Short-chain fatty acids influence host immunity, mucus secretion and microbial community structure to reduce enteritis

by

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Abstract

Optimal intestinal health is critical to overall host well-being, and acute and chronic enteric inflammatory diseases impart a significant detrimental impact on Canadians. The consumption of dietary fibre (DF) has long been associated with providing a health benefit to individuals, and regulatory organizations including Health Canada have legislated that scientific evidence be required to validate health claims. Short-chain fatty acids (SCFA) are produced by the microbial fermentation of DF in the colon, with acetate, propionate and butyrate being the most abundantly produced in the colon. Studies analyzing the effect of DF fermentation in pathogen-challenge models are limited. Thus, the overarching goal of this research was to determine how DFs, and in particular the by-products of DF fermentation, impact enteric inflammation and overall host health using an intestinal pathogen to incite inflammation. Two studies were conducted using *Citrobacter rodentium* to incite acute Th1/Th17 inflammation (i.e. enteritis). In the first study, the impact of butyrate on the host-microbiota relationship was examined in mice ± enteritis. Rectal administration of 140 mM butyrate to mice increased fecal concentrations of butyrate, and increased food consumption and weight gain in mice with enteritis. Histological scores of colonic inflammation 14 and 21 days post-infection (p.i.) were lower in infected mice administered 140 mM butyrate. In mice without enteritis, butyrate administration elevated the expression of *IL-10*, *TGFβ*, and *Muc2* in comparison to mice not administered butyrate. Infected mice administered butyrate displayed elevated expression of genes necessary for pathogen clearance (i.e. *IL-17A, IL-1β*), and epithelial barrier repair and restoration (i.e. *Relmβ, Tff3, Myd88*). Butyrate supplemented to inflamed colons increased *Proteobacteria* and *Lachnospiraceae,* and reduced the abundance of *Clostridiaceae* species. Mice with enteritis that were administered butyrate

exhibited increased accumulation of mucus in the colonic lumen and within goblet cells. In the second study, the impacts of the DFs, WB and RS on host enteric health were measured in mice ± enteritis. Diets enriched for RS increased weight gain in mice inoculated with *C. rodentium* compared to mice consuming a conventional control (CN) diet. Cecal and distal colonic SCFA quantities were higher in mice consuming DFs, and DF consumption increased butyrate concentrations in the distal colons of mice with enteritis. Histopathologic sores of inflammation in the proximal colon on day 14 (peak infection) and 21 p.i. (late infection) were lower in mice consuming DF-enriched diets compared to the CN diet. Consumption of WB reduced the expression of Th1/Th17 cytokines. Alternatively, the expression of bacterial recognition and response genes such as *Relmβ*, *RegIIIγ*, and *TLR4* increased in mice consuming the RS-enriched diets. Furthermore, each diet selected for different bacterial communities in the cecum, proximal and distal colon, suggesting a link between DF fermentation, SCFA concentrations, and inflammation in the murine colon. Collectively, study two data indicated that the consumption of DF-rich diets ameliorate the effects of *C. rodentium*-induced enteritis by modifying the host microbiota to increase SCFA production, and bacterial recognition and response mechanisms to promote host health. In conclusion, SCFA are important to colonic health, and when administered directly to colons primarily influence host immunity through the activation of innate factors, while SCFA derived from DFs in diets modify the host microbiome to effectively maintain intestinal homeostasis and promote host health. Notably, this research provides foundational information to ascertain the effects of functional foods on intestinal health and host well-being.

Preface

This thesis is an original work by Janelle A. Jiminez. The research project, of which this thesis is a part, received research ethics approval from the Animal Care Committee at Agriculture and Agri-Food Canada for the following projects: "Dietary fibre by-products and the intestinal health of Albertans" ACC Protocol 1322, May 2013- Aug 2014; "Short-chain fatty acids from dietary fibre breakdown and the intestinal health of Albertans" ACC Protocol 1338, Dec 2014- Jun 2014; and "Dietary fibre and intestinal health of Albertans: Use of a murine model to elucidate the diet-intestine-pathogen-microbiota interaction" ACC Protocol 1405, Nov 2012- Sep 2014.

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I dedicate this thesis

to all of the positive female influences in

my life who continue to motive me to put 100%

into everything I do. Without their influence,

I would not be who I am today, and would

not be achieving my goals. Thankyou

Mom, SR, RM, KM, AA, LO,

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SCFA	Short-chain fatty acid
DF	Dietary fibre
WB	Wheat bran
NSP	Non-starch polysaccharides
RS	Resistant starch
BCFA	Branched-chain fatty acids
HCO_3^-	Bicarbonate
DNA	Deoxyribonucleic acid
HDAC	Histone deacetylase
IFN	Interferon
iNOS	Inducible nitric oxide synthase
GALT	Gut-associated lymphoid tissue
PP	Peyer's patch
TLR	Toll-like receptor
Ig	Immunoglobulin
NFκB	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerization domain
APC	Antigen presenting cell
NK	Natural killer
MYD88	Myleoid differentiating factor 88
AMP	Antimicrobial peptide
CD	Cluster of differentiation
MHC	Major histocompatibility complex
TNFα	Tumour necrosis factor
Th	Helper T-cell
IL	Interleukin
TGF	Transforming growth factor
STAT	Signal transducer and activator of transcription
Foxp3	Forkhead box p3
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CrD	Crohn's disease
TFF	Intestinal trefoil factor
RELM	Resistin-like molecule
EPEC	Enteropathogenic *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
LEE	Locus of enterocyte effacement
TMCH	Transmissible murine crypt hyperplasia
CFU	Colony forming unit
UC	Ulcerative colitis
PAS	Periodic acid, Alcian Blue Schiffs stain
FISH	Fluorescent *in situ* hybridization
MCT	Monocarboxylate transporter
GPR	G-couple protein receptor
NLRP	Nucleotide-binding domain, leucine rich containing protein

Chapter 1. Literature Review[1]

1.0 Introduction

In 2010, Health Canada redefined dietary fibre (DF) to introduce consumers to new standards regarding foods considered to be beneficial for intestinal health. At that time, the 1985 definition was outdated and inadequate, and highlighted that the amount of fibre consumed by Canadians failed to provide added benefits to consumer health. As organizations such as the US Food and Nutrition Board Institute of Medicine (IOM), the American Association of Cereal Chemists (AACC), the European Food Safety Authority (EFSA), and the Codex Alimentarius Commission recognized materials such as oligosaccharides, synthetic fibres and resistant starches (RS) as forms of DF, Health Canada followed these organizational standards and realigned the definition to include these products (Health Canada, 2010). Redefining DF also meant that manufactures needed to demonstrate improved health from the consumption of DF-enriched diets. Subsequently, research examining the mechanisms involved in improving intestinal health following DF consumption became more important. In particular, research began to focus on the health benefits of DF fermentation within the colon, and how the production of the bioactive short-chain fatty acids (SCFA) acetate, propionate and butyrate interact with the intestine (Bach Knudsen, 2015; Donohoe et al., 2014).

The human intestine is a well-organized ecosystem of complex microbial communities. These communities interact with one another and the host, and are heavily influenced by diet and nutrient availability. To date, approximately $10^{13\text{-}14}$ bacteria with over 1000 species have been identified in the human intestine (Gill et al., 2006). Many of these species provide energy to the colon through their production of SCFA, which are produced from the fermentation of non-digestible food materials that resist digestion by stomach acid and small intestinal enzymes (Lawley and Walker, 2013). In the large intestine, resident populations of bacteria provide the host with a variety of nutrients, and also assist with regulating the host immune system to protect

[1] Portions of this chapter have been published in the article "Animal models to study acute and chronic intestinal inflammation in mammals," 2015, Gut Pathogens, 7:29.

against mucosal injury from intestinal pathogens (Eckburg et al., 2005). These resident microbes also directly support the growth of other commensal bacteria and inhibit the growth of competing pathogens (Sassone-Corsi and Raffatellu, 2015). For example, resident microaerophilic, facultative anaerobes, and anaerobic bacterial species co-exist within the intestine, and provide substrates for other resident commensal bacteria to grow and colonize in the host (Rios-Covian et al., 2016). Moreover, robust colonies of resident bacteria also compete for nutrients and release bioactive products through colonization resistance mechanisms that inhibit the growth and establishment of transient, pathogenic species (Stecher and Hardt, 2008).

The intestinal microbiome is essential to maintain the health and function of the intestine. As such, the microbial population is maintained in a relatively balanced state of homeostasis. Dysbiosis can occur when an imbalance exists within the microbiota, and consequences to the host can be profound, as dysbiosis can induce both acute and chronic intestinal inflammation (Tabas and Glass, 2013). Furthermore, fulminant dysbiosis in some incidences may be fatal in people. Although the beneficial effects of various bacterial species within the host microbiome are recognized, many of the bacterial species involved in this process are still being identified and characterized.

Fermentation of DF leads to the increase in SCFA quantities in the intestine and this increase in SCFA induces changes within the colon to reduce the induction of the intestinal pro-inflammatory immune response. The intestinal microbiome is a complex structure and research shows that changes in species diversity and abundance can affect the overall health status of an individual (The Human Microbiome Project, 2012). For instance, an increase in the abundance of *Bacteroidetes* in the intestine of individuals with Type 2 diabetes occurs in concert with the reduction of the *Firmicutes* population, (Qin et al., 2012). In contrast, obese individuals with Type 2 diabetes have large populations of *Firmicutes* in comparison to *Bacteroidetes* populations (Ley et al., 2005). Other bacterial species have been associated with improved intestinal health, including species that colonize intestinal mucus. For example, *Christensenella minuta* has been isolated in high numbers from the colons of lean humans, and its presence post human fecal transplantation suggest that this bacterium interacts with host metabolism to minimize weight gain in individuals (Goodrich et al., 2014).

Furthermore bacterial species such as *Akkermansia muciniphila* and *Mucispirillum schaedleri* are present in high numbers within the gut of healthy individuals (Dao et al., 2015; Hartstra et al., 2015; Wong et al., 2012).

Various bacterial species also promote the production of SCFA, and research has shown that bacterial species ferment select forms of DF such as resistant starches (RS), wheat brans (WB), and inulin, and these promote the production of specific SCFA that can reduce inflammation (den Besten et al., 2013). As an example, inulin stimulates the production of propionate (Levrat et al., 1991), while RS increases butyrate production (Birt et al., 2013). Diets enriched in RS or those with high WB content select for specific bacterial species. For instance, RS-rich diets can increase intestinal populations of *Bifidobacterium* spp., while *WB-rich* diets increase *Ruminococcus* spp., *Eubacterium* spp., and *Lactobacillus* spp. bacterial populations in monogastric animal models (Nielsen et al., 2014). In general, most bacterial species can influence inflammation by producing products that effect intestinal immune function. Several individual bacterial species, however, have a greater influence on intestinal health compared to others. *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia* and *Deferribacteres* have been shown to impact intestinal health, and their growth can be affected by diet-derived substrates. These bacteria facilitate the production of metabolites that can be beneficial to host health (Backhed et al., 2005). Therefore, the addition of DF to diets that stimulate the growth of specific bacterial phyla and individual species could be an effective strategy to improve intestinal health and limit intestinal disease.

It is proposed that the microbiome can improve gut health by increasing the production of mucin, a known component of the acellular innate immune system. Current research suggests that intestinal SCFA can increase mucus production within the gut, and importantly, can prevent bacteria from penetrating the mucosal epithelium and activating pro-inflammatory cytokine pathways within the gut-associated lymphoid tissue (GALT) that induce tissue inflammation. As an example, butyrate can increase *Muc2* gene expression in the murine colon (Gaudier et al., 2009) and MUC2 protein release in human colon cancer cell lines (Hatayama et al., 2007), however this response has not been demonstrated in the human colon (Hamer et al., 2010).

Numerous studies show that mice lacking the intestinal mucin MUC2 are more susceptible to developing intestinal inflammation, suggesting that the intestinal mucus layers minimize interactions between pathogenic bacteria and the host (Bergstrom et al., 2010; Hasnain et al., 2010; Van der Sluis et al., 2006). Determining a causal link between the consumption of dietary products that increase the production of intestinal mucus could therefore provide an effective strategy to mitigate intestinal inflammation.

Gaining foundational information on the mechanisms of these products on intestinal health permits further development of products that could mitigate intestinal disease. The first study examines the impact of rectally administered butyrate on intestinal inflammation, mucin production and changes to the microbiota in a pathogen-challenged mouse model. The second and final study examines the effects of diets rich in fermentable RS and WB on intestinal inflammation, mucin production and changes to the microbiota in a pathogen-challenged mouse model. The focus of this literature review is to provide background information on the following: 1) DF and its beneficial roles in host health; 2) chemical and structural composition of butyrate, RS and WB; 3) the intestinal microbiome; 4) the effects of RS and WB on intestinal physiology and immune function, and the intestinal microbiota; 5) the host intestinal immune system and anatomy; 6) the use of animal models to investigate intestinal disease; and finally 7) the use of *Citrobacter rodentium* as biological incitant of intestinal inflammation.

1.1 Health Canada dietary fibre definitions

The definition of DF proposed by Health Canada has evolved over the last 30 years. An early definition of DF indicated that 'endogenous components of plant material' resistant to digestion by human enzymes such as non-starch polysaccharides, lignin and associated substances were the main constituents of DF (Health Canada, 2010). Three years later, the definition was adjusted to include newly developed novel products. These 'novel fibres' were not traditionally used for human consumption, and had either been extensively chemically and physically processed, or were composed of highly concentrated plant material (Health Canada, 1988, 2010). This adjustment in the definition aligned its requirements for DFs to previously established standards

used by other countries: the United States, New Zealand, Australia, France, and other European Union members. Subsequently, another modification to the definition was added, that included fructo-oligosaccharides and galacto-oligosaccharides, resistant starches, other insoluble products derived from starch, undigested animal fibres, and synthetically produced indigestible products (Health Canada, 1988, 2010).

Finally, the latest definition of DF also required the verification of 'improved health', measured by the physiological effects that result from DF consumption. These physiological effects included: improved stool movements by the increase in fecal bulking, reduced total amounts of low density lipoprotein and cholesterol, reduced post prandial blood glucose and insulin levels, and increased production of energy-rich metabolites following DF fermentation (Health Canada, 2012). Thus, the current Health Canada definition of DFs states: 'DF are carbohydrates with a degree of polymerization of three or more saccharide units, and occur naturally in foods of plant origin, or are novel fibres with ingredients manufactured or synthetically produced, and have at least one physiological effect demonstrated by accepted scientific evidence' (Health Canada, 2012). In summary, these progressive changes in the definition of DF not only improved the standard requirements of the constituents of DF, but also regained public confidence in food safety and health, by requiring food manufactures to demonstrate 'proven' health benefits of DF consumption.

1.1.1 Beneficial health claims associated with dietary fibre in diets

Over the years, there has been a steady increase in research demonstrating that DF can improve the health of individuals. As examples, the consumption of DF such as cereal fibres (8-11 g) and whole grains (30-40 g) has been associated with a reduction in the incidence of Type 2 diabetes, cardiovascular disease (CVD) and obesity (Cho et al., 2013). Data from both animal models and people show that diets rich in DF can reduce the incidence of constipation, ulcerative colitis (UC), colon cancers, and improve colonic function (Martinez et al., 2010). Furthermore different types of DF such as RS and WB fractions reduce cholesterol, glucose, and insulin collected from the blood (Boll et al., 2015; Health Canada, 2012; Raigond et al., 2015).

Cardiovascular disease (i.e. cerebral vascular and coronary vascular disease), obesity and diabetes are leading causes of death throughout the world, and it has been shown that certain diets reduce the prevalence and severity of these diseases (Otles and Ozgoz, 2014; Sanchez-Muniz, 2012; World Health Organization, 2015). As examples, diets rich in DF lowered the prevalence of coronary heart disease, stroke, diabetes, and obesity by 34%, 26%, 29% and 30% respectively (Anderson et al., 2009; Lairon et al., 2005). In the intestine, enteritis is a common manifestation and it has been shown that the incidence of disease can be altered through DF consumption. Using a rat model of chemically-induced colitis, Videla et al. (2001) demonstrated that oral supplementation of inulin reduced neutrophil infiltration in the mucosa, and improved histological scores of intestinal injury (Videla et al., 2001). In other experiments, the administration of DF isolated from *Plantago ovata* seeds increased colonic butyrate concentrations, and facilitated recovery in patients convalescing from UC (Fernandez-Banares et al., 1999). Similar findings were also observed in people with active UC provided diets rich in germinated barley foodstuff (GBF). In this clinical trial, fermented GBF increased the content of luminal SCFA, decreased inflammation, and reduced mucosal injury (Kanauchi et al., 2003). The ingestion of DF has not, however, provided conclusive observations of reduced inflammation in people with Crohn's disease (CrD). In contrast, increasing the consumption of diets rich in DF found in fruits and vegetables can reduce the risk of CrD in women. Interestingly, this finding, however, was not observed in women with CrD given diets rich in DF derived from whole grains and legumes (Ananthakrishnan et al., 2013). The use of RS and WB to treat chemically-induced intestinal inflammation in animal models consistently shows a reduction in intestinal injury due to SCFA production (Butzner et al., 1996; Sina et al., 2009). As an example, rats supplemented RS *per os* displayed increased levels of butyrate and improved epithelial cell regeneration in the proximal colon (Jacobasch et al., 1999). Moreover, other researchers demonstrated that providing potato fibre to mice with enteritis increased intestinal SCFA concentrations and decreased incidence of leukocyte infiltration into the intestinal mucosa (Panasevich et al., 2015). Alternatively, diets rich in cellulose failed to increase SCFA production, reduce tissue injury, or improve the health of mice with

active enteritis (Goto et al., 2010). In summary, although treatment with DF is beneficial, it is evident that the specific form of DF and the type of disease present in the host (i.e. UC and CrD) influences the effectiveness of DF to reduce intestinal inflammation in animals and people (Ananthakrishnan et al., 2013; Galvez et al., 2005; Jacobasch et al., 2008).

1.1.2 Effects of dietary fibre on intestinal health

Fermentation by-products of DF can affect various physiological functions within the host. Dietary fibres are responsible for increasing the total amount of branched-chain fatty acids (BCFA) and SCFA in the intestine, and RS and WB have been identified as food components that have putative effects on increasing the general health of people (Bach Knudsen, 2015; Birt et al., 2013; Boll et al., 2015; Nielsen et al., 2014). It is widely accepted that DF reduces the severity of intestinal inflammation and the incidence of colon cancer (Bultman, 2014; Cho et al., 2013; Kaczmarczyk et al., 2012). As an example, DFs that are metabolized into β-glucan contribute to improved *tumour* surveillance and immunomodulation in the large intestine (Agrawal et al., 2010). Treatment with DF and the production of β-glucan has been used as an adjuvant to enhance chemotherapy and immunotherapy in colon cancer (Vannucci et al., 2013). Furthermore β-glucan from DF fermentation promotes tumour cell death by increasing nitric oxide activity, the induction of cytotoxic T-cells, and activation of compliment proteins that initiate phagocytosis and lysosomal degranulation (Ross et al., 1999). β-glucan also modulates intestinal immunity by binding to dectin ligands on granulocytes and antigen presenting cells, and subsequently increasing the priming and activation of both $CD4^+$ and $CD8^+$ T-cells (Vannucci et al., 2013). The binding of dectin ligands to intestinal B-cells is also involved in the activation and production of immunoglobulins within the intestine (Agrawal et al., 2010). The enhanced movement of ingesta through the intestine is also a mechanism to improve intestinal health, and helps to reduce intestinal inflammation and the development of intestinal cancers. This is observed by increasing the amount of WB consumed within the diet. The increased fermentation of WB increases stool frequency and reduces the 'contact time' between the intestinal epithelium and bile acid fermentation by-products, products that are often mutagenic in the colon (Hamer et al., 2008; Reddy et al., 2000).

1.2 The chemical and structural composition of dietary fibre

Dietary fibres in general represent a group of non-digestible food products that vary in water solubility, fermentation, fluid viscosity, and their effect on the host microbiome (Englyst et al., 2007). More specifically, DF consist of non-starch polysaccharides (NSP), resistant oligosaccharides, resistant starches, and non-carbohydrate based polymers such as lignins, chitin and hyaluronans (Hamaker and Tuncil, 2014). Dietary fibre is further characterized by the number of saccharide units, overall chain length, type of chain linkages, and the type of sugar units it possesses (Hamaker and Tuncil, 2014). A defining feature of DF is its resistance to digestion by host enzymes within the small intestine. The products that by-pass digestion in the host small intestine are subsequently metabolized in the colon by resident intestinal bacteria into bioactive molecules, including SCFA (Table 1.1) (Eswaran et al., 2013). Intestinal SCFA are necessary energy sources for colonocytes, and illustrate the importance of the symbiotic relationship between the microbiome, the host, and its environment (i.e. diet) (Al-Lahham et al., 2010; Bach Knudsen, 2015; Butzner et al., 1996; den Besten et al., 2013).

1.2.1 Structural composition and functions of wheat bran

Non-starch polysaccharides are a large category of DF that are largely present in WB, and NSP fermentation results in the production of arabinoxylan, β-1,3/1,6 linked D-glucose (i.e. β-glucan) and cellulose (Eswaran et al., 2013). These products are highly insoluble, non-digestible in the upper intestinal tract, and slowly fermented in the colon (Kumar et al., 2012), and as such affect the transit time of ingesta within the intestine. These carbohydrates increase the rate of protein digestion, reduce phenol and ammonia production, and importantly, increase the release of molecules that promote the feeling of satiety (Bach Knudsen, 2015; Birt et al., 2013). The consumption of WB-rich diets has been linked to increasing amounts of SCFA within the intestine, which may be associated with reduced fecal transit time and increased fecal bulking (Wong et al., 2006).

