrate and the inflammatory stimuli used. We used the specific SCFA ratios to mimic the physiological concentrations found in a human being (Cummings, 1981; Cummings *et al.*, 1987). To be able to mimic the aspect of consuming higher-fiber diet, we decided to double the concentration of butyrate in the SCFA mix, because

fermentation of dietary fiber leads to increased production of butyrate. The inflammatory stimulus that we used was a mixture of TNF α , IL-1 β and INF- γ for basolateral side and LPS for the apical side, to represent acute inflammatory reactions and mimic chronic and severe inflammatory bowel disease (Chavez *et al.*, 1999; Qiang *et al.*, 2011).

The LDH activity results also showed that inflammation, higher butyrate containing SCFA mix and increasing amount of time of exposure to SCFA increased the LDH activity, indicating that these factors led to more cell-damage. LDH cytotoxicity test is a measurement of the amount of cytosolic lactate dehydrogenase in the supernatant that is released from cells that are damaged. This assay has been used by several researchers as a test of cytotoxicity or cell viability (Carrasco-Pozo *et al.*, 2010; Kadota *et al.*, 2013; Pedersen, 2015). Huang *et al.* did not report the results but mentioned that Caco-2 cells treated with 20 mM butyrate led to increased toxicity. Peng *et al.* also found that Caco-2 cells exposed to 8 mM butyrate for 72 hours, led to a 5-fold increase in the number of cells that were floating (indicator of dead cells) in comparison with Caco-2 cells that were not treated with butyrate. They did not find a similar apoptosis rate in cells that were exposed to 2 mM butyrate for 72 hours (Peng *et al.*, 2007). These results suggest that the concentration of butyrate used in the mixture or the presence of other SCFA could have influenced the amount of cell damage incurred by the Caco-2 cells.

CONCLUSION

Although our hypothesis - when inflamed Caco-2 cells are exposed to an increased ratio of butyrate to physiological concentrations of acetate and propionate (as produced after fermentation of dietary fiber), intestinal integrity is improved and IL-8 secretion is reduced was disproved, several lessons can be learned from this study. Firstly, butyrate may behave

differently in the presence of other SCFA. So for future studies it is important to study the effects of the combination of SCFA as opposed to butyrate alone. Secondly, the ratio and concentration of SCFA used should be carefully selected such that it is physiologically relevant and not highly toxic to cells *in vitro*. This would be extremely important while simulating the effects of consumption of high-fiber diets that would lead to increased butyrate concentration in the colon. Future work is required to determine the concentrations of SCFA and change in the concentration of SCFA in the colon due to high-fiber consumption and the effect of these changes on the intestinal epithelial cells.

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CHAPTER 3: GENERAL CONCLUSIONS

Several studies have been conducted to determine the effect of dietary fiber and short chain fatty acids on general health and in diseased states. While there is evidence from epidemiological studies regarding the inverse relationship between dietary fiber intake and the risk of developing inflammatory bowel diseases, more research needs to be done to prove if dietary fiber acts as a preventative measure or a therapeutic one. Also, one should note that there are different types of dietary fibers that have different gastrointestinal fate. Hence, the protective or reparative effects of dietary fibers need to be characterized by its type.

An increased concentration of butyrate due to higher fermentation of dietary fiber is one of the mechanisms that has been attributed towards its protective effect. Several studies have been conducted to determine the effect of butyrate on intestinal epithelial cell *in vivo* and *in vitro*. However, it is important to note that the colonic lumen contains other byproducts of microbial fermentation that may impact the effect of butyrate. Hence, future studies should be mindful about determining the effect of butyrate in the presence of other luminal products like short chain fatty acids like acetate and propionate which are also to be found at high concentrations in the lumen. Moreover, the ratio and concentrations of these SCFA should be carefully selected such that it is physiologically relevant and not highly toxic to cells *in vitro*. This would be extremely important for *in vitro* studies that would like to simulate the effects of consumption of high-fiber diets that leads to an increase butyrate concentration in the colon.

Future work is required to determine the *in vivo* concentrations of SCFA and change in them due to high-fiber consumption and the effect of these changes on the intestinal epithelial cells in healthy and diseased states.