1.2.2 Structural composition and functions of resistant starch

Resistant starch is composed of chains of starch molecules rich in amylose and

amylopectin, and carbohydrates linked by α-1,4/α-1,6 glucosidic bonds (Louis et al., 2007). The digestibility and gelatinization (i.e. irreversible digestion of starch molecules in water) of RS is highly dependent on the number of amylopectin chains within RS (Birt et al., 2013). Resistant starches with greater numbers of α-1,4/α-1,6 glucosidic linkages are less digestible than those with lower numbers of α-1,4/α-1,6 glucosidic linkages (Birt et al., 2013; Englyst et al., 2007). Four distinct classes (i.e. Type 1 RS- Type 4 RS) can be used to categorize RS, and these are differentiated by the chemical composition and digestibility of starch molecules (Englyst et al., 2007). The level of 'digestion resistance' is associated with the accessibility of starch molecules for enzymatic hydrolysis by few host pancreatic amylases, and amylases primarily produced by bacteria (Macfarlane and Englyst, 1986; Novak and Vetvicka, 2008). The four distinct types of RS are as follows: Type 1 RS is a highly resistant form of RS, as the carbohydrate molecules are well protected from enzymatic digestion by a prominent protein matrix and thick cell wall (Birt et al., 2013). These RS are only digestible following the milling process (Raigond et al., 2015). Type 3 RS is composed of large quantities of retrograded (i.e. starch that becomes gelatinized at cool temperatures) starch and amylose (Birt et al., 2013; Witt et al., 2010). At low temperatures, the amylose fractions aggregate to form stable B-type crystalline molecules that are required to maintain the gelatinized structure (Raigond et al., 2015). Type 4 RS is a chemically synthesized, low gelatinized starch, and can be modified in a manner that converts, substitutes, or cross-links carbohydrates to limit digestion (Raigond et al., 2015). For instance, this starch can contain chemically synthesized 'distarch phosphodiester cross-links' that reduce gelatinization and decrease digestion by amylases (Kahraman et al., 2015; Thompson, 2000).

For the purpose of this thesis, Type 2 RS will be examined in more detail. Type 2 RS are natural starch granules that commonly form compact crystalline structures (Raigond et al., 2015). These starches are naturally occurring, and unlike Type 4 RS, do not require chemical and physical processing to reduce the gelatinization properties that make the starch more accessible for enzymatic digestion. Examples of naturally derived Type 2 RS include: uncooked potato starch, green banana starch, and high-amylose maize starches (Birt et al., 2013; Topping and Clifton, 2001). Type 2 RS also

contains large quantities of ungelatinized starch chains (Martinez et al., 2010), and high-amylose maize contains both a high number of amylose carbohydrate units, and long amylopectin chains. Type 2 RS are relatively inexpensive, abundant, and naturally occurring products that importantly, have been shown to effect the host intestinal microbiome (Hamaker and Tuncil, 2014; Zhu et al., 2013). There are numerous studies that have highlighted the beneficial effects of RS-rich diets to alter the host microbiota and SCFA production to improve intestinal health. Martinez et al. (2010) demonstrated that Type 2 and Type 4 RS selectively promoted the growth of specific bacterial populations beneficial to intestinal health in people (Martinez et al., 2010). Feeding pigs diets rich in Type 2 RS resulted in the increase of total intestinal SCFA, and reduced the expression of genes involved in the adaptive immune response and epithelial cell turn over (Haenen et al., 2013a). Notably, most studies show that diets containing RS increase the presence of bacterial species involved in the formation of butyrate within the intestine. In summary, properties of RS such as the chemical structure, enhanced solubility, and ease of digestion make RS an ideal DF to consume to improve the intestinal health of people.

1.3 Short-chain fatty acids in the intestine

Following the ingestion of food, soluble and insoluble fibres pass through the small intestine and accumulate in the cecum and colon, and subsequently the fermentation of DF into SCFA occurs primarily in the cecum and proximal colon, while BFCA fermentation mainly occurs in the distal colon (Bach Knudsen, 2015). Fermentation of DF produces large quantities of SCFA, and the proportions of acetate, propionate and butyrate occur in a 60:20:20 ratio (Cummings, 1981). Production of BCFA such as isovalerate and isobutryate within the distal intestine are much lower than the quantities SCFA produced in the same region (Cummings et al., 1987). In the healthy intestine, total concentrations of SCFA within the cecum and proximal colon are approximately 100 mM, and this concentration decreases as ingesta approaches the caudal aspect of the distal colon (Hamer et al., 2008). The production of SCFA is important in energy metabolism, immune function and pH equilibrium within the intestine (Topping and Clifton, 2001; Tremaroli and Backhed, 2012). As an example, the pH of the intestine becomes more neutral as it progresses from the cranial aspect of

the small intestine to the distal colon (i.e. cecum pH 5.4-5.8; colon pH 6.5-6.7) and SCFA produced by DF fermentation become anionic molecules that directly regulate intestinal pH (Bach Knudsen, 2015; Bergman, 1990; Velázquez et al., 1997).

Acetate is the highest produced SCFA from microbial fermentation of DF, and constitutes approximately 60-80% of the total amount of SCFA within the intestine (Wong et al., 2006). Once acetate is formed, it is delivered to the liver via the portal vein and synthesized into cholesterol and other lipids, and as such acetate is present in higher quantities in portal blood and liver as compared to other SCFA (Wong et al., 2006). Acetate is also a component in the production of butyrate, as it is an important substrate for the butyrate biosynthetic pathway, as well as a major product of butyrate oxidation (Figure 1.1) (Birt et al., 2013; Stams and Plugge, 2009). Propionate is the second most abundant SCFA produced within the cecum and large intestine, and represents approximately 20-30% of the total SCFA produced following DF fermentation (Bergman, 1990). This SCFA is metabolized within the liver and is an important substrate for glucose production (Wong et al., 2006). Finally, butyrate is the lowest produced SCFA, and normally represents approximately 10-20% of the total SCFA concentration. Notably, this amount of butyrate can change depending on the type of DF fermented (Bach Knudsen, 2015), and it has been suggested that amounts of butyrate measured in the cecum and proximal colon may underestimate the total levels of butyrate within the intestine (Hamer et al., 2008). Moreover, butyrate is readily absorbed and used as an energy source within colonocytes, therefore measurements of butyrate from blood, tissue, and feces may result in inaccurately low measured values (Hamer et al., 2008; Topping and Clifton, 2001).

As mentioned above, SCFA are used by the host for various physiological processes and as such SCFA must be absorbed from the intestine into systemic circulation. In general, absorption of butyrate occurs at the apical membrane of colonocytes (Cummings et al., 2004), and SCFA present in low pH environments (i.e. small intestine) form protonated free acids that passively diffuse across the cell membrane (Wachtershauser and Stein, 2000). In contrast, the pH of the cecum and colon are near neutral, and therefore SCFA form weak acids and are transported into the cell by a weak acid/HCO_3^- exchanger (Cummings, 1981; Wachtershauser and

Stein, 2000). Another method of SCFA transport across the apical and basolateral membrane of colonic epithelial cells uses electrochemical neutral Na^+ Cl^- exchange (Vidyasagar and Ramakrishna, 2002). This exchanger moves Na^+ ions into the cell, and facilitates water uptake by the colon (Vidyasagar and Ramakrishna, 2002). The bulk movement of water helps draw dissolved SCFA into the intestine. Finally, Monocarboxylate 1 (MCT-1) is also an important transporter for SCFA uptake into colonocytes. The expression of this transporter is upregulated in the presence of SCFA, and its upregulation helps increase the amount of SCFA that can enter the cell (Cuff et al., 2005).

1.3.1 Physio-chemical properties of butyrate

As previously stated, intestinal pH is regulated through the production of SCFA by DF fermentation. Butyric acid (pka of 4.81) in the presence of a neutral pH forms a weak acid by dissociating into anions and producing a deprotonated form of butyrate (Topping and Clifton, 2001; Velázquez et al., 1997). Although butyrate forms a relatively weak acid in the intestine, butyric acid is often administered to animals as a neutralized salt (i.e. sodium butyrate), as this chemical form of butyrate is less irritating and induces less tissue injury to the host compared to the weak acid form (Figure 1.1). For example, administration of butyrate *in vivo* in its free acid form resulted in marked tissue injury characterized by erosions in the intestinal mucosa, and disruptions to the epithelial cell osmotic pressure and electrochemical gradients (Wachtershauser and Stein, 2000). Delivery of high concentrations of sodium butyrate to the colon is important in studying the effects of butyrate on the intestinal mucosa and colonic health (Velázquez et al., 1997). Delivery of butyrate salt often occurs with either rectal enemas or intracolonic infusion, as these methods provide more reproducible and consistent results as compared to butyrate delivery *per os* (Wachtershauser and Stein, 2000; Welters et al., 1996).

1.3.2 Biological effects of butyrate on the host

Production of butyrate from RS fermentation is linked to a number of physiological effects on the host, summarized in Table 1.2. Briefly, butyrate is the preferred energy source in healthy (i.e. non-inflamed or neoplastic) colonocytes, providing up to 70%

of their energy requirements (Cummings and Macfarlane, 1997). Butyrate also participates in the regulation of colonocyte differentiation and turnover (Guilloteau et al., 2010), and contributes to increasing nutrient absorption within the colon (Bach Knudsen, 2015). Within the cell, butyrate undergoes β-oxidation ($\Delta G^{\circ\prime}$= +48 kJ; $\Delta G'$= -22 kJ) (Stams and Plugge, 2009), and produces acetyl-CoA metabolites, and these products are further metabolized within the tricarboxylic acid cycle (Donohoe et al., 2012). Butyrate oxidation also generates hydroxybutryate, acetoacetate, and acetate. These water soluble ketone bodies are transported to the liver, and used as energy sources in the liver and other tissues (i.e. brain) (Laffel, 1999).

Although the colonocyte generally uses the oxidative phosphorylation pathway to metabolize butyrate to generate energy, there are periods of increased cell turnover when glycolysis is the preferred biochemical process for energy production. Notably, cellular glycolysis occurs more frequently when cells are undergoing inflammatory or neoplastic changes (Donohoe et al., 2012). This biochemical change also allows butyrate to collect near the nucleus, and this event can affect cell turnover (Donohoe et al., 2012). Researchers demonstrated that within cancerous cells, butyrate will inhibit histone deacteylase (HDAC) activity and notably reduce cell proliferation and the expression of genes involved in apoptotic events within the cell (Zimmerman et al., 2012). Pro-inflammatory gene expression can also be attenuated by HDAC inhibition (Vinolo et al., 2011) leading to the subsequent reduction in NFκB activation and iNOS production (Zimmerman et al., 2012). Other investigators reported different cellular processes that reduce intestinal inflammation following treatment with butyrate. Recent studies have demonstrated that butyrate improves the intestinal epithelial barrier by increasing the expression of proteins that form tight junctions (Ploger et al., 2012). Furthermore, butyrate can also improve mucin secretion; however the results are contradictory and the cellular mechanisms that increase mucin production following treatment are poorly understood (Barcelo et al., 2000). As examples, Gaudier et al. (2009) examined the effect of butyrate on mucus expression and demonstrated that membrane-bound mucin gene expression increased, following the rectal administration of sodium butyrate to mice (Gaudier et al., 2009). Other investigators have also shown increased *MUC2* gene expression in human cancer cell

lines and increased MUC2 protein production in cells similar to human goblet cells following the addition of low concentrations of butyrate (Burger-van Paassen et al., 2009; Hatayama et al., 2007). In contrast, Hamer et al. (2010) investigated mucin secretion in healthy human beings, and found that individuals in convalescence from UC demonstrated no change in *MUC2* expression, mucus secretion, or the amount of sialomucins within the gut following butyrate enema treatment (Hamer et al., 2010). Despite these varying results, treatment with butyrate remains a possible strategy for reducing intestinal inflammation and improving intestinal health.

1.4 The intestinal microbiome and bacterial functions in the colon

Within the intestinal tract, diverse and large populations of bacteria (i.e. 500-1000 species) reside in the lumen and are often associated with the thick intestinal mucus layer loosely overlying the epithelium (Eckburg et al., 2005; Morgan and Huttenhower, 2014). With this marked variation in intestinal bacterial species, there is increasing interest in research that investigates the role of the intestinal microbiome, host health, and the development of inflammation associated with intestinal bacterial communities. Metagenomics is becoming an increasingly useful tool to study the effects of bacterial taxa on intestinal community structure and host health, and in combination with proteomics, transcriptomics, and metabolomics has provided researchers with numerous strategies to study host health. Moreover these metagenomic-based studies can also investigate the impact of host physiological and immune responses (Foxman and Martin, 2015). Metagenomics also aids in the categorization of the intestinal microbiome into distinct groups. These groups are defined by physiochemical and metabolic properties, the functions of these communities within the host intestine, and the interactions between the bacterial species occupying similar regional niches (Backhed et al., 2005). Selection pressure from either the host or competing microbial populations induces genetic and metabolic adaptations within bacteria, allowing the bacteria to become more competitive within their specific niches (Stecher and Hardt, 2011). Some of these selection pressures include: competition for local niches and metabolic substrates between other microorganisms (i.e. strict anaerobic bacteria vs. facultative bacteria); the anatomical location of the microbes within the gut (i.e. proximal intestine vs. distal intestine,

intestinal lumen vs. intestinal crypt); the adaptability of bacteria to the genetic profile (i.e. unique individual physiological functions); and intestinal immune responses of the host (Sassone-Corsi and Raffatellu, 2015).

The intestine is in a constant state of 'balanced' physiologic and immunologic activity, with constant interaction occurring between the host intestine and the microbiome (Vaishnava et al., 2011). Bacterial species can be either harmful to the host (i.e. pathogenic) or have a neutral and or beneficial effect on the host (i.e. commensal). Commensal bacteria are usually innocuous and rarely elicit significant inflammatory responses within the intestine of immunocompetent hosts. Importantly, these organisms can be beneficial to the host by providing essential nutrients and protecting the host by 'competitively excluding' pathogenic bacteria within the gut (Stecher and Hardt, 2011). In the human and murine intestine, the most numerous and diverse commensal bacterial phyla are *Firmicutes* and *Bacteroidetes*, with smaller populations of *Verrucomicrobia*, *Deferribacteres*, *Proteobacteria*, *Actinobacteria*, and Archaea species (Eckburg et al., 2005; Wong et al., 2012). Notably, there is marked variability in the bacterial species within the intestine of individuals, and conditions of health, diet, and species interaction within niches influence the presence of the various bacterial species (Backhed et al., 2005; Lozupone et al., 2012). For example, investigations examining the effect of host genetics on the intestinal microbiome, found associations between populations of intestinal bacteria and people with similar ethnic backgrounds living in similar ecological regions (Lozupone et al., 2012). Finally, the existence of an established microbiome can provide host benefits such as: the increase of nutrients available following fermentation, a balanced intestinal pH, increased oxygen availability for epithelial cells, and positive effects towards immune function (Louis et al., 2007).

1.4.1 Microbes associated with DF fermentation

There are various microbial species within the gastrointestinal tract involved in the fermentation of carbohydrates into SCFA and BCFA. Bacterial species within the *Clostridia* cluster XIVa of the *Firmicutes* phyla have many species involved in DF fermentation (Louis et al., 2007). *In vivo* studies using monogastric animal models fed

diets rich in RS increased the abundance of *Bifidobacterium* spp., *Parabacteroides* sp., *Ruminococcus intestinalis*, *R. bromii*, and *Eubacterium rectale* in the large intestine (Haenen et al., 2013b; Martinez et al., 2010). Other studies have demonstrated that *R. brommii* is an important fermenter of RS in humans (Kovatcheva-Datchary et al., 2009). This species is responsible for increasing acetate production, which is then used by *E. rectale* to produce butyrate in the colon (Kovatcheva-Datchary et al., 2009). Similarly, diets rich in arabinoxylan and other NSPs of WB, preferentially increase the abundance of *Clostridia* cluster XIVa species, and importantly bacteria from the *Clostridia* cluster XIVa are rich in species (i.e. *Faecalibacterium praznutzii* and *Roseburia intestinali*) that produce large quantities of butyrate (Nielsen et al., 2014). Other butyrate-producing bacteria that are not members of the *Clostridia* cluster XIVa, namely *Lactobacillus* spp. and *Bifidobacterium* spp. are also important in DF fermentation. These bacteria increase in numbers in the presence of diets rich in DF (Nielsen et al., 2014). Collectively, these studies demonstrate that various populations of bacteria within the microbiome ferment DF and promote intestinal SCFA production (Backhed et al., 2005).

1.5 Intestinal morphology and immunity

1.5.1 Morphology of the human mammalian intestine

The mammalian intestine is a tubular structure that originates at the pylorus of the stomach and terminates at the distal aspect of the anus. Morphologically the intestinal mucosa is composed of an epithelial layer overlying the lamina propria. Beneath the lamina propria is the submucosa surrounded by the muscularis externa and serosa (Lacy, 2010). At the apical border of the epithelium is the brush border embedded within its protective glycoprotein rich mucus layer (Figure 1.2) (Pelaseyed et al., 2014). The intestine is imperative for the digestion of foodstuffs, and the subsequent absorption of carbohydrates, amino acids, lipids, nucleic acids, vitamins, and other micronutrients into systemic circulation (Boudry et al., 2010). The intestine is also important in water absorption and the excretion of toxic metabolites in bile salts (Mader, 2007).

Within the intestine, bacteria either reside within the luminal ingesta, or within the mucus layer overlying the epithelial cells. The intestinal mucosa is an important

barrier that not only prevents bacterial invasion into the host epithelium, but contains the GALT, an important component of the host immune system. Gut-associated lymphoid tissue within the intestine is divided into two parts: aggregated tissues consisting of solitary lymphoid follicles and Peyer's Patches (PP), and non-aggregated tissue that includes cells within the mesentery, lamina propria, and intraepithelial lymphocytes (Schley and Field, 2002). Histologically a PP is composed of a variety of cellular components involved in maintaining its structure and the generation and propagation of host immunity. Peyer's Patches within the intestinal mucosa form dome-like structures, and these are lined with small numbers of specialized epithelial cells (i.e. M cells) that facilitate transepithelial transport of bacteria. Using Toll-like receptors (TLR) attached to M cells on their apical and basolateral membranes, viable bacteria, or remnants of bacteria, can be transported to juxtaposed dendritic cells and subsequently induce an adaptive immune response. This response can lead to the production of immunoglobulins (IgA), activate of B and T-cells, secrete cytokines and chemokines, and recruit macrophages and granulocytes (Figure 1.2) (Bergstrom et al., 2012; Boulard et al., 2010; Danese et al., 2007; Ermund et al., 2013; Hayden et al., 2006; Mowat, 2010; Schley and Field, 2002).

1.5.2 Intestinal immunity

1.5.2.1 Innate immunity

The intestinal immune system is a complex and integrated physiologic system. It combines coordinated responses between the innate and adaptive immune responses within the intestinal mucosa (Geremia et al., 2014; Janeway et al., 2001; Wallace et al., 2014) and is composed of both cellular and non-cellular components (Figure 1.2).

Non-cellular and cellular components of the innate immune system play a critical role in protecting the host from intestinal infections. Constituents of the non-cellular innate immune system include: 1) physical barriers such as the intestinal epithelial lining, tight junctions and the presence of the intestinal mucus layer; 2) chemical barriers including the production of stomach acid; 3) antimicrobial proteins such as cryptidins, β-defensin, α-defensin, heat shock proteins, and compliment; 4) cytokines and chemokines produced from both immune cells and cells not involved in immune functions; 5) TLRs and Nod-like receptors (NODs); and 6) digestive enzymes namely

peptidase, nuclease and lipase (Mader, 2007; Williams, 2011). Cellular components of innate immunity include macrophages, mast cells, granulocytes, natural killer (NK) cells, NK T-cells, γδ T-lymphocytes and dendritic cells (Turvey and Broide, 2010). Macrophages, and in particular dendritic cells, are antigen presenting cells (APC), that engulf pathogens and present their antigens to lymphocytes of the adaptive immune system, thereby linking the innate and adaptive immune systems in a coordinated manner (Jiang and Chess, 2004).

1.5.2.2 Mechanisms of adaptive immunity

Similar to the innate immune response, the non-cellular and cellular components of the adaptive immune response are essential in preventing and eliminating infection within the intestine. One of the primary functions of the adaptive immune response is the production of immunoglobulins (Ig). Immunoglobulins are produced by plasma B-cells and bind pathogens (Medzhitov and Janeway, 1997). The pathogens are either neutralized by agglutination with antibodies, or are targeted for destruction by the following methods: (1) activation of the compliment system, (2) opsonisation of pathogens for granulocyte digestion, and (3) release of cytokines to activate NK cells (Williams, 2011). Although all immunoglobulin isotypes can be produced by intestinal lymphocytes and plasma cells, the secretory immunoglobulin IgA is continuously secreted into the intestinal lumen. This constant production of immunoglobulin is important for the agglutination of bacteria, and prevents bacteria from binding to the epithelium, which is a key element for protecting the mucosa from invasion of pathogenic organisms (Macpherson et al., 2008).

The primary lymphocytes involved in the cellular component of the adaptive immune response are $CD8^+$ and $CD4^+$ T-cells. Major histocompatibility complex (MHC) class I receptor restricted cytotoxic $CD8^+$ T-cells recognize and destroy pathogens through the release of cellular proteins: perforins and granzymes. These proteins destroy infected cells and *tumour* cells by forming pores within the plasma membrane, or inducing apoptosis with the cell (Janeway et al., 2001). Cytotoxic $CD8^+$ T-cells also enhance the release of effector cytokines such as interferon-γ (IFN-γ), and tumour necrosis factor-α (TNFα), which collectively attract macrophages and granulocytes to sites of infection, and activate intracellular bactericidal (i.e.

myeloperoxidase) processes (Janeway et al., 2001). $CD4^+$ T-cells, also known as, helper T-cells are important lymphocytes involved in the direction of immune responses through the release of pro-inflammatory and anti-inflammatory cytokines. These $CD4^+$ cells interact with host derived MHC-II receptors on APC and are categorized into the four main subsets of $CD4^+$ helper T-cells (Th1, Th2, Th17, and Treg) summarized in Table 1.3.

In the intestine, the most prominent pro-inflammatory helper T-cell responses that protect against infection are Th1, Th2 and Th17 cells (Schley and Field, 2002) and will be briefly discussed. Type 1 helper T-cells are induced in response to intracellular pathogens, and elicit pro-inflammatory cytokines to eliminate these pathogens (Schley and Field, 2002; Yoneyama et al., 2000). Th1 immune responses are generally identified by the secretion of IFN-γ from effector Th1 T-cells, and importantly this immune response is required to protect and resolve the initial stages of a *C. rodentium* induced infection in mice (Costa et al., 2011). Th2 immune responses are necessary to protect the host from extracellular pathogens such as endoparasites and ectoparasites. Th2 immune responses facilitate release of vasoactive products that attract and activate eosinophils and mast cells, and this response aids in the production of subclasses of immunoglobulins and anti-inflammatory molecules. The Th2 response has been identified by the release of four main cytokines, being IL-4, IL-5, IL-13 from effector Th2 T-cells (Schley and Field, 2002; Yoneyama et al., 2000). Th17 immune responses are characterized by the secretion of IL-17, and facilitate the release of cytokines in the Th22 response (Maynard et al., 2012). Th17 cells are essential in the protection and resolution of *C. rodentium* infections in mice through the release of IL-22 in late stages of infection (Costa et al., 2011; Zheng et al., 2008).

Collectively, both the innate and adaptive immune responses operate in concert to prime and maintain a functional immune system within the mammalian intestine. Understanding interactions between innate and adaptive immune responses is essential for investigating the mechanisms that induce intestinal injury and disease.

1.6 Chemical, structural and biological properties of intestinal mucus

In the intestine, the mucus layers play a valuable role in protecting the epithelial lining from bacterial, viral, protozoan, helminth and fungal invasion. As previously

mentioned, intestinal mucus serves as a physical barrier and inhibits pathogens from breaching the epithelial layer (Atuma et al., 2001). Studies investigating the role of colonic mucus in host protection are becoming more important, as the emergence of new, highly-pathogenic intestinal disease has become more prevalent (Bergstrom et al., 2010; Johansson et al., 2011a). The importance of the intestinal mucus layer to act as a barrier to prevent intestinal injury has been shown experimentally in *Muc2*$^{-/-}$ knockout mice. The *Muc2* gene is important for the production of secreted mucin (i.e. MUC2) that loosely lines the epithelium, forming an effective viscous barrier between the intestinal lumen and epithelium (Ermund et al., 2013). Notably, mice lacking the MUC2 protein develop spontaneous colitis that is accentuated following challenge with enteric pathogens. For example, it has been demonstrated that pathogen-challenged *Muc2*$^{-/-}$ mice develop spontaneous colitis, and the severity of colitis is markedly greater than pathogen-challenged wildtype mice with intact mucus layers (Jakobsson et al., 2015; Morampudi et al., 2016; Van der Sluis et al., 2006). Similarly, other studies showed that impaired mucin production or altered chemical composition of mucin increased the severity of intestinal injury (Bergstrom et al., 2010; Lu et al., 2011; Van der Sluis et al., 2006). Thus, these studies provide evidence that intestinal mucus is required to minimize epithelial damage, and is an important barrier to prevent the attachment of luminal pathogens.

The mucus layers are important structures in intestinal health and function. Intestinal mucus is comprised of 98% water and 2% mucin, and the mucins are composed of small, cysteine-rich glycoproteins mainly released from goblet cells (Linden et al., 2008). In the small intestine, mucus exists as a continuous, loosely adherent layer of mucin released from both goblet cells and Paneth cells at the intestinal crypts (Pelaseyed et al., 2014). In contrast, mucus in the large intestine is divided into two layers (Figure 1.3): a firmly attached layer located directly above the epithelial lining called the glycocalyx, and the relatively loosely-adherent mucus layer located directly above the glycocalyx, that is similar to the small intestine loosely-adherent mucus layer (Atuma et al., 2001; Inglis et al., 2012; Johansson et al., 2011b). It is within the loosely-adherent layer that most commensal bacterial populations reside (Backhed et al., 2005). The tightly-adherent mucus layer (i.e. glycocalyx) is

stratified and overlies the epithelium of the colon, and is variable in thickness and species specific. As an example, in mice this mucus layer is approximately 50 µm, while it in humans it is ~200 µm (Johansson et al., 2014; Johansson et al., 2010). The tightly-adherent glycocalyx also contains the majority of the transmembrane non-secreted mucin proteins produced, and these mucins include: MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17 (Pelaseyed et al., 2014).

As indicated, within the intestine mucins are either secreted into the lumen, or bound directly to the epithelium. MUC2 is the main secretory mucin glycoprotein in the lumen, and is composed of two amino acid-rich domains that are flanked by an N-terminus cysteine-rich domain, and a C-terminus cysteine-rich domain (Pelaseyed et al., 2014). The amino acid-rich domain at the center of the molecule is the PTS domain, and consists of a Proline-Threonine-Serine amino acid repeats (Ambort et al., 2011; Johansson et al., 2011a). During synthesis, MUC2 undergoes post-translation modification, and is dimerized by cross-linking the C-terminal disulphide bonds. Following this, *O*-linked glycosylation of the PTS domain occurs, allowing the glycoprotein to become a trimer (Ermund et al., 2013). The chemical composition of mucin also enables the creation of disulphide cross-linkages that further polymerize mucins and form stronger glycoprotein complexes (Gouyer et al., 2015). Importantly, the structural arrangement of the glycoprotein forms a densely-packed, net-like polymer in goblet cells, and becomes an effective and relatively impenetrable loosely-adherent mucus layer (Ambort et al., 2012).

1.6.1 Mucus-associated cofactors and bacteria associated with mucus

As mentioned, goblet cells are the primary intestinal cells that produce and release mucin from intracellular storage vesicles. These cells also secrete other molecules that assist in maintaining epithelial barrier integrity and immune competence (Figure 1.4). As examples, trefoil factors (TFF) and resistin-like molecules (RELM) are also secreted from goblet cells, and both proteins are involved in the regulation of immune function within the intestines of mice and people (Krimi et al., 2008). There are four members in the RELM family, and all contain cysteine-rich molecules expressed in different areas of the body. The RELMβ protein is exclusively expressed from goblet cells in the intestine (He et al., 2003). The physiological roles of RELMβ in the host

are poorly understood, however several studies suggest that RELMβ mediates insulin resistance in mice, and could be associated with the development of diabetes in animals fed high-fat diets (Kushiyama et al., 2005). Expression of *Relmβ* increases within inflamed intestinal tissue following challenge with bacterial pathogens and endoparasites (Artis et al., 2004; He et al., 2003). Interestingly, increased expression of *Relmβ* is associated with improved histological scores in intestinal tissue from mice challenged with chemical inducers of inflammation (Krimi et al., 2008). The mechanisms RELMβ uses to prevent intestinal injury from pathogens remain unknown. Studies provide contradictory observations, as RELMβ can reduce chemically-induced inflammation in wild-type mice (Krimi et al., 2008), as well as accentuate tissue injury associated with pathogen-challenge in *Muc2*$^{-/-}$ gene knock-out mice (Morampudi et al., 2016).

Trefoil factors are members of the trefoil protein family, and are small, protease resistant, cysteine-rich proteins (Taupin and Podolsky, 2003). In the intestine, trefoil factor 3 (TFF3) is secreted from goblet cells, and can induce epithelial repair, inhibit cellular apoptosis and improve mucosal barrier function (Taupin and Podolsky, 2003). TFF3 also increases the expression of pro-inflammatory cytokines *TNFα*, *IL-6* and *IL-8 in vitro* (Graness et al., 2002) and contributes to the cellular composition of the mucus layer (Albert et al., 2010). As an example, TFF3 has often been associated with MUC1 and MUC2 proteins in intestinal mucus, suggesting that these molecules can be co-secreted from goblet cells (Poulsom and Wright, 1993). In summary, RELMβ and TFF3 are proteins involved in forming intestinal mucus and work in concert to preserve barrier function and maintain a healthy intestine (Pelaseyed et al., 2014).

In the intestine, bacteria can be found associated with the ingesta in the lumen, or associated within the loosely-adherent mucus layer. Among the many species present within the mucus layer, there are multiple species that inhabit this layer. For instance, species such as *Mucispirillum schaedleri* selectively colonize host mucus, and other species colonize mucus as well as degrade host mucins as an energy source (Hansson, 2012; Johansson et al., 2011b). These mucolytic species belong to various phyla and include: *Bacteroides thetaiotaomicron*, *Fecalibacterium prausnitzii, Ruminococcus gnavus*, *Bifidobacterium longum*, *B. bifidum*, and *Akkermansia muciniphila* (Belzer

and de Vos, 2012; Png et al., 2010; Robertson et al., 2005; Wrzosek et al., 2013). Of note, *F. prausnitzii* and *R. gnavus* are also known to increase intestinal butyrate concentrations through DF fermentation. As examples, it has been proposed that *F. prausnitzii* can work together with other species such as *B. thetaiotamicron* to modulate goblet cell differentiation and mucus O-glycosylation in response to increased acetate and butyrate levels in the colon (Wrzosek et al., 2013). Furthermore, *A. muciniphila* has been attributed to increasing mucosal barrier function by increasing the concentrations of intestinal SCFA, and stimulating mucus secretion from goblet cells (Belzer and de Vos, 2012; Derrien et al., 2004). As such, mucus is important in the intestine to reduce damage to the mucosa, and to facilitate growth and colonization of microbial species that may contribute to the maintenance of homeostasis and host health.

1.7 Non-human models of intestinal inflammation

Understanding the mechanisms involved to induce intestinal injury is necessary to develop mitigating strategies to prevent disease. Studies using tissue samples collected from affected humans would provide the most reliable data; however, there are challenges in acquiring human tissue for research. These challenges include the ethical use of harvested human tissue for experimentation; the small sample size of collected tissue, and the pronounced genetic variability between individuals (Jiminez et al., 2015). As such, comparative whole animal models are employed to investigate intestinal disease in people. Importantly, many of these models provide large tissue sample sizes and are obtained from animals with genetically identical backgrounds (i.e. genetically engineered rodent models). At present, no perfect animal model exists that investigates intestinal inflammation, and all suitable models have limitations. Despite this, dependant on what is investigated, a suitable animal model exists that can provide investigators with relevant and applicable information.

Rodents, and in particular mice, are the most commonly used animal models for investigating intestinal disease and impact of the microbiome on intestinal health and function (Jiminez et al., 2015). Mice are considered a good animal model as their intestinal microbial architecture (Figure 1.5) and function as well as their genetics and immune function are similar to people (Mouse Genome Sequencing et al., 2002). As

examples: 1) mice are good models to study the microbiota and host intestinal interactions as both human and murine intestinal communities have the same diversity of species within the *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla (Figure 1.5) (Dethlefsen et al., 2007). 2) The mouse gastrointestinal tract is also anatomically and functionally similar to the human gastrointestinal tract, and importantly mice have many features analogous to the adaptive immune response present in people (Figure 1.6) (Mestas and Hughes, 2004). 3) Mice share many specific intestinal genes with humans, and mapping of the mouse genome and comparative genomic studies concluded that over 90% of human and mouse genes are shared among species, and approximately 80% of the mouse genes have a human orthologue (Bryda, 2013; Mouse Genome Sequencing et al., 2002). A final advantage to using mice to study intestinal function is that mice are highly fecund animals with relatively short gestation periods and estrous cycles, and are still able to produce large litter sizes in this short time span (Nguyen and Xu, 2008). Although mice do not provide large amounts of tissue to researchers, especially from the colon, their high fecundity and ease of maintenance make the pooling of tissue a method that is commonly used to obtain representative data.

1.7.1 Injury caused by inciting acute colitis in intestinal models with *C. rodentium*

Mice infected with *C. rodentium* develop a progressive but self-limiting proliferative colitis. The lesions are characterized by: the elongation of the colonic crypts; hyperplastic epithelium; epithelial cell loss; increased mitotic activity of germinative cells; a reduction of goblet cells; and a marked transmural mixed cellular infiltrate (Koroleva et al., 2015; Mundy et al., 2005). The development of TMCH disrupts barrier function, and activates similar cellular signalling pathways that have been importantly recognized in patients with inflammatory bowel disease (Luperchio and Schauer, 2001). Linden et al. (2008) noted that *C. rodentium* infection impaired goblet cell morphology, resulting in the reduction of mucus production and exacerbation of *C. rodentium* infection and tissue damage (Linden et al., 2008). In summary, *C. rodentium* is an excellent biological incitant for measuring the effects of butyrate, RS, and WB on physiological, immunological, and microbiota changes associated with acute enteritis in the murine model.

1.7.2 Functional similarities between *C. rodentium* and Enteropathogenic *Escherichia coli* and Enterohemorrhagic *E. coli*

In humans, two common foodborne enteric pathogens: Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) induce diarrhea in immunocompetent individuals, and on occasion can cause severe intestinal illness and death in immunocompromised humans. In contrast, these bacteria do not readily induce intestinal injury or disease in mice (Frankel et al., 1998). As such, *C. rodentium*, a murine pathogen known to induce transmissible murine crypt hyperplasia (TMCH) in mice is often employed to investigate the mechanisms EPEC and EHEC induced tissue injury (Bhinder et al., 2013; Lupp et al., 2007). Infection with *C. rodentium* in rodents, and EPEC and EHEC in people share a similar enterocyte of effacement (LEE) pathogenicity island, which encodes for genes necessary for bacteria to be infectious and pathogenic (Deng et al., 2010). Genes critical for the pathogenicity of these species include: a regulator for LEE gene expression; a type III secretion system; the intimin outer membrane adhesin; the Tir receptor; EspA-G effector proteins; and open reading frames (Deng et al., 2001). The most notable feature of pathogenicity associated with the LEE pathogenicity island is the formation of attaching and effacing lesions. Effector molecules encoded on the LEE pathogenicity island facilitate the initial attachment of bacteria to the epithelial cell, followed by the effacement of the brush border microvilli, leading to the formation of the pedestal-like structure (Collins et al., 2014). Following attachment, a type III secretion system is deployed allowing the insertion of effector molecules into the cell and these molecules are responsible for the pathophysiology of disease (Deng et al., 2010; Luperchio and Schauer, 2001). These effectors include: 1) the Tir and intimin molecules that maintain bacteria-epithelial attachment, 2) Esp effectors to assist in epithelia cell death and pedestal formation, 3) non-LEE encoded effectors that can enhance *C. rodentium* colonization (Garcia-Angulo et al., 2008), and 4) the mitochondrial-associated protein that enhances *C. rodentium* colonization by reducing epithelial barrier integrity (Collins et al., 2014; Mundy et al., 2005).

1.8 Summary

Eating diets rich in DF is important to maintain the intestinal health of Canadians. As such, it has become increasingly important that products with proven beneficial effects to the intestine following DF fermentation are accurately represented in the food industry. It is clear that microbial fermentation of DF produces important products in the intestine that affect barrier function, immune responses and influence the presence of bacterial populations within the microbiota. Short-chain fatty acids are important compounds produced following DF fermentation, and butyrate in particular has been shown to enhance intestinal health. Butyrate improves intestinal health by: providing energy to colonocytes, attenuating intestinal inflammation, modulating the production of host mucus, and affecting the abundance and diversity of bacterial species within the microbiome. Infectious and non-infectious intestinal diseases are becoming more prevalent, and natural therapeutics as alternatives to antibiotics provide another option to mitigate intestinal inflammation and improve intestinal health. Therefore the focus of this research was to study how beneficial products such as butyrate and fermentable DF can improve intestinal health.

1.9 Hypothesis and Objectives

Hypothesis: If diets rich in DF are supplemented to mice with and without enteritis, then an increase in SCFA concentrations in the mouse intestine will occur, and these fermentation products will modulate the intestinal microbiome, regulate inflammation and tissue repair, and enhance mucin production.

The objective of the research for this project were to identify mechanisms involved in the reduction of intestinal inflammation following the addition of butyrate, and the effects of DF fermentation by the intestinal microbiome.

1.10 Tables and Figures

Table 1.1 Different types of DF and proposed health benefits to the colon. (Bach Knudsen, 2015; Birt et al., 2013; Eswaran et al., 2013)

Fibre Type	Solubility	Chain Length	Fermentation Rate†	Potential Health Benefit
Fructo-oligosaccharides Galacto-oligosaccharides	Soluble	Short-chain	High	• Delay of gastric emptying and increase fecal bulking and bowel movement frequency • Regulates colon transit time • Selective growth of *Bifidobacteria* spp. • Enhancement of *Clostridia* cluster XIVa bacteria when mixed with inulin
Resistant starch Pectin Guar gum Inulin	Soluble	Long-chain	High	• Increase in intestinal SCFA content • Regulates colon transit time, slows down small intestine absorption time • Inulin stimulation of *Bifidobacteria* sp. and *Lactobacilli* sp.
Psyllium Oats	Intermediate	Long-chain	Intermediate	• Laxative effect • Increased abundance of bacterial species • Moderate gas and SCFA production
Wheat bran Lignin Fruit and vegetables	Insoluble	Long-chain	Slow	• Increase fecal bulking and bowel frequency • Regulates colon transit time • SCFA slowly fermented • Increased overall abundance of bacterial species
Cellulose Methylcellulose	Insoluble	Long-chain	Non-fermentable*	• Laxative effect • Less of an effect on gas production • Potential for treating constipation

†Fermentation rate determined by ability of dietary fibre to be degraded by bacterial enzymes in the large intestine. *poorly and incompletely fermented by human intestinal flora.

Table 1.2 Summary of the reported effects of butyrate on colonic health measured in cell lines and animal models.

Property	Reference
Under cell stress functions as an histone deacetylase inhibitor at the nucleus	(Zimmerman et al., 2012)
Increased mucus synthesis *in vivo* and *in vitro*	(Gaudier et al., 2009)
Regulation of tight junction proteins and overall barrier function	(Lewis et al., 2010; Ma et al., 2012)
Immunomodulatory by the inhibition of NFκB	(Inan et al., 2000)
Increase epithelial cell turnover and absorption	(Guilloteau et al., 2010)
Increase apoptosis of malignant cells, decreased apoptosis of normal cells	(Jacobasch et al., 1999)

Table 1.3 T-cell responses (Janeway et al., 2001; O'Shea and Paul, 2010; Schmitt and Ueno, 2015; Weaver et al., 2013)

T cell subtype	Co-stimulatory receptors	Transcription Factors	Activation Factors	Associated Cytokines	Immune Response
Cytotoxic	$CD8^+$, MHCI	-	$Ca2^+$	Granzymes, perforins,	Cytotoxic, apoptosis inducing
		-	Fas ligand	Caspase 8/Caspase 3 activation	
		-	-	IFNγ, TNFα, TNFαβ	Activates macrophages and increases MHCI expression, inhibits viral replication
Helper, Th1	$CD4^+$MHCII	T-bet	STAT4	IL-6, IL-8, IL-12p70	Pro-inflammatory
			STAT1	IFNγ*	
Helper, Th2	$CD4^+$, MHCII	Gata3	STAT6	IL-4, IL-5, IL-9, IL-13	Anti-inflammatory/ allergenic
Helper, Th17	$CD4^+$, MHCII	Rorγt	STAT3, TGFβ, IL-1β, IL-6, IL-21	IL-17A, IL-17F, IL-23, IFNγ	Immunoregulatory Pro/Anti-inflammatory
Helper, Th22			IL-23, IL-12, IL-6. TNFα	IL-22	
Helper, Th9	$CD4^+$, MHCII	Gata3, IRF4, PU.1	STAT6, IL-4, TGFβ, IL-21*	IL-9	Allergy, autoimmunity and parasite response (Schmitt et al., 2014)
Regulatory, Treg	$CD4^+$ MHCII	$Foxp3^+$	STAT5, $CD4^+CD25^+$	IL-10, TGFβ	Regulatory
Follicular, Tfh	CD4+ MHCII	Bcl-6	STAT3	IL-21, CXCR5, IL-6	Induction of B-cells to produce antibodies

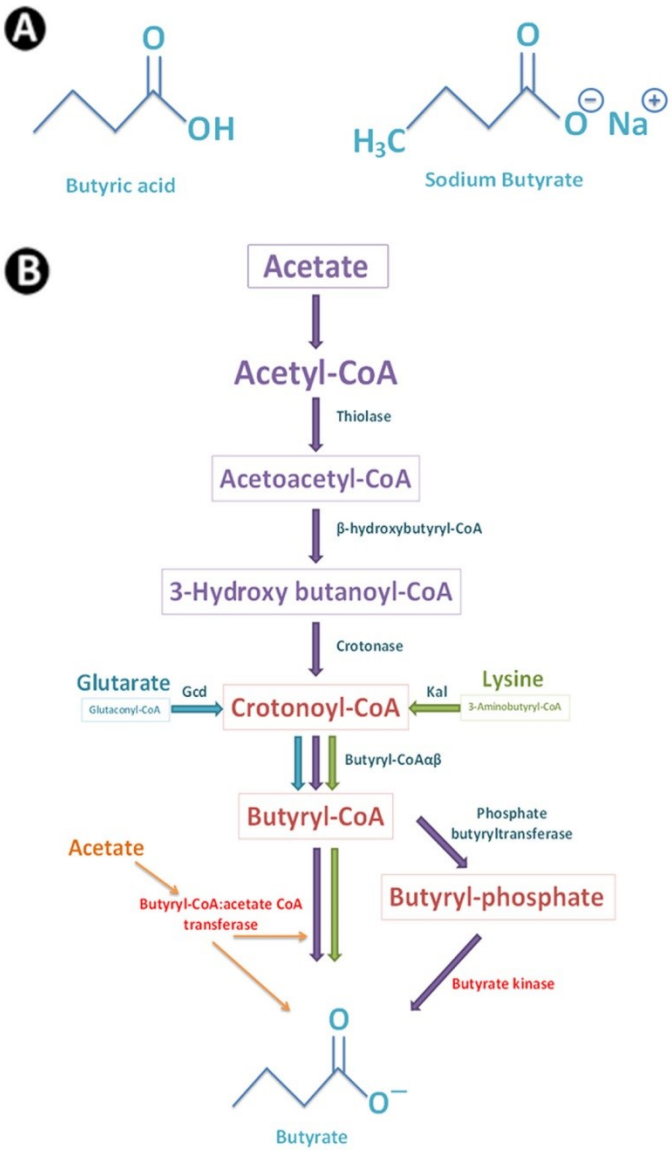

Figure 1.1 The chemical structures of butyrate and the common butyrate synthesis pathways. **(A)** Chemical structures of butyric acid (left) and sodium butyrate (right). **(B)** Common butyrate synthetic pathways initiating from multiple precursor compounds: Acetate, glutarate, and lysine. Purple boxes identify ketone bodies. Enzymes required for catalyzing substrates into intermediate products (outlined with boxes) are shown in blue. Red enzymes are required for the terminal substrate catabolism into butyrate. Gcd, glutaconyl-CoA decarboxylase (α, β subunits); Kal, 3-aminobutyryl-CoA ammonia lyase. Adapted from (Vital et al., 2014).

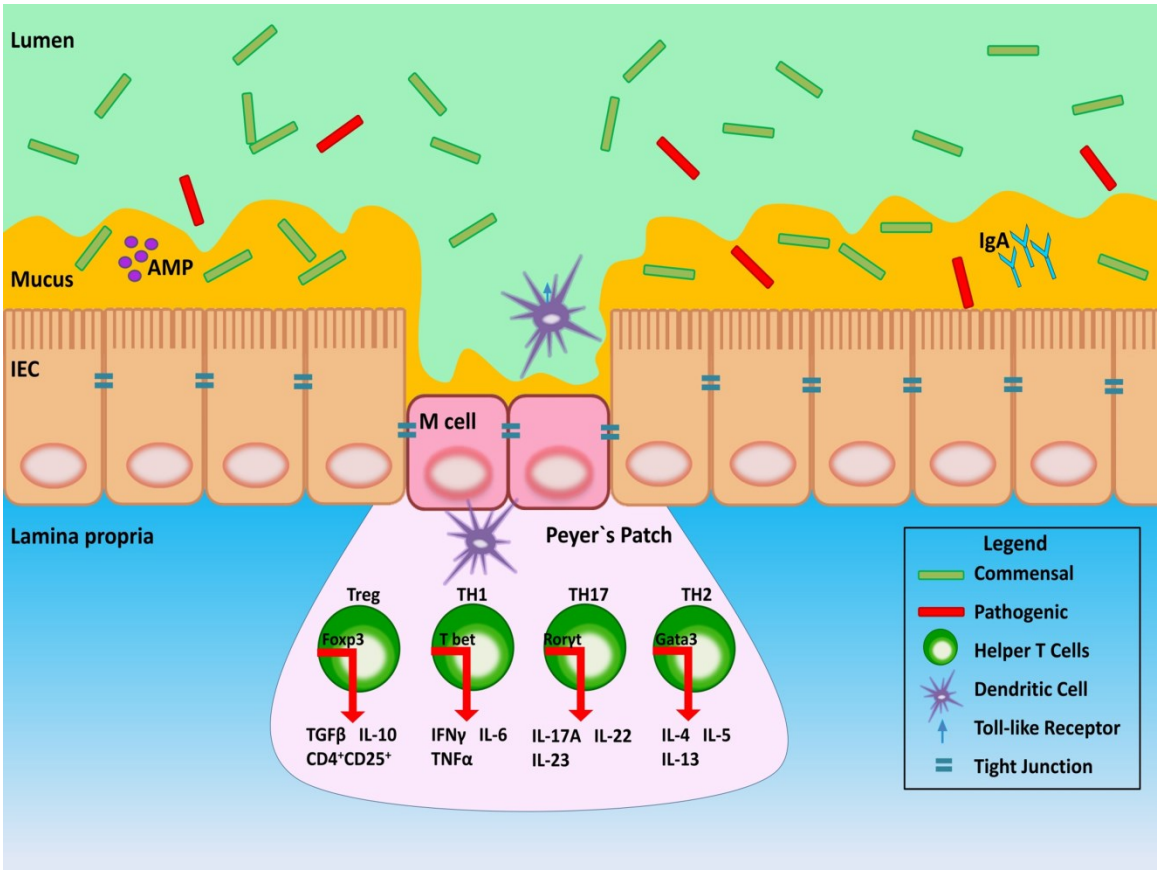

Figure 1.2 Organization of small intestinal architecture and the main immune responses in the intestine. Important components of inflammation in the intestine are represented with the major intestinal helper T-cell responses identified. Transcription factors necessary for the release of cytokines are followed by the red arrows for each response, and the most common cytokines released in each response are highlighted. Immune activity is centered in Peyer's patches within the lamina propria, and dendritic cells move through M cells to link information between the immune cells and the lumen. In the lumen, mucus, antimicrobial peptides and secretory IgA act as host derived defenses against pathogenic bacteria, while TLRs on the surfaces of epithelial cells, APCs and dendritic cells help stimulate an appropriate immune response. Commensal bacteria are considered to be important in immunoquiescence, and reside within the lumen and mucus. AMP, antimicrobial peptide; IgA, immunoglobulin A; IEC, intestinal epithelial cell; TLR, toll-like receptor. Image reprinted from (Jiminez et al., 2015).

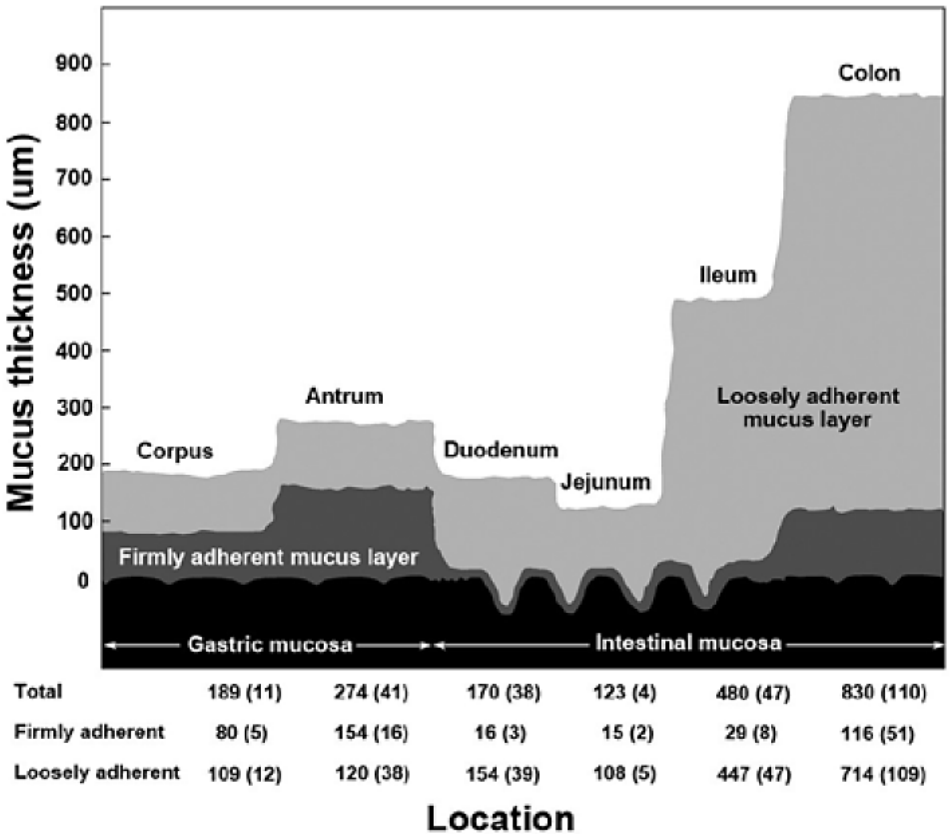

Figure 1.3 Characterization of mucus in the gastric and intestinal mucosa. Two separate layers of mucus are identified, which vary in mucus thickness and composition relative to the bacterial load in the designation region of the intestine. Image adapted from (Inglis et al., 2012).

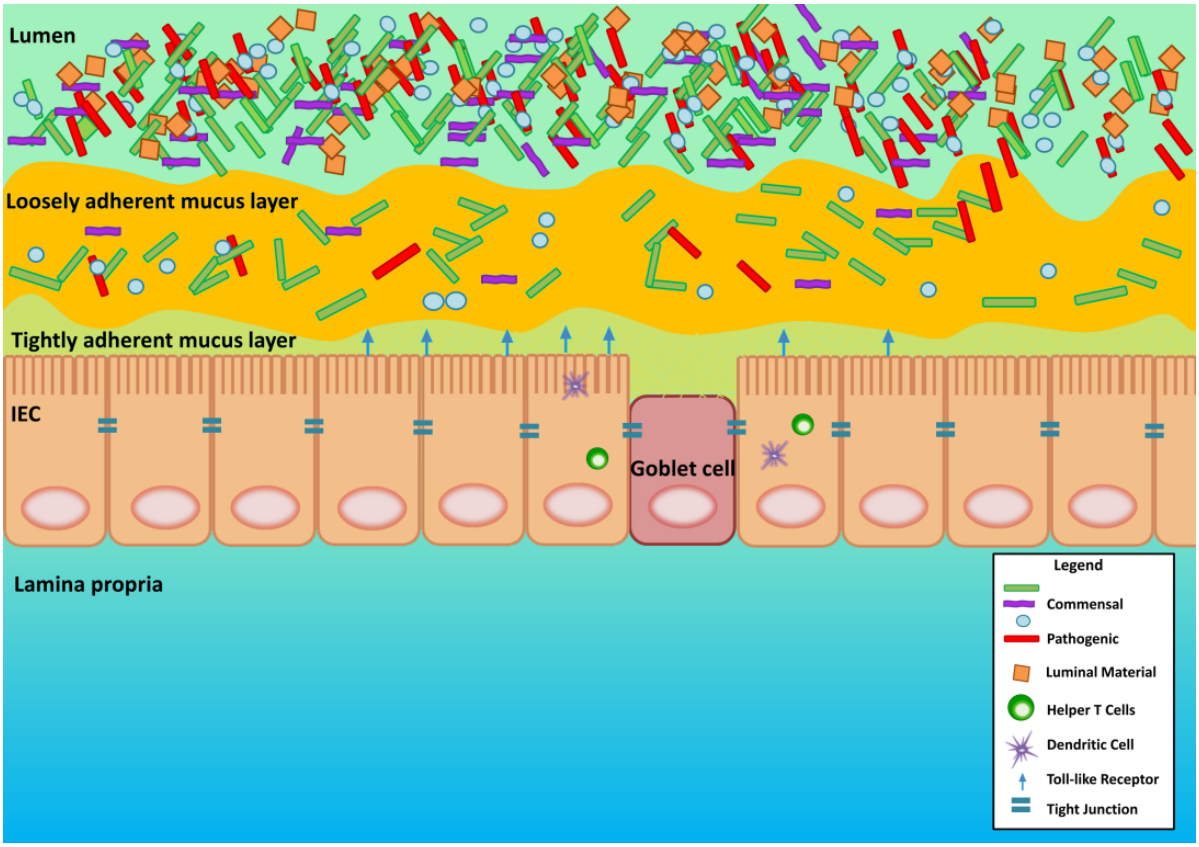

Figure 1.4 Structural organization of the mucus layers within the large intestine. Above the epithelium, the glycocalyx or tightly adherent mucus layer is a sterile (Johansson et al., 2008), stratified layer that provides the initial barrier. This layer is mainly comprised of transmembrane bound mucins. Directly above the tightly-adherent layer, the thick, loosely-adherent layer is located, comprised mostly of MUC2 (with lesser amounts of other mucins) and the mucus-associated bacteria. Host factors such as antimicrobial peptides and immunoglobulins also exist within the layers, and in conjunction with the host immune response are responsible for maintaining homeostasis within the intestine.

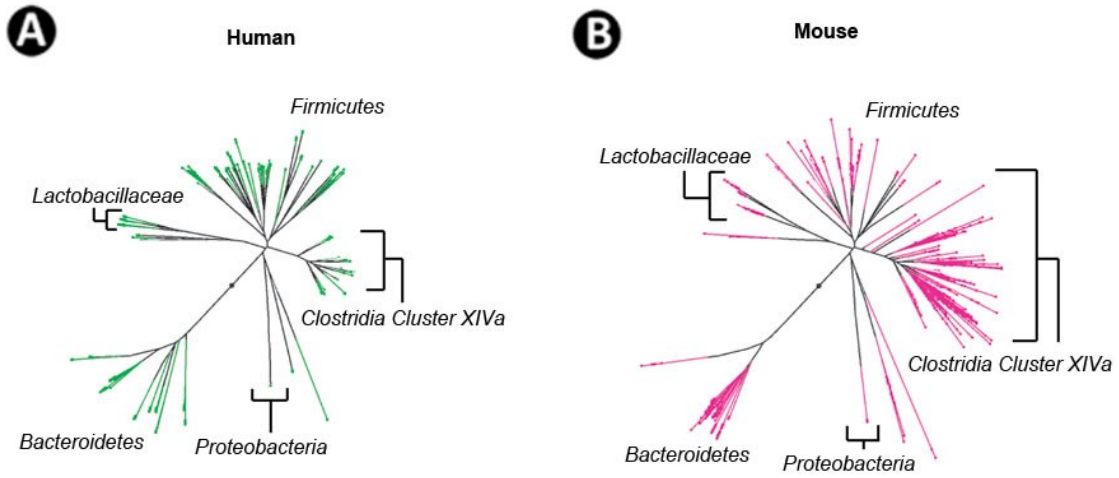

Figure 1.5 Comparison of human and murine intestinal bacteria from 16S rRNA gene sequences. **(A)** Human phylogenetic tree of common microbial phyla. **(B)** Mouse phylogenetic tree of common microbial phyla. Individual microbiomes are represented as neighbor-joined phylogenetic trees. The figure demonstrates the similarities between the two intestinal communities. For each tree, the central black branch indicates similar phyla and class shared among multiple hosts. The colored branches represent the relationship between similar phyla and classes that exists between the species. Image adapted from (Dethlefsen et al., 2007).

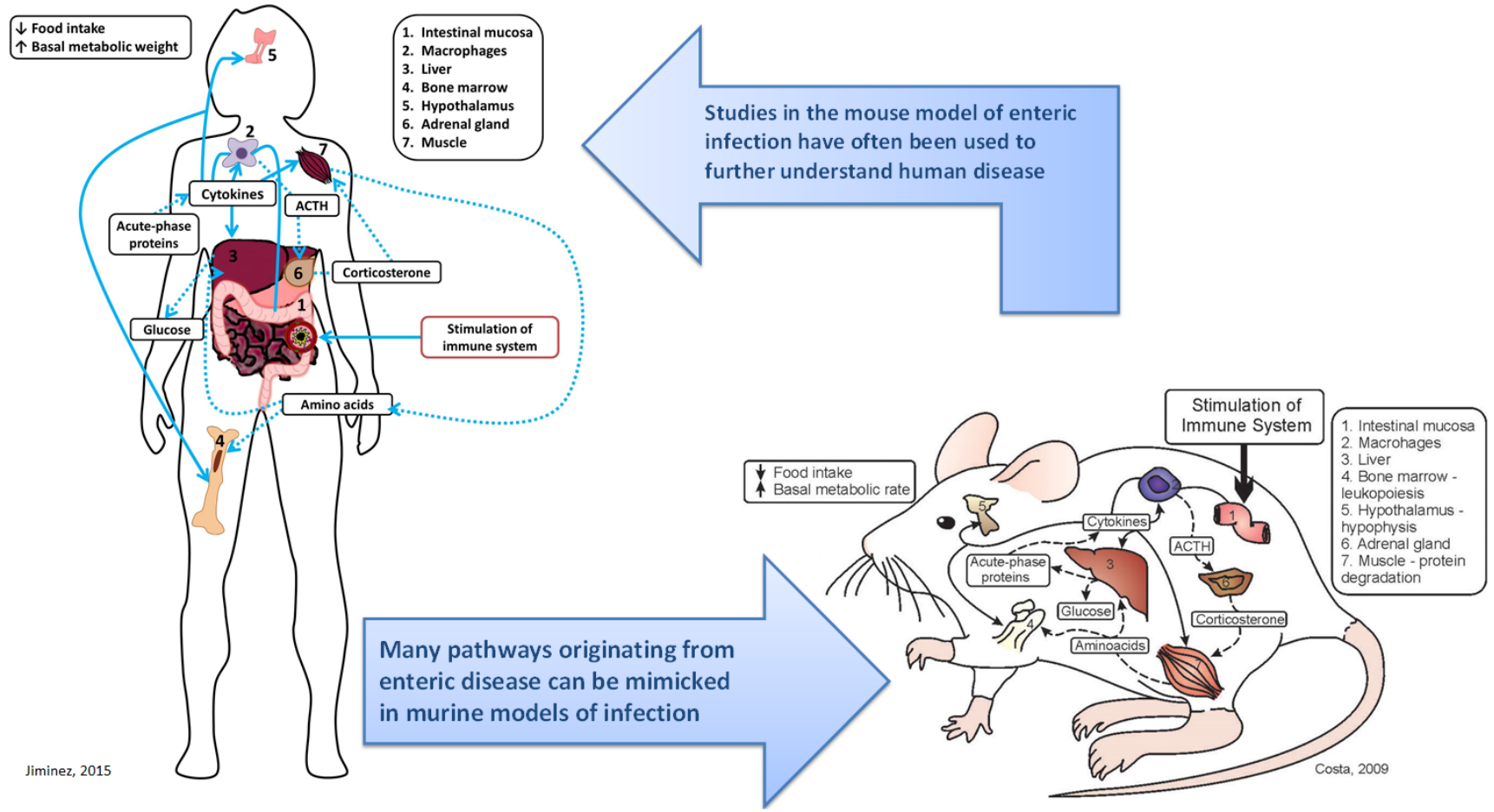

Figure 1.6 The systemic effects of intestinal inflammation compared between the human and murine physiology. An infection within the host intestine induces the activation of multiple integrated systemic metabolic pathways. The image demonstrates the similarities between these metabolic pathways in the human and mouse models. Image adapted from (Jiminez et al., 2015) and from (Costa, 2010).

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Chapter 2: Butyrate ameliorates intestinal inflammation and modulates mucin secretion in *Citrobacter rodentium* infected mice

2.1 Introduction

Butyrate is a SCFA produced by the fermentation of DF in the large intestine, and it is purported to confer a variety of health benefits. Butyrate provides energy to colonocytes, and is the preferred energy source as compared to other SCFA produced within the colon (Lin et al., 2012). In people, concentrations of colonic butyrate can range from 10-20 mM (Cummings et al., 1987), and approximately 95-99% of all SCFA produced in the colon are rapidly absorbed and metabolized into energy sources by colonic cells (Cummings, 1984). A failure to utilize butyrate as an energy source can lead to intestinal disease. As an example, individuals with ulcerative colitis often have metabolic deficiencies in butyrate transport systems, suggesting a reduced ability to absorb butyrate (Leonel and Alvarez-Leite, 2012). These individuals also have impaired butyrate oxidation pathways in colonocytes, further contributing to intestinal disease (Simpson et al., 2000). There has been controversy as to what is considered the optimal concentration of butyrate within the host. Studies that use experimental models of colitis in rodents have reported administering butyrate at concentrations ranging from 40 mM to 130 mM (Barcelo et al., 2000; D'Argenio et al., 1996; Gaudier et al., 2009; Pacheco et al., 2012; Shimotoyodome et al., 2000). Furthermore, concentrations of butyrate administered to people with ulcerative colitis via enemas have ranged from 80 mM to 150 mM (Hamer et al., 2008). A limited number of researchers have investigated changes in mucus secretion as a result of butyrate enemas in animal models, and have also administered butyrate at a high concentration (e.g. 100 mM) in their models. Therefore the high absorptive rate of intestinal SCFA necessitates that higher concentrations be used in scientific investigations (Gaudier et al., 2009; Pryde et al., 2002).

Butyrate also contributes to intestinal health beyond the provision of energy to colonocytes. The addition of butyrate directly to human colons (Hamer et al., 2010), colonic epithelial cells (Russo et al., 2012), and carcinoma epithelial cells (Fung et al., 2012) has been shown to be beneficial in reducing intestinal inflammation (Canani et al., 2011; Ploger et al., 2012; Zimmerman et al., 2012). *In vitro* analyses have shown that

butyrate can reduce expression of pro-inflammatory cytokines and enhance epithelial barrier function, and *in vivo* studies conducted in mice without enteritis showed that butyrate increases mucus synthesis in goblet cells (Gaudier et al., 2009; Hamer et al., 2008). Furthermore, several studies using intestinal cell lines have shown that the administration of butyrate to the intestinal cells can down-regulate NF-κB and reduce the expression of pro-inflammatory cytokines (Hofmanova et al., 2014; Hyzd'alova et al., 2008; Usami et al., 2008), and this is also observed in mouse models of chemically-induced enteritis (Liu et al., 2016; Song et al., 2006).

The mechanisms by which butyrate influences intestinal health, however has not be extensively studied in animal models, particularly the effects of butyrate in response to pathogen induced inflammation, including the impact on dysbiosis and intestinal injury. Some evidence also indicates that butyrate plays a role in maintaining the intestinal barrier by increasing the expression of mucins such as MUC2 and enhancing mucus production (Guilloteau et al., 2010). Intestinal mucins are composed of transmembrane or secretory glycoproteins released from goblet cells, and are involved in forming two protective mucus layers, with MUC2 being the major secretory glycoprotein in the colon (Atuma et al., 2001; Pelaseyed et al., 2014). Importantly, the mucus layer is thought to inhibit bacteria from binding to the epithelium, entering the gut-associated lymphoid tissue (GALT), and inducing pro-inflammatory responses (Johansson et al., 2011). Although mucus is involved in deterring bacteria from disrupting the epithelium, there are bacterial species that utilize mucus as an energy source and colonize the mucus layers as well. *Akkermansia muciniphila* is a well-known mucus degrading organism, and is often isolated from the colons of mice and people (Belzer and de Vos, 2012). Another mucus colonizing bacteria is *Mucispirillum schaedleri,* which selectively colonizes intestinal mucus in a variety of organisms (Robertson et al., 2005). Thus, high densities of mucus-associated bacteria such as *A. muciniphila* and *M. schaedleri* in the colon can be considered as indicators of abundant mucus. Few studies have examined the mechanisms by which butyrate affects mucus secretion, and those investigations often report conflicting results. For instance, Gaudier et al. (Gaudier et al., 2009) demonstrated that rectal administration of butyrate decreased colonic mucus thickness, and increased mucin synthesis in the proximal colon. Conversely, Hamer et al. (Hamer et al., 2010)

reported that the rectal administration of butyrate did not affect mucin production, or mucus secretion in human patients suffering from ulcerative colitis (UC). More recently, studies investigating the effect of butyrogenic bacteria on the intestinal mucus barrier, showed that changes in intestinal bacterial populations influence the production and secretion of mucins from goblet cells (Wrzosek et al., 2013). Moreover, treatment with butyrate also altered bacterial populations of *Bacteroidetes*, *Firmicutes*, *Deferribacteres* and *Proteobacteria* in the large intestine (Kumar et al., 2015; Martinez et al., 2010; Nielsen et al., 2014; Schwab et al., 2014). I hypothesized that butyrate supplementation to acutely inflamed colons will contribute to a temporal and spatial increase in MUC2 and overall mucus secretion, and concomitantly reduce pro-inflammatory signaling and inflammation. To test this hypothesis, I incited acute inflammation with *C. rodentium* (± rectally administered butyrate) and temporally measured a variety of variables including: food consumption and weight gain; intestinal SCFA concentrations; colonic cell damage and injury; the expression of genes involved in pro-inflammatory immune responses and repair; and the overall changes in the colonic bacterial community structure. I also measured the changes to intestinal mucus accumulation between the lumen and at the mucosal surface, including the overall secretion of MUC2 in response to butyrate supplementation to inflamed and non-inflamed colons, with the goal of ascertaining the effects dietary fibre fermentation by-products have on mucus layer maintenance and function.

2.2 Materials and Methods

2.2.1 Experimental design

The experiment was arranged as a completely randomized design with four levels of butyrate concentration (0 mM, 80 mM, 100 mM, and 140 mM), two levels of immunological stress (± *C. rodentium*), and three levels of time post-inoculation (p.i.) (14, 21, and 28 days p.i.). Each replicate included 24 mice, and four replicates were performed on separate occasions (96 animals in total).

2.2.2 Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and

approved by the Lethbridge Research and Development Centre (LRDC) Animal Care Committee (Animal Use Protocol Review 1322), and the LRDC Biosafety and Biosecurity Committee before commencement of the research.

2.2.3 Mouse maintenance

Specific pathogen free (SPF) C57BL/6J female mice were obtained from Charles River Laboratories (Montreal, QC) at 3-weeks of age. For each replicate, mice were group-housed with six mice per cage upon arrival, and were given 10 days to adapt to the animal facility environment under a 10:14 hr dark:light cycle. After the adaptation period, mice were transferred to individually ventilated cages (one mouse per cage) operated in containment mode. Mice were provided a low fibre diet (AIN-93G 103455GI, Dyets Inc., Bethlehem, PA), and were permitted to eat and drink *ad libitum*. Sterile shredded paper was provided for bedding. The health status of each mouse was monitored daily using a quantitative scoring system (Council, 2009). Cages including bedding, food, and water were replaced weekly. Initial body weights were taken a day before the initial enema and gavage inoculations, and again at the time of euthanization. Overall weight gain and feed consumption were measured.

2.2.4 Butyrate administration

A stock solution of butyric acid (>99%; Sigma-Aldrich, Oakville, ON; MW=88.11 g/mol; 100 mL) was diluted with 1X phosphate buffered saline (PBS; 0.01 M NaH_2PO4, 0.04 M Na_2HPO4, 0.07 M NaCl, Sigma-Aldrich; pH 7.4) to attain final concentrations of 80 mM, 100 mM, and 140 mM butyrate; the pH was adjusted to 7.4 ± 0.2 with 10 M sodium hydroxide. The butyrate solution was prepared the day prior to administration, and stored at 4°C until used. Solutions were warmed to room temperature (RT) for 30 min before administration. Phosphate buffered saline (PBS) served as the butyrate control treatment. Butyrate treatments were administered via enemas (300 µL) at 2-day intervals throughout the experimental period. To administer enemas, mice were inverted at a 45° angle, and a 22G X 2.5 cm-long gavage needle with a 1.25 mm ball tip was gently inserted into the colon, the liquid was slowly injected, and mice were maintained in an inverted position for 30 sec after administration of the enema. Animals were monitored for discomfort/pain for 4 hr after the enemas were administered.

2.2.5 *C. rodentium* inoculation

Green fluorescent protein-labelled *C. rodentium* DBS100 (ATCC 51459) was used to incite acute inflammation. The bacterium was grown aerobically on Lysogeny Broth agar (LA) with 30 µg/mL chloramphenicol at 37°C for 24 hr. To differentiate GFP-labelled *C. rodentium* from non-labelled *C. rodentium*, a chloramphenicol resistance gene was incorporated into the genome, which required chloramphenicol to be used for growth and isolation techniques. Biomass was removed from the surface of the agar and transferred into sterile Lysogeny Broth (LB) containing 15 µg/mL chloramphenicol (Sigma-Aldrich). Cultures were maintained for 2 hr at 37°C at 100 rpm, until an $OD_{600} > 0.1$ was obtained. Cultures were centrifuged at 2,256 x g for 15 min, supernatants were removed, and *C. rodentium* cells were re-suspended in 3.0 mL PBS. To confirm densities of viable cells, inoculum was diluted in a 10-fold dilution series, 100 µL of each dilution was spread in duplicate onto LA, cultures were incubated at 37°C, and the number of *C. rodentium* colonies were counted at the dilution yielding 30 to 300 CFU after 24 hr. Cell densities were adjusted to 3×10^9 CFU/mL with PBS. For each replicate, 12 mice were gavaged on two consecutive days with *C. rodentium* cells in PBS (100 µL) or with PBS alone (100 µL) using a 22G X 2.5 cm-long gavage needle with a 1.25 mm ball tip.

2.2.6 Isolation of *C. rodentium* from feces

Fecal samples from mice were collected at 3 day intervals, homogenized in 1.0 mL LB, the homogenate diluted in a 10-fold dilution series, and 100 µL aliquots of each dilution spread in duplicate on MacConkey agar (Becton, Dickinson and Company, Mississauga, ON) containing 15 µg/mL chloramphenicol (Sigma-Aldrich). Cultures were incubated at 37°C for 24 hr, and colonies of *C. rodentium* were enumerated at the dilution yielding 30 to 300 CFU. To confirm the identity of *C. rodentium*, colony PCR was performed on arbitrarily-selected colonies. The EspB protein is responsible for *C. rodentium* attachment to host membranes to cause infection, and this effector molecule was chosen as a target gene for identification (Newman et al., 1999). Primers specific for the *espB* gene, (F:5'-GCTTCTGCGAAGTCTGTCAA-3', R:5'-CAGTAAAGC-GACTTAACAGATT-3') were used to confirm the identity of *C. rodentium* isolates (Newman et al., 1999). PCR conditions commenced with one cycle of 15 sec at 95°C,

followed by 35 cycles of 45 sec at 95°C, 1 min at 57°C, 1 min at 72°C, and a final cycle of 5 min at 72°C. The amplicon was 270 bp in size.

2.2.7 Animal euthanization and intestinal sample collection

On days 14, 21 and 28 p.i., one randomly selected mouse from each treatment was anesthetized with isoflurane followed by euthanasia by cervical dislocation under anesthesia. Immediately after death, a mid-line laparotomy was used to exteriorize the intestine, and a gross pathological assessment of the intestine was completed. The colon was longitudinally incised, and ingesta was collected and stored at -20°C for analysis of SCFA. Sections of distal colon (≈4 mm long) were weighed and placed at -20°C for DNA analyses, and in RNAlater™ (Qiagen Inc., Toronto, ON) at -20°C for mRNA extraction. Tissue from the distal colon was also collected for histopathologic and mucus analyses, as well as for fluorescent *in situ* hybridization (FISH).

2.2.8 Histopathology

Harvested colonic tissue was fixed in Surgipath® 10% neutral buffered formalin (Leica Biosystems, Concord, ON) for 24 hr. Formalin-fixed tissues were dehydrated in ethanol and placed in Histo-Clear (Diamed Lab Supplies, Mississauga, ON) prior to embedding in paraffin at 60°C. Sections (5 µm) were deparaffinized with xylene, and stained with hematoxylin and eosin (H&E). Tissues were scored for mucosal damage by an experienced veterinary pathologist blinded to the treatments using an established scoring guide (Costa et al., 2011) that ranked common characteristics of mucosal damage from 0 to 4, with 4 being pronounced damage and 0 representing minimal to no damage (Table A1). Sections were also scored (0 - 3 or 4) for epithelial cell wall hyperplasia based on mild to severe increases in cells found within crypt columns; for crypt height based on mild to severe increases in height; for epithelia cell injury noting the degree of focal erosions and cell shedding; the degree of inflammation based on the number of neutrophils and mononuclear cells present in the lamina propria; goblet cell depletion based on the number of goblet cells and mucin droplet size; and the degree of mitotic activity based on how much of the epithelial cell displayed increased activity (Table A1).

2.2.9 Characterization of mucus and MUC2

Colonic tissue samples were fixed overnight in Methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) (Johansson and Hansson, 2012) prior to dehydration with ethanol and Histo-Clear (Diamed Lab Supplies). Sections (5 μm) were deparaffinized for 5 min on a 60°C heating bed, cleared with xylene, and rehydrated in a decreasing ethanol gradient (100%, 90%, 70%, 50%) according to a standard protocol (Abcam, Toronto, ON). To visualize mucus, sections were stained with Alcian Blue (pH 2.5; American MasterTech, Lodi, CA) for 30 min, 0.5 % Periodic Acid (American MasterTech, Lodi, CA) for 5 min, and Schiff's solution (American MasterTech, Lodi, CA) stored at 4°C for 15 min. To visualize MUC2, an adjacent section to the PAS stained section was used. Deparaffinized and rehydrated sections were immersed in 10 mM sodium citrate (0.05% Tween 20, pH 6.0) at 96-100°C for 20 min, and cooled to RT. A portion of the section was circled with a hydrophobic pen, and blocking solution (IHC® Select; Millipore Ltd., Etobicoke, ON) was applied for 2 hr. Rabbit anti-MUC2 primary antibody (Abcam Inc., Toronto, ON) was diluted in Tris-Buffered Saline (TBS) containing 1% bovine serum albumin (BSA) to a final concentration of 1:50, and incubated overnight at 4°C. Slides were then washed with 0.025% Triton-TBS solution, and conjugated goat anti-rabbit IgG diluted at 1:1500 with 1% BSA in TBS (Alexa Fluor 568, Abcam Inc.) was applied to the sections for 2 hr. Slides were then washed with the 0.025% Triton-TBS wash solution, and mounted with ProLong® Gold Antifade solution with 4',6'-diamidino-2-phenylindole (DAPI) DNA stain. Slides were stored in a light-omitting box at 4°C, and a confocal microscope (Fluoview™ FV1000, Olympus Canada Inc., Richmond Hill, ON) was used to visualize mucus and MUC2 associated with colonic tissues.

2.2.10 SCFA analysis

To quantify SCFA levels, feces were weighed within 30 min of collection and homogenized in PBS at a 1:9 ratio (w/v). Meta-phosphoric acid (Sigma-Aldrich) was added to the homogenate at a 1:4 ratio (v/v), and incubated at RT for 30 min. Samples were then centrifuged for 75 min at 16,100 x *g*, and the supernatants were collected and stored at -20°C. Acetate, butyrate, and propionate concentrations were quantified with a gas chromatograph (Model 6890N with 7683 Series Injector; Agilent Technologies,

Mississauga, ON) according to an established protocol (Cottyn and Boucque, 1968; Playne, 1985).

2.2.11 Characterization of ingesta and mucosal-associated bacterial communities

Mucosa-associated bacterial genomic DNA was extracted from distal colonic samples using DNeasy Blood and Tissue extraction kit (Qiagen Inc.). Genomic DNA was also extracted from the distal ingesta using the QIAamp® Fast DNA stool extraction kit (Qiagen Inc.). Extracted DNA was processed using an Illumina protocol for creating 16S rRNA gene metagenomic sequencing libraries (Illumina, 2013). Extracted DNA was normalized to 5 ng/μL in 10 mM Tris (pH 8.5). Following this, 2.5 μL of purified DNA was PCR amplified with 5 μL of each amplicon primer, spanning the V3 and V4 regions of the 16S rRNA gene (F:5'TCGTCG-GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; R:5'GTCTC-GTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Illumina, 2013) and 12.5 μL of 2x KAPA HotStart Ready mix (Kapa Biosystems, Inc., Wilmington, MA) for a final volume of 25 μL. The resulting 550 base pair product underwent a PCR clean-up using AMPure XP beads (Beckman Coulter Canada Inc., Mississauga, ON) on a magnetic stand to isolate the DNA, and the product was washed with 80% ethanol and eluted with 10 mM Tris pH 8.5. An indexing PCR reaction was used to add forward and reverse indices to each sample. Conditions included 5 μL of DNA, 5 μL of each index primer (specific non-repeating pair per sample) and 25 μL of 2x KAPA Hifi HotStart Ready mix and Nuclease-free water (Qiagen Inc.) to a final volume of 50 μL per sample. A final PCR clean-up was performed on the 630 bp product. Indexed DNA libraries were quantified and normalized to 4 nM with 10 mM Tris pH 8.5, and 5 μL of each normalized library was pooled into one sample for sequencing using a MiSeq (Illumina, San Diego, CA). A PhiX control was run in parallel with the normalized DNA libraries, and both were denatured and diluted to 4 pM prior to loading onto the MiSeq cartridge.

Forward reads were assembled using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) software package (Caporaso et al., 2010b), resulting in a total of 13,125,994 sequences. Barcodes were extracted from each sample FASTQ file, and

joined with its corresponding forward read. Libraries were split according to barcode, and sequences were filtered to include only those sequences with a base calling accuracy Phred value (Q) of 20 or greater, indicating the probability of 1 in 100 base calls being incorrect. Sequence reads were filtered to exclude reads with more than three consecutive low quality base calls, less than 75% of the read length containing consecutive high base calls, and/or the barcode having more than 1.5 errors present. These sequences (n=5,390,077) were then chimera checked using USEARCH 6.1 software, and the resulting chimeras were filtered out prior to picking operational taxonomic units (OTU) from the Greengene reference database. In total, 371,065 OTUs were identified using a 97% similarity parameter, and the most common sequence was used to define the groups of similar OTUs. After, OTUs were aligned using the NAST algorithm (Caporaso et al., 2010a) to the Ribosomal Database Project (RDP) classifier of 0.5 (sequences having at least 50% similarity to reference database sequences). Taxonomy was assigned to each sequence cluster using UCLUST (Edgar, 2010) and classified using the Greengenes reference database (McDonald et al., 2012). An OTU table was produced, and all samples were rarified so that 3,450 OTUs were randomly chosen and compared between each sample for analysis (OTU per biological sample ranged from 3,450 to 100,000). Diversity among species (β-diversity) was examined using Bray-Curtis, weighted and unweighted UniFrac analyses (Lozupone et al., 2011).

2.2.12 Visualization of intestinal bacteria

The presence and localization of bacteria within the colon was determined using FISH. Preparation of colonic samples for FISH was performed using the method described above for PAS and MUC2 staining. Distal colonic tissue cross-sections were circled with a hydrophobic pen, and sections were incubated in the dark overnight at 37°C, with either the Alexa Fluor 555-conjugated total bacteria probe, EUB338 (Life Technologies, Burlington, ON) (5'-GCTGCCTCCCGTAGGAGT-3'), or the Alexa Fluor 555-conjugated γ-*Proteobacteria* probe, Gam42a (Life Technologies) (5'-GCCTTCCCACATCGTTT-3'). Probes were stored in 0.25 µg/µl stocks and diluted (1:100) with hybridization buffer (0.9 M NaCl, 0.1 M, Tris pH 7.2, 30% formamide, 0.1% SDS) prior to adding the solutions to the sections. The sections were stored in a dark, humidifying box. After incubation with the fluorescent probe, sections were washed

with hybridization buffer in the dark for 15 min, and then with wash buffer (0.9 M NaCl, 0.1 M Tris pH 7.2). Sections were mounted with ProLong® Gold Antifade solution with DAPI (Bergstrom et al., 2010). Sections were analyzed with the Zeiss Axioskop II plus (Carl Zeiss Canada, Ltd., North York, ON) microscope using the Zen2 (Blue edition) core imaging software.

2.2.13 Quantification of gene expression

The gene expression of immune related cytokines (Table A2) was analyzed from total RNA that was extracted using an RNeasy® Mini kit (Qiagen Inc.). The concentration and quality of the total RNA extracted was analyzed using a RNA 600 Nano LabChip and a 2100 bioanalyzer (Agilent Technologies). Using 1000 ng of total RNA, reverse transcription was performed using the QuantiTect® Reverse Transcription Kit (Qiagen Inc.). The reference genes used to normalize the measured C_t values were *Ppia*, *Hprt*, and *GusB*. Quantitech SYBR Green Mastermix (Qiagen Inc.) was used as an indicator of double stranded DNA and product amplification. Individual PCR reactions consisted of: 1 µL of cDNA; 3 µL of dH_2O; 0.5 µL of 10 µM forward primer; 0.5 µL of 10 µM reverse primer; and 5 µL SYBR green (Table A2). Reactions were run in triplicate per cDNA sample. Quantitative PCR reactions were run on a 384-well ABI 7900HT qPCR thermocycler (Life Technologies), with an activation step of 95°C for 15 min, and 40 cycles of 94°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, followed by 1 cycle of 95°C for 15 sec, 55°C for 15 sec and 95°C for 15 sec. Normalized gene expression was calculated using qbasePLUS (Biogazelle, Zwijnaarde, Belgium) based on geNorm and qBase quantification models (Hellemans et al., 2007; Vandesompele et al., 2002).

2.2.14 Statistical analyses

The majority of the statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). Continuous data was checked for normality, and analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Where applicable (i.e. samples were not independent), collection time was treated as a repeated measure; the appropriate covariance structure was utilized according to the lowest Akaike's Information Criterion. In the event of a significant main effect, the least squares means test (LSM) was used to compare treatments within factors. Categorical data (i.e. histopathology) was analyzed

using the GLIMMIX procedure. For bacterial community analyses, both SAS and Primer 7 were used. In Primer 7, PERMANOVA (permutational ANOVA) and principle co-ordinate analyses (PCoA) were used to analyze β-diversity, whereas, analysis of variance (MIXED procedure) with a protected LSM test were used to analyze α-diversity. Differences in the abundance of microbial OTUs were also analyzed using the MIXED procedure with a protected LSM test. For both parametric and non-parametric statistical analyses, P-values ≤0.050 were considered to be statistically significant, whereas P values >0.050 and ≤0.100 were considered to be a significant trend.

2.3 Results

2.3.1 *C. rodentium* incited enteritis in C57BL/6 mice

Citrobacter rodentium was not detected in feces of mice not gavaged with the bacterium. In contrast, all mice with enteritis shed the bacterium in their feces. Regardless of butyrate treatment, shedding of *C. rodentium* peaked on day 14 p.i. (4×10^7 CFU/mL to 3×10^9 CFU/mL), and densities of the bacterium decreased thereafter (Day 21, 7×10^4 CFU/mL to 7×10^7 CFU/mL; Day 28, 3×10^4 to 1.20×10^6 CFU/mL). Mice that were not gavaged with *C. rodentium* did not show symptoms of infection, nor did they exhibit overt evidence of enteric epithelial cell hyperplasia, epithelial cell injury, mitotic activity, goblet cell presence or crypt height, with total average scores of less than 1.30 ± 0.25. Alternatively, all mice gavaged with *C. rodentium* presented symptoms and signs of enteritis and exhibited substantially higher histopathologic scores (P≤0.001) (Figure 2.1A-D) relative to mice without enteritis (Figure 2.2) for all of the categories examined. The degree of tissue injury was highest on day 14 p.i. (i.e. peak infection), and decreased by day 21 (i.e. late infection) and was minimal by day 28 (i.e. clearance).

2.3.2 High butyrate concentrations decrease epithelial cell hyperplasia, epithelial cell injury and goblet cell depletion in mice with enteritis

Mice with enteritis exhibited total histopathologic scores ≥ 14.0 ± 2.7 at peak infection, ≥ 9.3 ± 3.5 at late infection, and ≤ 6.5 ± 0.1 at clearance (Figure 2.1A). Scores of total histopathologic changes did not differ between peak and late infection (P=0.19), but were lower (P≤0.009) at clearance. Averaged over time, total pathologic changes (P=0.020), goblet cell depletion (P=0.047), epithelial cell injury (P=0.031), and epithelial

cell hyperplasia (P=0.023) were lower in mice with enteritis and administered butyrate at a concentration of 140 mM (Figure 2.1A-D) compared to mice not administered butyrate. Increased mitotic activity (P=0.054) was also observed in mice with enteritis and administered butyrate at 80 mM at peak infection when compared to mice with enteritis not administered butyrate (Figure 2.3).

2.3.3 Fecal butyrate concentrations are higher in mice administered butyrate at 140 mM during peak and late infection

There was no difference in concentrations of butyrate measured in feces (P=0.59) due to butyrate administration (Figure 2.4A-B). Furthermore, there was no effect (P=0.56) of time p.i. on the overall concentration of butyrate measured in feces. However, mice with enteritis exhibited a trend for higher quantities of butyrate (P=0.073) in their feces compared to mice without enteritis. The rectal administration of butyrate also did not affect total SCFA concentrations (P=0.84) measured in feces. However, in mice inoculated with *C. rodentium*, SCFA concentrations measured in feces were higher (P=0.020) relative to mice inoculated with PBS (Figure 2.4D). Furthermore, total SCFA concentrations were higher (P=0.047) in mice with enteritis at late infection compared to peak infection.

2.3.4 Butyrate increases weight gain and feed consumption in mice with enteritis during peak infection

Butyrate administration did not affect feed consumption (P>0.95) or weight gain (P>0.44) in mice without enteritis at any of the three sample times (Figure 2.5). At peak infection, mice with enteritis and administered butyrate consumed more (P=0.003) food than control mice that were not administered butyrate (Figure 2.6). During late infection (P=0.38) and clearance (P=0.34), there was no difference in food consumption between control mice and those supplemented with butyrate. Mice with *C. rodentium* induced enteritis that were administered butyrate gained weight faster (P≤0.002) than mice not administered butyrate at peak infection (Figure 2.7A), but not at late infection or clearance (P≥0.53) (Figure 2.7B).

2.3.5 Mucus localizes to the lumen and goblet cells with butyrate supplementation at peak infection

At peak and late infection, mice with enteritis and administered butyrate at a concentration of 140 mM exhibited increased accumulation of mucus in the colonic lumen and within goblet cells relative to mice administered PBS enemas (Figure 2.8A-D, 2.9A-D). Immunostaining was used to examine localization of MUC2 within the distal colon of mice with enteritis, and no conspicuous differences were observed among the PBS and butyrate treatments at peak infection (Figure 2.10A-B). However, at late infection, an increase in MUC2 localization was observed in the lumen of mice administered butyrate relative to mice administered PBS (Figure 2.10C-D). This corresponded to butyrate stimulation of mucus accumulation in the distal colon.

2.3.6 Butyrate supplementation affects enteric gene expression in mice with enteritis

In mice without enteritis, the administration of butyrate had no effect ($P \geq 0.16$) on the expression of Th1 (*IFNγ*), Th2 (*IL-4*), and Th17 (*IL-17A*, *IL-22*) associated cytokines, and *Myd88*, *Prg3*, *RegIIIγ*, *Tff3*, and *TLR9* relative to mice that were not administered butyrate (Figure 2.11). In contrast, in mice administered butyrate at a concentration of 80 mM exhibited a trend of increased ($P=0.062$) expression of Th2 (*IL-4)* cytokines, and significantly ($P \leq 0.050$) increased expression of Th1 (*IFNγ*, *TNFα*), and Th17 (*IL-22*) cytokines in mice with enteritis (Figure 2.12); in mice administered butyrate at concentrations of 100 mM and higher also exhibited increased ($P \leq 0.050$) expression of *IL-1β*. The administration of butyrate at 80, 100, and 140 mM to mice with enteritis increased ($P \leq 0.036$) the expression of genes involved in bacterial recognition and defense including *Myd88*, *TLR2*, and *Ltb4r1*, but only butyrate administered at 80 mM ($P=0.005$) and 100 mM ($P=0.004$) exhibited increased expression of *TLR9*. Butyrate administered at 80 mM ($P=0.044$) and 100 mM ($P=0.076$) but not 140 mM ($P=0.99$) exhibited increased expression of *Prg3*. Butyrate administration at 100 mM also increased ($P=0.028$) expression of *RegIIIγ*, and administration of 100 mM ($P=0.007$) and 140 mM ($P=0.012$) butyrate increased expression of *RELMβ*, while 80 mM ($P=0.002$) and 100 mM increased ($P= 0.045$) expression of *Tff3* in mice with enteritis. There was no difference ($P \geq 0.36$) between the PBS and *C. rodentium* treatments in the expression of the Treg response cytokines, *IL-10* and *TGFβ* in mice not administered butyrate (Figure 2.13A-D). In mice

administered butyrate at 80 and 100 mM, expression of *IL-10* was increased (P≤0.028), and all concentrations of butyrate stimulated an increase (P=0.049) in *TGFβ* expression in mice with and without enteritis (Figure 2.13A-D). Regardless of butyrate administration, mice with enteritis displayed a trend of decreased (P=0.053) *Muc2* expression relative to mice without enteritis. Administration of butyrate at concentrations of 80 and 100 mM increased *Muc2* expression in mice without enteritis (P<0.023) and demonstrated a trend for increased (P<0.088) *Muc2* expression with enteritis (Figure 2.13E-F).

2.3.7 Butyrate supplementation reduces the abundance of *Firmicutes* and butyrate producing bacteria in the distal colon

Based on Uni-Frac comparisons of community similarities, the structure of the mucosa-associated bacterial community was more variable, and differed (P=0.087) from the community structure of the ingesta in the distal colon (Figure 2.14). The administration of 140 mM butyrate altered the community structure within ingesta but not within the mucosa-associated community in mice without enteritis (Figure 2.15A-D). Butyrate affected (P≤0.050) the abundance of bacteria within the *Bacteroidetes* (i.e. *Paraprevotelaceae*), *Firmicutes* (i.e. *Bacilli*; *Clostridiaceae*; *Erysipelotrichales*; *Peptostreptococcaceae*) and *Tenericutes* (i.e. *RF39*) phyla (Figure 2.16A-B). During peak infection, the administration of butyrate showed a trend for reduced (P=0.063) the abundance of *Firmicutes*, and averaged over both time points, the densities of mucosa-associated *Lachnospiraceae* were reduced (P=0.022) in in mice with and without enteritis (Figure 2.17, Figure 2.18A). At late infection, mice inoculated with *C. rodentium* exhibited a lower abundance (P=0.022) of species within the *Bacteroidetes* phylum; however, in mice without enteritis, butyrate increased (P=0.022) these bacteria, especially *Parabacteroides* sp. (P=0.023) associated with the mucosa averaged over time (Figure 2.17, Figure 2.18A). An increase (P=0.005) in the abundance of γ-*Proteobacteria* was also observed during late infection in the distal mucosa in mice with enteritis administered 140 mM butyrate, and in mice without enteritis, butyrate increased (P=0.052) the presence of *Bilophila* sp. in association with the mucosa (Figure 2.17). Within the ingesta, butyrate administration altered bacterial communities during late infection (Figure 2.17). For example, mice without enteritis and administered 140 mM

butyrate displayed a depletion in *Firmicutes* (P=0.025), namely *Clostridiales* (P=0.029) species, and showed a trend of reduced (P=0.067) *Ruminococcaceae* species abundance during late infection. Alternatively, mice with enteritis exhibited a higher abundance of *Firmicutes* (P=0.025), *Clostridiales* (P=0.029), and *Lachnospiraceae* species (P=0.014) relative to mice with enteritis not administered butyrate at late infection (Figure 2.17). In mice without enteritis *Parabacteroides* spp. increased (P=0.045) in abundance with butyrate supplementation (Figure 2.18) averaged over time. Although significant differences between the treatments in the abundance of mucus-associated species were not evident regarding the abundance of mucus-associated species, butyrate administration caused a trend of reduced *A. muciniphila* abundance within the ingesta collected from the distal colon in mice with and without enteritis (Figure 2.18B). In contrast, butyrate administration was associated with a general trend of increased abundance of *A. muciniphila* associated within the mucosa (Figure 2.18A). Similarly in mice without enteritis, a trend of increased abundance of the mucus dwelling bacterium, *Mucispirillum schaedleri* in the mucosa-associated community was observed (Figure 2.18A), as well as within the ingesta of mice with enteritis (Figure 12.18B). Overall, butyrate administration decreased the abundance of bacteria in the ingesta and associated with mucosa. However, in ingesta collected from the distal colon, butyrate effectively increased the abundance of members of the *Lachnospiraceae* family during infection.

2.3.8 FISH visualization displays a high abundance of *Proteobacteria* in butyrate supplemented tissue

High densities of γ-*Proteobacteria* and *Enterobacteriaceae* were associated with the mucosa in the distal colon of mice infected with *C. rodentium* when compared to mice without enteritis, and high densities of γ-*Proteobacteria* were observed during late infection in mice with enteritis supplemented with 140 mM butyrate compared to those supplemented with 0 mM butyrate (Figure 2.19A-B). It is noteworthy that in mice with enteritis, high densities (P=0.036) of mucosa-associated *Pseudomonas* spp. were also observed in mice administered butyrate in the distal ingesta as compared to those not given butyrate (Figure 2.20). *Proteobacteria* were more often observed within intestinal colonic crypts in mice rectally administered butyrate at a concentration of 140 mM (Figure 2.19D, F) relative to mice not administered butyrate (Figure 2.19C, E). Butyrate

supplementation at a concentration of 140 mM increased the total abundance of γ-*Proteobacteria*, especially in mice infected with *C. rodentium*.

2.4 Discussion

2.4.1 Butyrate increases food consumption and weight gain through metabolic signaling

Food consumption and weight gain are characteristics of good intestinal function and host health, and it was observed that infection with *C. rodentium* caused a reduction in food consumption and weight gain, which has been previously reported (Bhinder et al., 2013; Costa et al., 2011). It was also observed that the rectal administration of butyrate resulted in an increase in food consumption and weight gain in infected mice, indicating that butyrate plays an important role in improving feeding behaviour and enhancing growth during periods of active enteritis. In rodents, butyrate within the intestine utilizes cyclic-AMP dependent mechanisms to stimulate gluconeogenesis, and high glucose levels in the portal vein activate brain stimuli that promote the metabolism of glucose, improve feed intake, and subsequently promote growth (Boets et al., 2015; De Vadder et al., 2014). Thus, it is possible that butyrate administered to mice challenged with *C. rodentium* ameliorates bacterial induced colitis by enhancing intestinal gluconeogenesis.

2.4.2 Butyrate influences Th1 and Th17 responses within the inflamed colon

Infection with *C. rodentium* resulted in a strong activation of Th1 and Th17 associated cytokines. These responses are necessary for pathogen clearance (Costa et al., 2011; Weaver et al., 2013). Similarly to other studies, I observed elevated levels of epithelial cell hyperplasia, in addition to increased expression of *TNFα, IL-1β, IL-17A* and *IL-22* in the distal colon of *C. rodentium* infected mice (Costa et al., 2011; Luperchio and Schauer, 2001). In the current study, butyrate treatment further increased the expression of cytokines involved in the Th1 and Th17 immune response, which potentially attenuated the infection and promoted epithelial cell restoration (Costa et al., 2011; Wang et al., 2014). This is underscored by the prominent increase in the expression of *IFNγ*, *TNFα, IL-1β*, and Th17 associated cytokines, which are generally used as indicators of active intestinal inflammation (Kojouharoff et al., 1997; Weaver et al., 2013). The expression of *TNFα*, *IFNγ*, and *IL-1β* can induce macrophage activation and epithelial cell derived nitric oxide (NO), and increased quantities of NO have been observed in colonic

epithelial cells displaying marked tissue damage and inflammation (Kojouharoff et al., 1997; Obermeier et al., 1999). Nitric oxide has been identified as an inducer of inflammation, however, Th1 induced NO production has also been observed in mice with attenuated inflammation during acute colitis (McCafferty et al., 1997; Obermeier et al., 1999). Furthermore, increases in cytokines such as IL-22 and IL-23 have been linked to the reduction in levels of intestinal inflammation in the dextran sulfate sodium-induced colitis in mice, which is important in the maintenance of homeostasis (Sugimoto et al., 2008). Collectively, coordinated Th1 and Th17 immune responses are critical to the clearance of *C. rodentium* (Costa et al., 2011), and butyrate further stimulated the expression of cytokines involved in resolving the infection (Collins et al., 2014).

2.4.3 Butyrate stimulation of the Th17 response triggers bacterial recognition factors

Expression of the cytokines *IL-17A* and *IL-17F* are in important in the clearance of *C. rodentium* infection, and it has been proposed that these cytokines facilitate the induction of antimicrobial peptides to reduce inflammation (Tanoue and Honda, 2015). Specifically, Th17 cytokines including IL-22 have been shown to increase REGIIIγ production during *C. rodentium* induced colitis in mice (Zheng et al., 2008). I observed that gene expression of the *Myd88* signaling factor (Abreu, 2010) for the intestinal antimicrobial C-type lectin REGIIIγ was increased in mice with enteritis supplemented with butyrate, compared to mice with enteritis that were not treated with butyrate. *Myd88* expression is involved in the enterocyte caspase-1 inflammasome signaling pathway, and it is a required component for intestinal tissue repair (Saleh and Trinchieri, 2011). In contrast, REGIIIγ is a protein considered to be a maintenance factor in intestinal homeostasis by regulating the distance and interactions between resident intestinal bacteria and the epithelial surface (Vaishnava et al., 2011). Additionally, *RegIIIγ* gene expression in the current study was upregulated in mice with enteritis that were administered butyrate. It was demonstrated that butyrate administration restored the distance between the epithelial layer and bacteria in the lumen, and this corresponded to an increase in *RegIIIγ* expression. REGIIIγ binds directly to the peptidoglycan on the cell wall of Gram positive bacteria to induce antimicrobial activity (Cash et al., 2006; Vaishnava et al., 2011). In mice with enteritis and administered 140 mM butyrate, a

marked reduction of *Firmicutes* abundance was observed; the majority of *Firmicutes* members primarily possess a Gram positive cell wall structure (Vesth et al., 2013). Mice with and without enteritis and administered butyrate displayed an increase in the abundance of Gram negative *Proteobacteria*. This trend was also observed by Lupp et al. (Lupp et al., 2007), who reported an increase in *Enterobacteriaceae* bacteria during *C. rodentium*-induced colitis, and attributed this to a marked reduction in bacterial abundance caused by both the *C. rodentium* infection, and the impending host response. My research shows that butyrate treatment increases the expression of proteins involved in bacterial-host interactions (i.e. antimicrobial lectins targeting Gram positive cell walls) and suggests that these proteins are involved in altering various bacterial populations within the colon.

2.4.4 Butyrate affects Treg responses and mucus secretion

Regulatory T-cell responses are important for modulating and resolving inflammatory responses within the intestine. These responses in association with the stimulation of mucus secretion at the epithelial surface provide an effective strategy to reduce tissue injury following challenges with bacterial pathogens (Smith et al., 2013; Van der Sluis et al., 2006). Previous studies have shown butyrate increases CD4+ *Foxp3* expression from T-cells within the colonic mucosa, leading to the mitigation of intestinal injury and promoting inflammatory quiescence (Arpaia et al., 2013; Furusawa et al., 2013). Similarly, my findings demonstrated that butyrate administered at low and high concentrations increased the expression of *TGF-β* and *IL-10* in mice with and without enteritis. The maintenance of the physical mucus barrier is important in providing protection to the intestinal epithelium and maintaining intestinal homeostasis (Bergstrom et al., 2010; Bergstrom and Xia, 2013), and I observed that butyrate administration to mice increased *Muc2* gene expression, production of mucus within goblet cells, and deposition of mucus at the epithelial layer. The presence of SCFA within the intestine has the potential to upregulate mucin secretion (Kles and Chang, 2006), and butyrate in particular has been linked to increasing *Muc2* gene expression in cell lines and within the murine colon (Gaudier et al., 2009; Hamer et al., 2008; Hatayama et al., 2007). In contrast, data in support of the ability of butyrate to increase mucus secretion *in vivo* are inconsistent (Gaudier et al., 2004; Hamer et al., 2010; Van der Sluis et al., 2006).

Although this research strongly suggests a relationship between increased mucus production and the rectal administration of butyrate, the amount of butyrate measured in fecal samples remained unchanged among the butyrate concentration treatments. However, butyrate concentrations measured in feces from mice with enteritis were higher suggesting that SCFA were more rapidly absorbed by colonocytes in animals without enteritis (Cummings, 1984; Pryde et al., 2002).

The maintenance of a protective epithelial barrier is a complex process and includes other proteins involved in the production and secretion of mucus, and enterocyte development and turnover. As an example, accessory proteins such as TFF3 and RELMβ are involved in maintaining epithelial cell homeostasis and regeneration (Nair et al., 2008), and it was observed that the increased expression of genes encoding both proteins occurred following treatment with butyrate. TFF3 is a trefoil peptide secreted from intestinal goblet cells, and increases mucin viscosity to provide further protection to the mucosa, and can enhance epithelial repair in the small and large intestine (Kim and Ho, 2010; Kindon et al., 1995). RELMβ is primarily secreted from goblet cells, and is mainly found in localized foci of inflammation in mice and humans (Artis et al., 2004). The functions of *Relmβ* are poorly understood, however, this gene has been associated with the regulation of insulin resistance, and recent evidence suggests it is immunomodulatory in the colon and contributes to intestinal mucus secretion in mice (Bergstrom et al., 2015; Hogan et al., 2006; Krimi et al., 2008). It was shown that butyrate administration in mice with enteritis contributed to the increased expression of *Relmβ*, *Tff3*, and *Muc2*, and ultimately increased mucus secretion in the distal colon. My research demonstrates that butyrate may affect the regulation of mucus secretion during periods of intestinal inflammation through the modulation of proteins associated with mucus secretion, integrity, and epithelial cell repair and restitution.

2.4.5 Butyrate alters bacterial community structure

A general decrease in *Firmicutes* abundance was observed in inflamed intestines in the presence and absence of 140 mM butyrate. Shifts from *Bacteroidetes* to *Firmicutes* dominant communities in inflamed intestines have been reported previously, however, evidence is conflicting on whether higher densities of either *Bacteroidetes* or *Firmicutes*

correlate to negative or beneficial effects on intestinal health. These results correspond with Schwab et al.(2014), who observed that chemically induced injury to the colon increased the abundance of bacteria belonging to the *Bacteroidales* order, however, this shift also resulted in the reduction of bacteria in the *Clostridiales* order, an order that includes many butyrogenic bacterial species (Schwab et al., 2014). It was found that mice challenged with *C. rodentium* exhibited increases in *Deferribacteres* and *Proteobacteria* species, and these changes are similar to the observations of Hoffman et al. (Hoffmann et al., 2009). My observations parallel their findings only at the bacteria family level however. I also observed a butyrate dependent increase in *Lachnospiraceae* in ingesta within the distal colon, suggesting that butyrate was associated with the increase in abundance of this group of bacteria. Importantly, the *Lachnospiraceae* family includes *Coprococcus* spp., *Roseburia* spp., and *Butyrivibrio* spp., bacteria that are butyrate-producing species in the mammalian intestine (Meehan and Beiko, 2014). My findings suggest that under conditions of inflammation, butyrate increases the abundance of bacteria in the *Lachnospiraceae* family. Notably many of these bacteria synthesize butyrate in the distal colon, providing butyrate as a substrate to be metabolized by epithelial cells increasing cell differentiation and repair, and subsequently mitigating inflammation (Pryde et al., 2002). Furthermore, this data shows that butyrate is also an inducer of host mucin secretion, and the presence of mucus-associated bacteria may signify an increase in the availability of mucus for microbial metabolism. This data also showed trends of increased abundance of *A. muciniphila* at the mucosal surface in response to butyrate treatment. This bacterium uses host-derived mucus as its main carbon, nitrogen, and sulfur source for maintenance and growth (Derrien et al., 2011), and this feature is thought to allow *A. muciniphila* to thrive in intestinal lumens with limited availability of diet-derived carbohydrates (Derrien et al., 2004). The increase of *A. muciniphila* during periods of intestinal inflammation has also been linked to the increased expression of host mucus and immune modulating genes (Everard et al., 2013), suggesting that this bacterium induces mucin production and secretion during episodes of colitis. Collectively, my findings provide evidence that during infection, butyrate modulates the bacterial community in the inflamed distal colon to promote the growth of butyrogenic bacterial species, which may also stimulate the butyrate-driven production of

mucus by the host. Thus, changes in bacterial community structure and mucin production can potentially reduce intestinal inflammation and improve intestinal health.

2.4.6 Butyrate modulates inflammation

Although butyrate is a SCFA that has been implicated in improved barrier function, promotion of intestinal epithelial cell growth and repair, and enhanced host mucin secretion (Gaudier et al., 2009; Guilloteau et al., 2010; Hamer et al., 2008), it was observed that butyrate administration directly to the colon mildly exacerbated infection in mice with enteritis. In this regard, mice with enteritis and treated with a low concentration of butyrate tended to have modestly higher histological scores during peak and late infection that significantly subsided at the time of infection clearance. Histological scores also tended to be the highest in mice treated with butyrate at 80 mM, when compared to those treated with butyrate at a concentration of 140 mM, suggesting that butyrate administration works at a threshold, and is most effective at reducing inflammation when administered to mice at high concentrations *in vivo*. As indicated previously, butyrate-treated mice challenged with *C. rodentium* showed varied increases in gene expression of cytokines involved in the clearance of infection (*IL-17A, TNFα*), the reduction of mucosal inflammation (*TGFβ, IL-10*), and the improvement of host barrier function (*Muc2, Relmβ, Tff3*). These findings suggest that other processes that stimulate cytokine pathways that are not pro-inflammatory in nature may also contribute to modestly heightened levels of tissue inflammation. As an example, butyrate in the presence of *C. rodentium* can cause the activation of the promoter of the locus of enterocyte effacement (LEE) operon, and the expression of *ler* genes necessary for the expression of virulence factors that can exacerbate tissue inflammation (Franzin and Sircili, 2015; Takao et al., 2014). Moreover, morphological transformations in the mucosa following *C. rodentium* have been associated with a hyper-reactive epithelial reparative response that induces changes such as marked epithelia hyperplasia with increased production of undifferentiated enterocytes (Collins et al., 2014). This could also potentially increase inflammation scores (i.e. in my scoring system). In the current study, enhanced gene expression of epithelial cell regenerating proteins like MYD88, TFF3, and RELMβ in butyrate treated mice likely further elevated inflammation scores (i.e. increased epithelial hyperplasia, crypt height, and mitotic activity) in *C. rodentium*-

challenged mice, masking some of the anti-inflammatory effects and accentuating pro-inflammatory tissue responses. Finally, an increase in the mucus-degrading bacterium, *A. muciniphila* at the mucosal surface in butyrate treated mice was observed that was not observed in ingesta in the distal colon. As mentioned previously, *A. muciniphila* is known to colonize the intestinal mucus layer, and its presence has been associated with increased epithelial cell turn over, increased biosynthesis of epithelial cell components and enhancing mucin production (Derrien et al., 2011). Therefore, it can be postulated that the presence of *A. muciniphila* increased epithelial growth (i.e. hyperplasia), turnover, and the mitotic activity of colonocytes to increase total histological inflammation scores.

2.4.7 Conclusions

These findings provided evidence that butyrate can improve intestinal health during periods of inflammation by activation of coordinated physiological responses. These responses include the activation of the Th1 and Th17 immune response, tissue repair, and increases in mucus production and secretion. High concentration of supplemented butyrate improved the weight gain of infected mice, and during the clearance of the infection, improved histological scores in the distal colon. Generally, butyrate treatment under non-inflamed conditions reduced the abundance of *Firmicutes* and γ-*Proteobacteria*, and during inflammation contributed to the growth of *Proteobacteria*, *Lachnospiraceae*, and mucus-associated species. Collectively, my findings demonstrated that treatment with butyrate affected intestinal responses in the host and altered the intestinal bacterial populations in a dose dependent manner, underscoring the complexity of interactions among SCFAs, the host intestine, and the enteric microbiota. An association between butyrate supplementation, mucus accumulation, and *Muc2* gene expression was also observed. In addition, the increase in the abundance of mucus-associated species such as *A. muciniphila* and *M. schaedleri* in mice administered butyrate adds further evidence to support the study hypothesis that butyrate in the colon promotes host health through enhanced mucus secretion and cell maintenance factors. Notably, this research provides foundational information that can be used to determine the effects of prebiotics and other functional foods on the production of butyrate by enteric bacteria and their impact on intestinal health and host well-being.

2.5 Tables and Figures

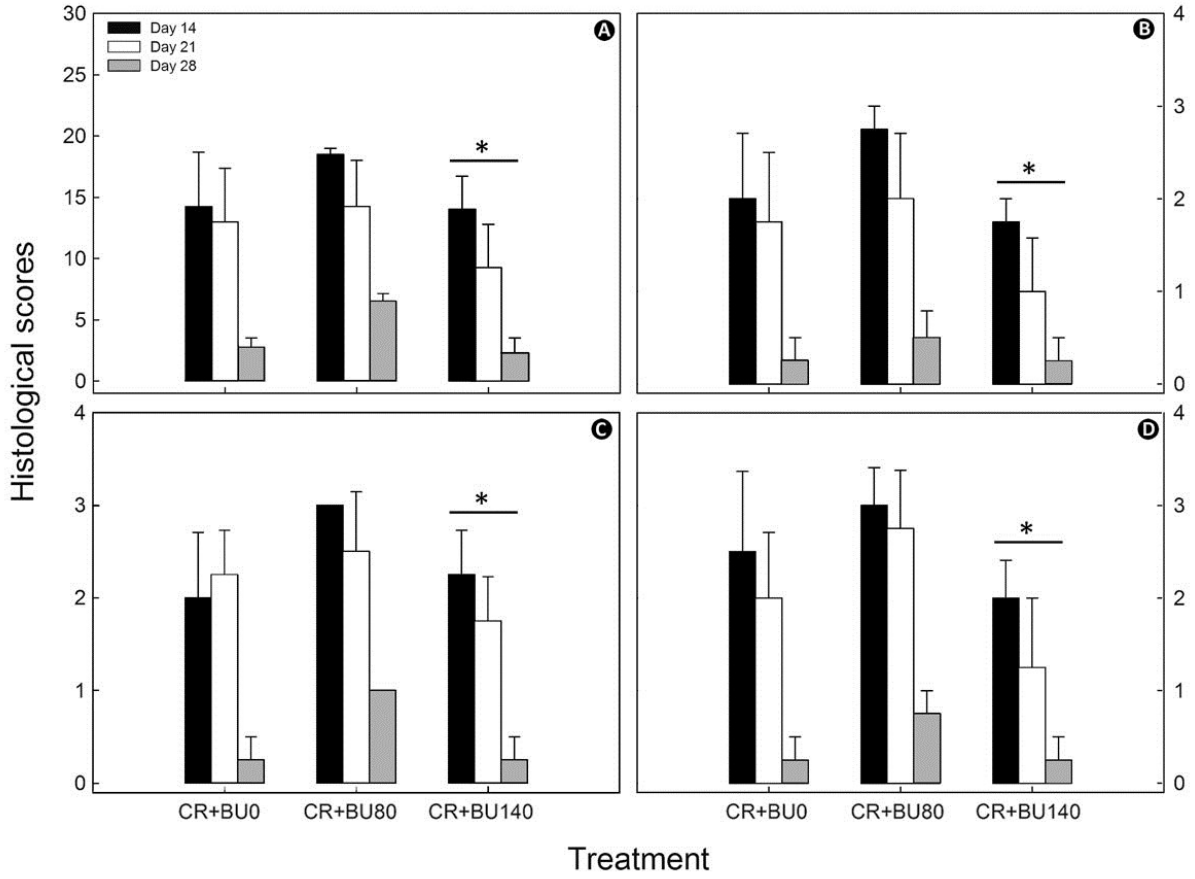

Figure 2.1 Histopathologic changes in colonic tissue from mice inoculated with *C. rodentium* (CR), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80) and 140 mM (BU140) averaged over time. **(A)** Total histopathologic scores. **(B)** Goblet cell depletion. **(C)** Epithelial cell injury. **(D)** Epithelial cell hyperplasia. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{*}P \leq 0.050$ relative to the BU0 treatment averaged over time points. For all treatments, infection with *C. rodentium* reduced over time ($P \leq 0.005$).

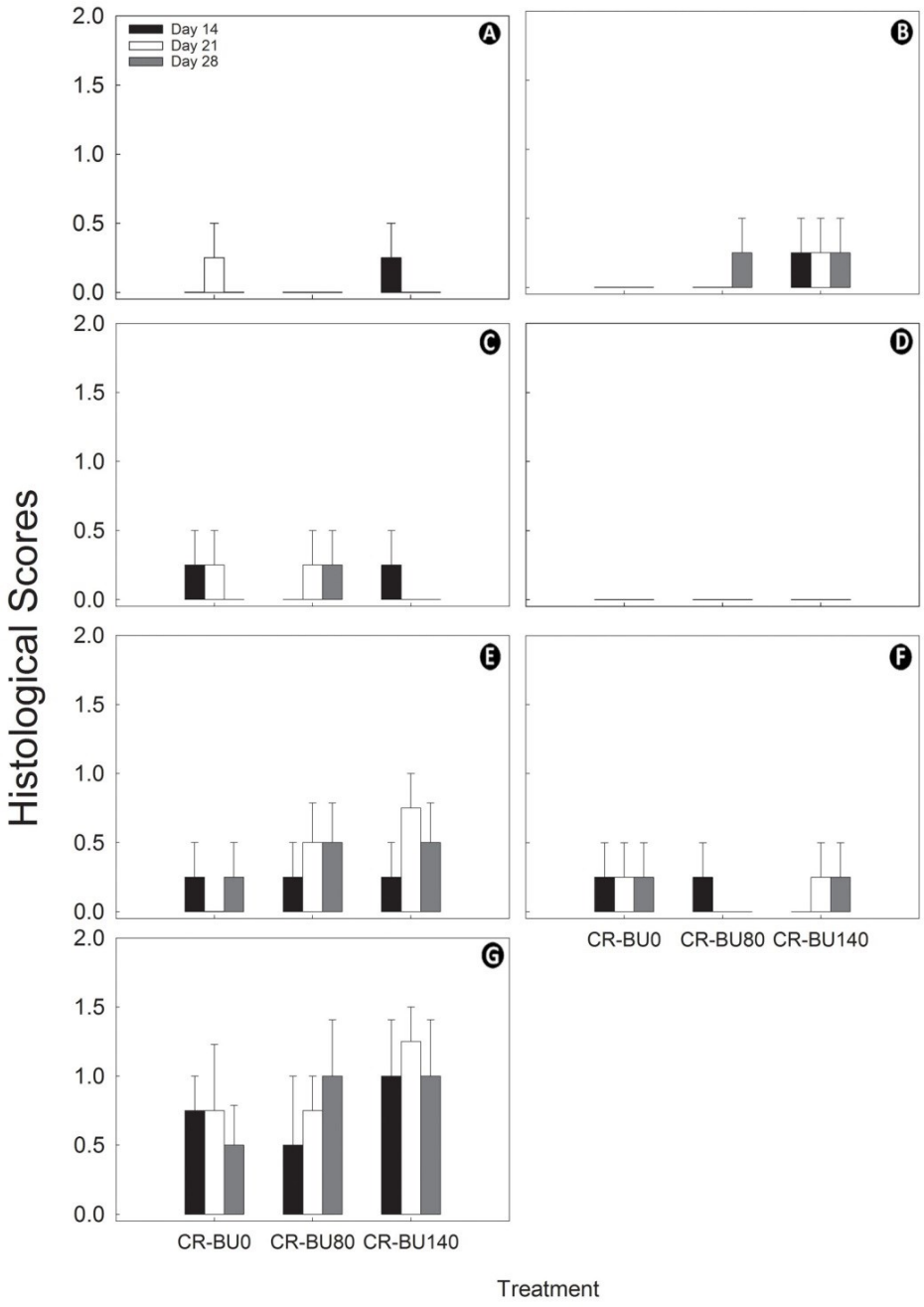

Figure 2.2 Histopathologic changes in colonic tissue from mice inoculated with PBS (CR-), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80) and 140 mM (BU140). **(A)** Epithelial cell hyperplasia. **(B)** Crypt height. **(C)** Epithelial cell injury. **(D)** Goblet cell depletion. **(E)** Inflammation. **(F)** Mitotic activity. **(G)** Total histopathologic scores. Vertical lines associated with histogram bars represent standard errors of the mean (n=4).

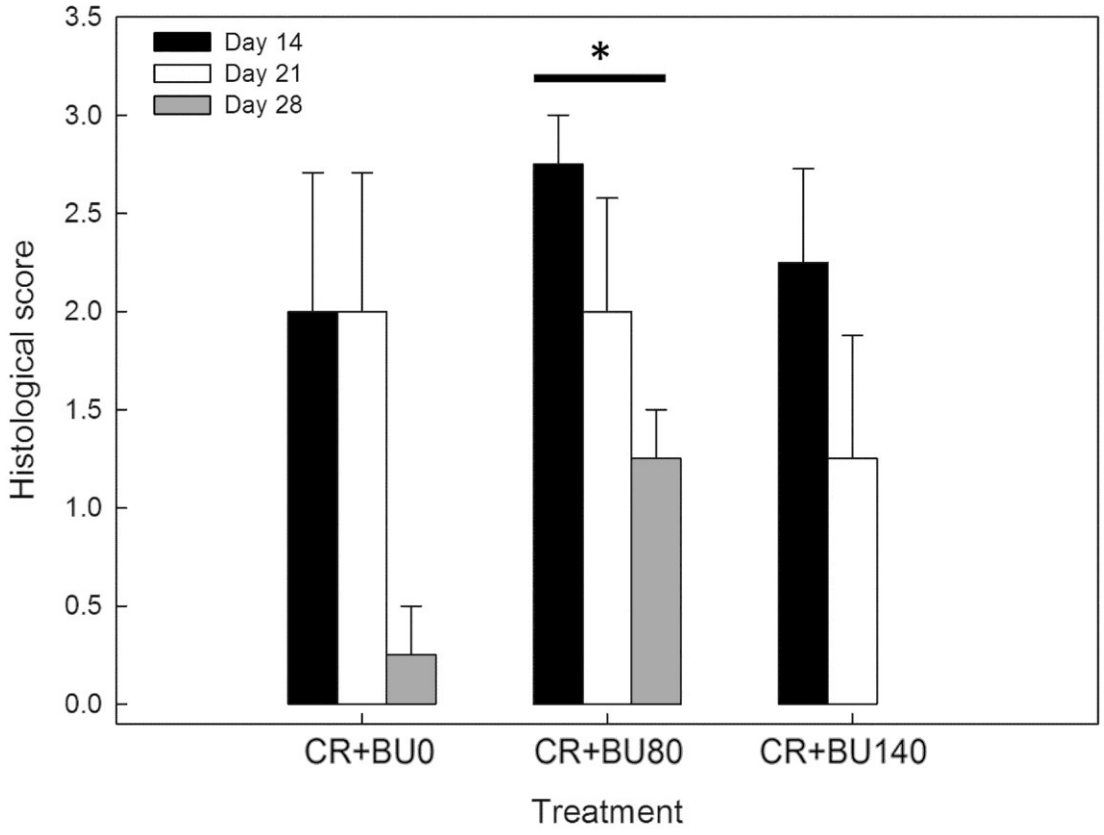

Figure 2.3 Mitotic cell activity in mice inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80) or 140 mM (BU140). Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{*}P \leq 0.050$ relative to the BU0 treatment averaged over time points. In all instances, infection with *C. rodentium* effects mitotic cell activity by time ($P \leq 0.005$).

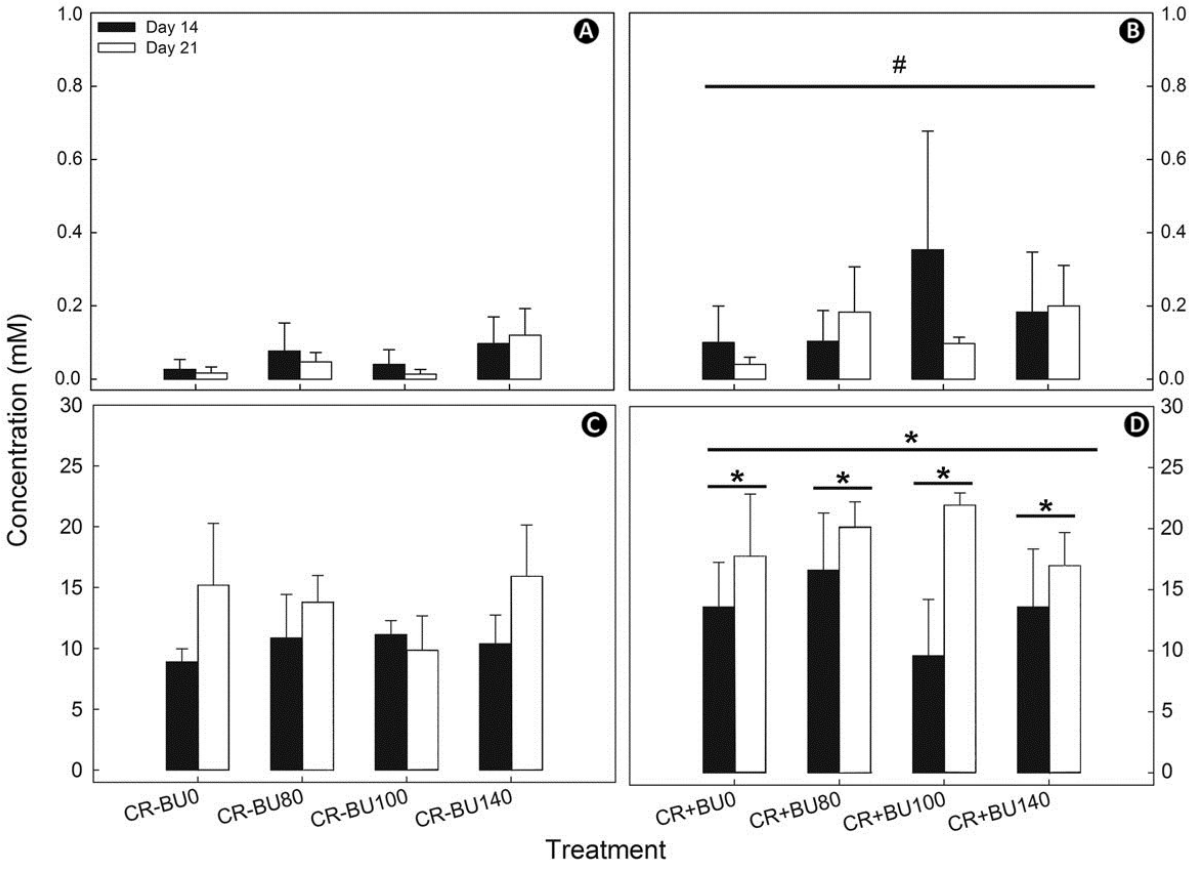

Figure 2.4 Concentrations of butyrate and total short-chain fatty acids (SCFA) measured in feces from mice gavaged with PBS (CR-) or inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80) and 140 mM (BU140). Samples were collected on days 14 and 21 p.i. **(A)** Butyrate concentrations in feces from CR- mice. **(B)** Butyrate concentrations in feces from CR+ mice. **(C)** Total SCFA concentrations in feces from CR- mice. **(D)** Total SCFA concentrations in feces from CR+ mice. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{\#}P \leq 0.100$ relative to CR- mice. $^{*}P \leq 0.050$ between days p.i. in mice with enteritis, and relative to CR- mice.

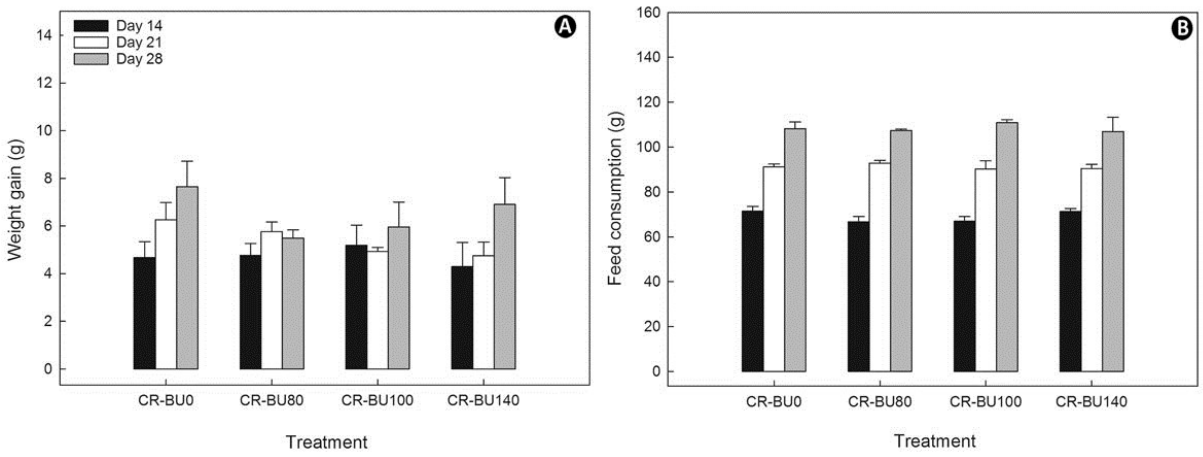

Figure 2.5 Weight gain and feed consumption in mice gavaged with PBS (CR-) and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140). **(A)** Weight gain measurements over 28 days. **(B)** Feed consumption measurements over 28 days. Vertical lines associated with markers represent standard errors of the mean (n=4). No statistical differences were observed relative to the BU0 treatment.

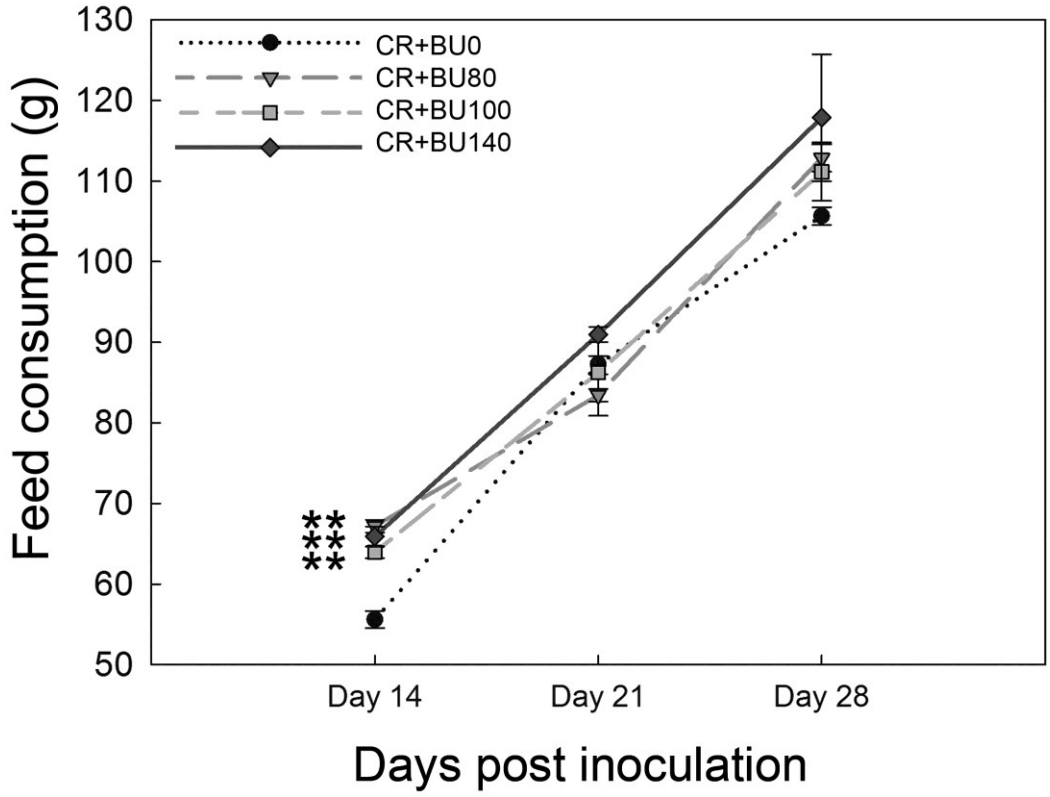

Figure 2.6 Daily food consumption in mice inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140). Vertical lines associated with markers represent standard errors of the mean (n=4), error is minimal where error bar is not evident and obscured by the marker. $^{**}P \leq 0.010$ relative to the BU0 treatment on Day 14 p.i.

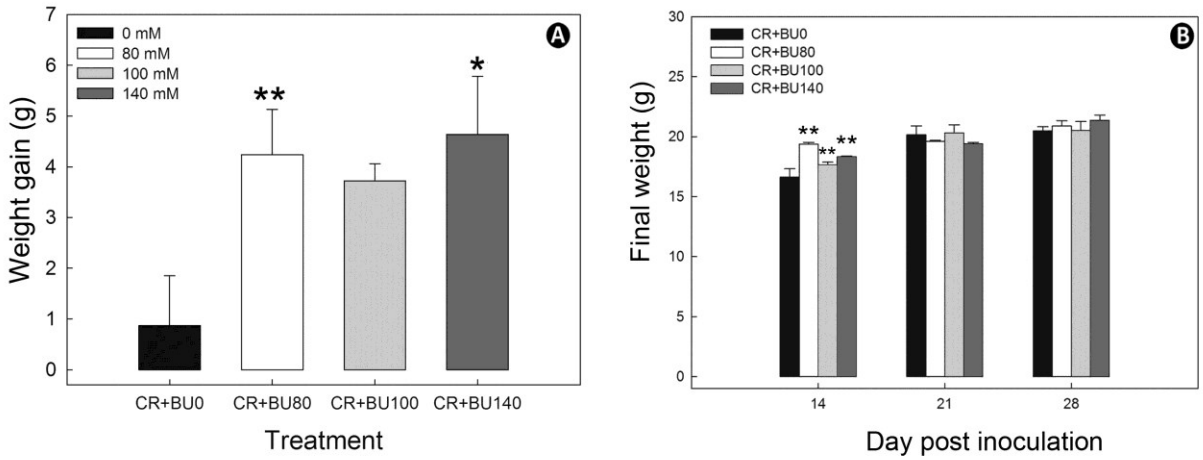

Figure 2.7 Weight gain and final weights in mice inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140) on day 14 p.i. **(A)** Compared to the CR+BU0 mM control, CR+ BU80 mM ($P<0.001$); CR+ BU100 mM ($P=0.13$); CR+ BU140 mM ($P=0.016$) during infection. **(B)** Compared to the CR+BU0 mM control, CR+ BU80 mM ($P<0.001$); CR+ BU100 mM ($P<0.001$); CR+ BU140 mM ($P<0.001$) during infection. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{*}P\leq0.05$, $^{**}P\leq0.010$ relative to the BU0 treatment.

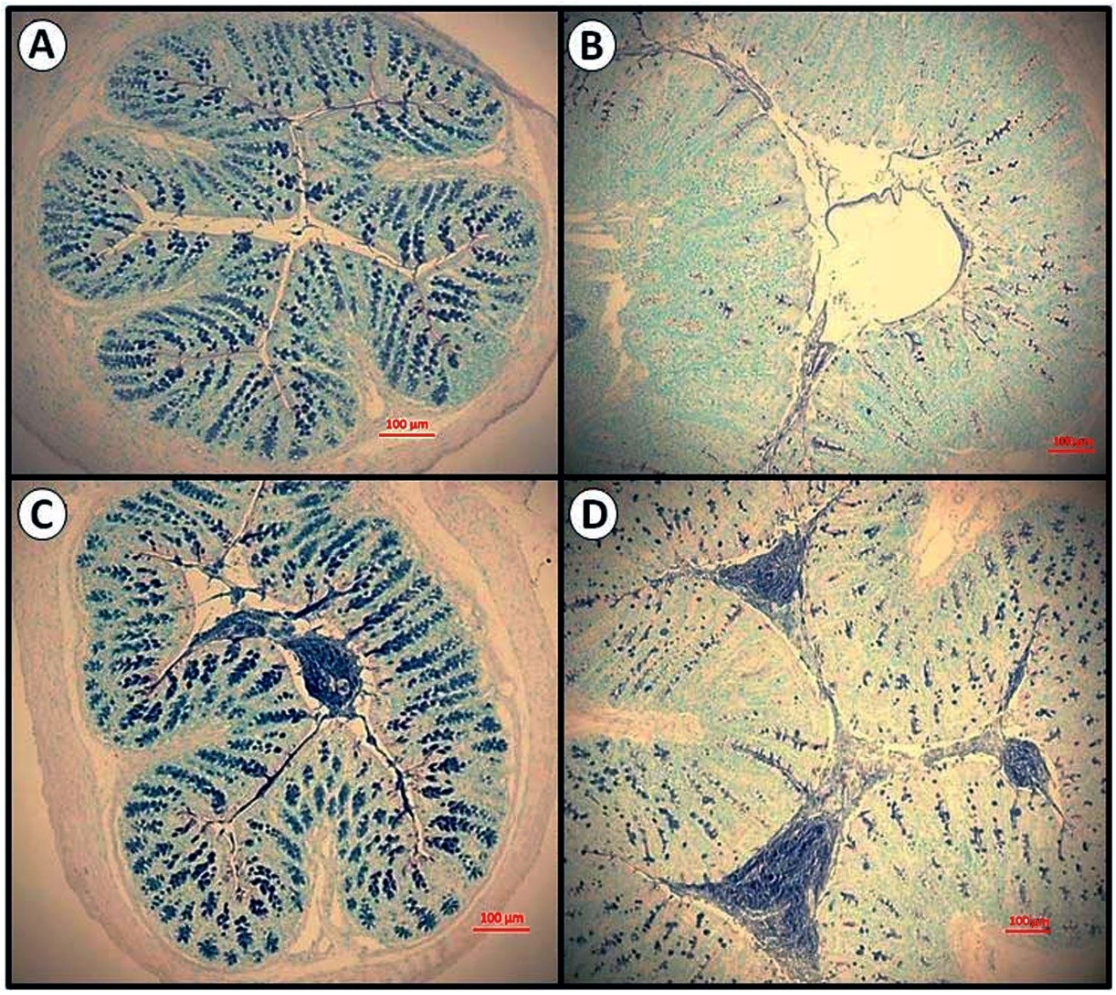

Figure 2.8 Mucus localization in Periodic Acid Schiff (PAS) stained sections from the distal colons of mice gavaged with PBS (CR-) or inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at a concentration of 140 mM (BU140) at day 14 p.i. **(A)** CR-BU0. **(B)** CR+BU0. **(C)** CR-BU140. **(D)** CR+BU140. Tissue from infected mice supplemented with butyrate exhibited a consistent increase in the density of mucus (blue stain) within goblet cells and the colonic lumen. Bar, 100 µm.

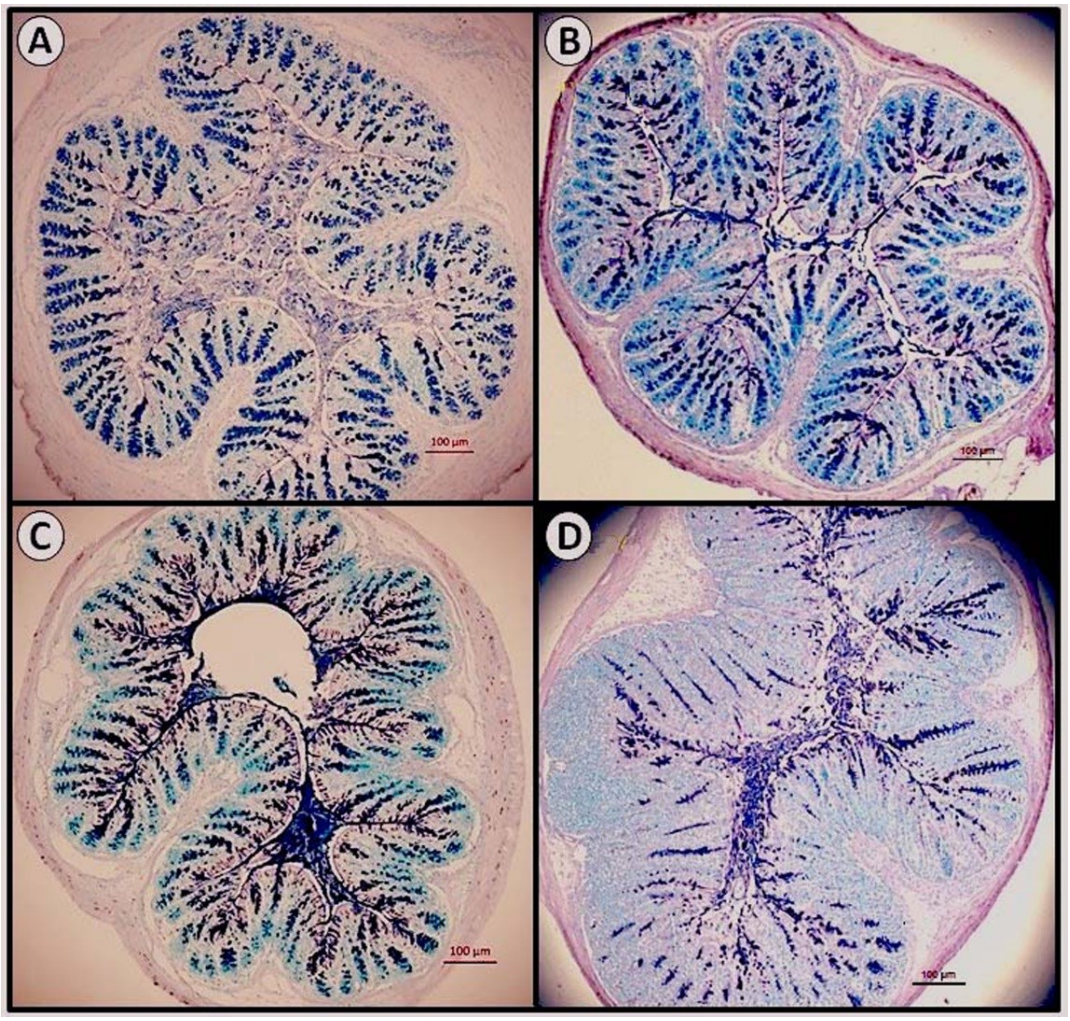

Figure 2.9 Mucus localization in Periodic Acid Schiff (PAS) stained sections of the distal colons of mice gavaged with PBS (CR-) or inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at a concentration of 140 mM (BU140) at day 21 p.i. **(A)** CR-BU0. **(B)** CR+BU0. **(C)** CR-BU140. **(D)** CR+BU140. Tissue from infected mice supplemented with butyrate exhibited an increase in the density of mucus (blue stain) within the colonic lumen. Overall, more mucus was observed in in the lumen on day 21 p.i. relative to 14 p.i. Bar, 100 µm.

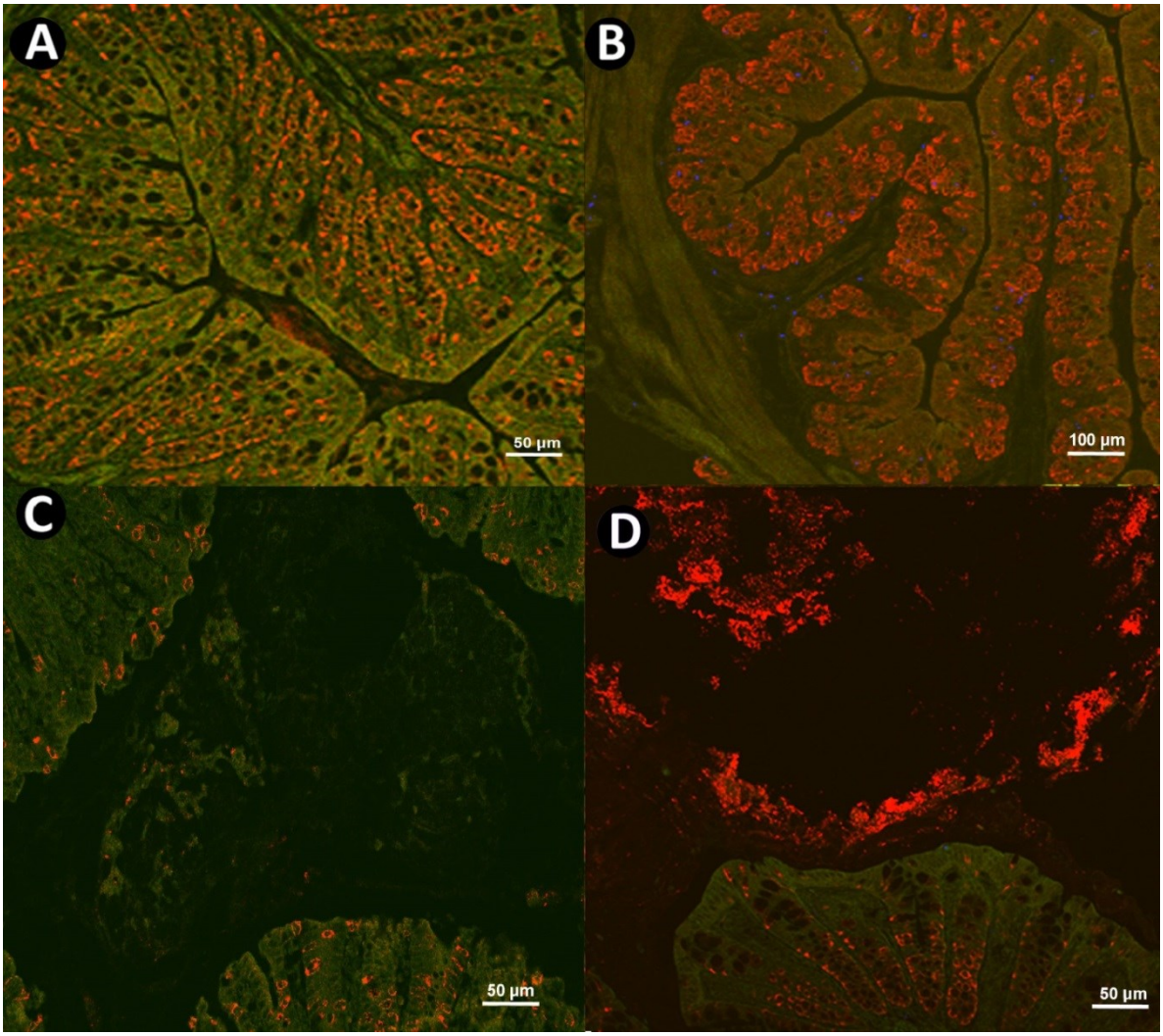

Figure 2.10 Localization of MUC2 in mice inoculated with *C. rodentium* (CR+) and rectally administered PBS (BU0) or butyrate (BU140) to distal colonic tissue sections and immunohistochemically stained with antibodies specific for MUC2 at Day 14 and Day 21 p.i. **(A)** CR+BU0 mM, Day 14 p.i. **(B)** CR+BU140 mM, Day 14 p.i. **(C)** CR+BU0 mM, Day 21 p.i. **(D)** CR+BU140 mM, Day 21 p.i. MUC2 (red) localizes mainly within intestinal goblet cells during peak infection (AB), and as late infection occurs MUC2 can be found more distributed in the lumen with butyrate supplementation (D).

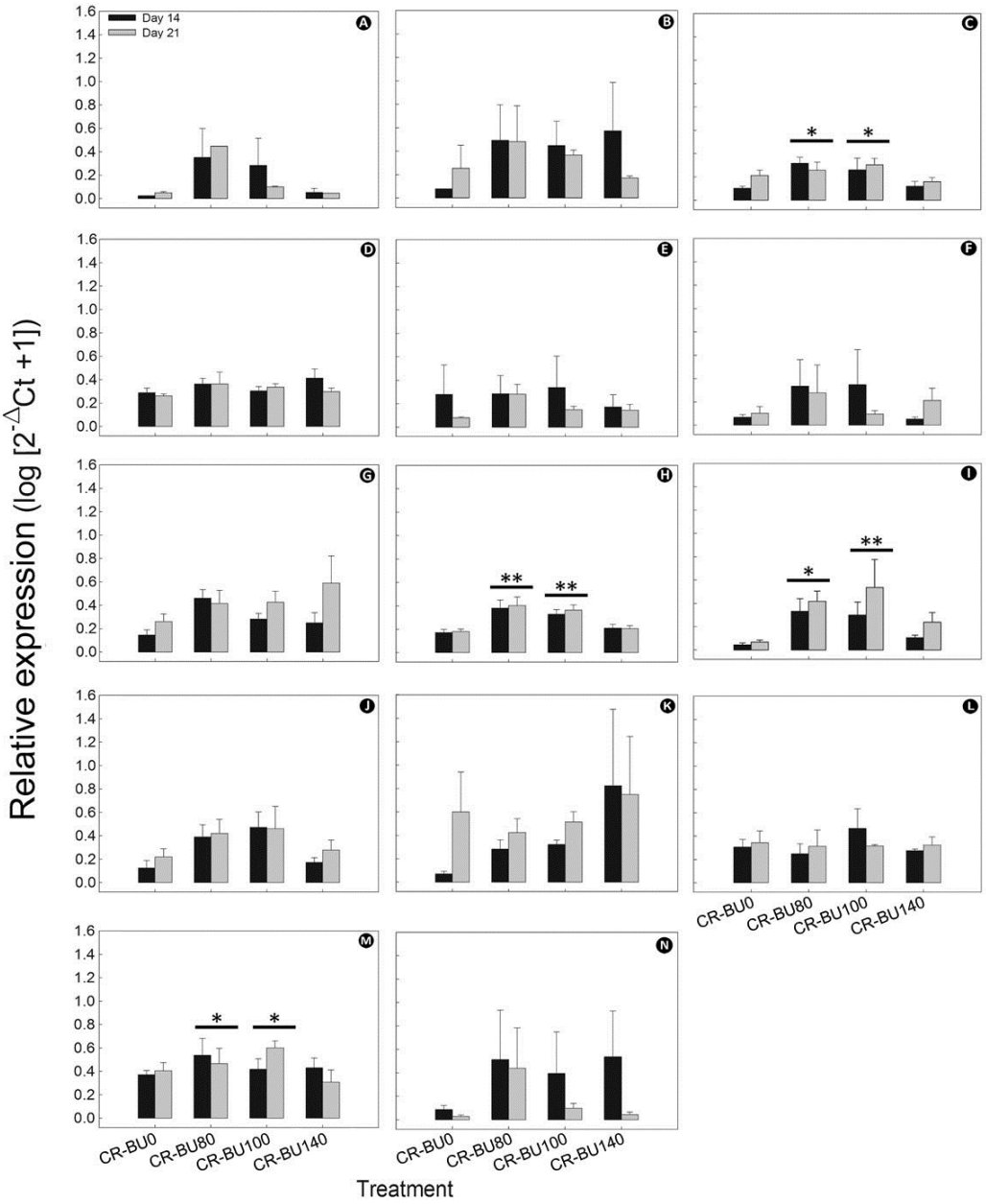

Figure 2.11 Relative mRNA gene expression of cytokines related innate barrier function, and host pathogen recognition genes measured in colonic tissue harvested from mice inoculated with PBS (CR-), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140). **(A)** *IL-17A*. **(B)** *IL-22*. **(C)** *IL-1β*. **(D)** *Myd88*. **(E)** *RegIIIγ*. **(F)** *IFNγ*. **(G)** *TLR9*. **(H)** *TLR2*. **(I)** *TNF*α. **(J)** *Ltb4r*1. **(K)** *Prg3*. **(L)** *IL-4*. **(M)** *Tff3*. **(N)** *Relmβ*. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{\#}P\leq0.100$, $^{*}P\leq0.050$, and $^{**}P\leq0.010$ relative to the BU0 treatment averaged over time points.

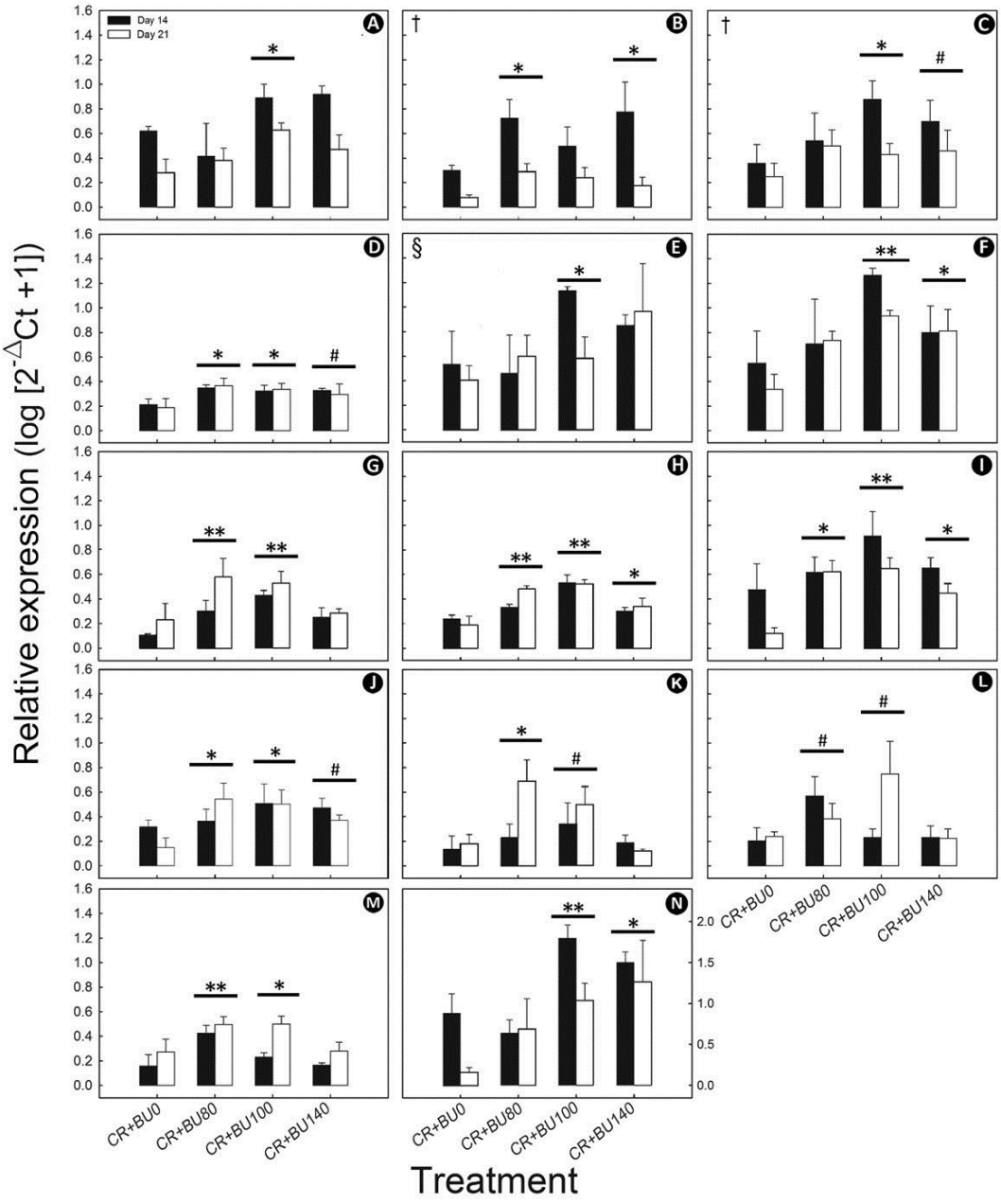

Figure 2.12 Relative mRNA gene expression of cytokines related innate barrier function, and host pathogen recognition genes measured in colonic tissue harvested from mice inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140). **(A)** *IL-17*A. **(B)** *IL-22*. **(C)** *IL-1β*. **(D)** *Myd88*. **(E)** *RegIIIγ*. **(F)** *IFNγ*. **(G)** *TLR9*. **(H)** *TLR2*. **(I)** *TNFα*. **(J)** *Ltb4r1*. **(K)** *Prg3*. **(L)** *IL-4*. **(M)** *Tff3*. **(N)** *Relmβ*. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{\#}P \leq 0.100$, $^{*}P \leq 0.050$, and

[**]P≤0.010 relative to the BU0 treatment averaged over time points. [†]Statistical value represents difference determined by excluding a butyrate treatment, and comparing CR+BU0 to only two of the three other treatments; [§]Statistical value represents comparison between CR+BU0 treatment and CR+BU100 only.

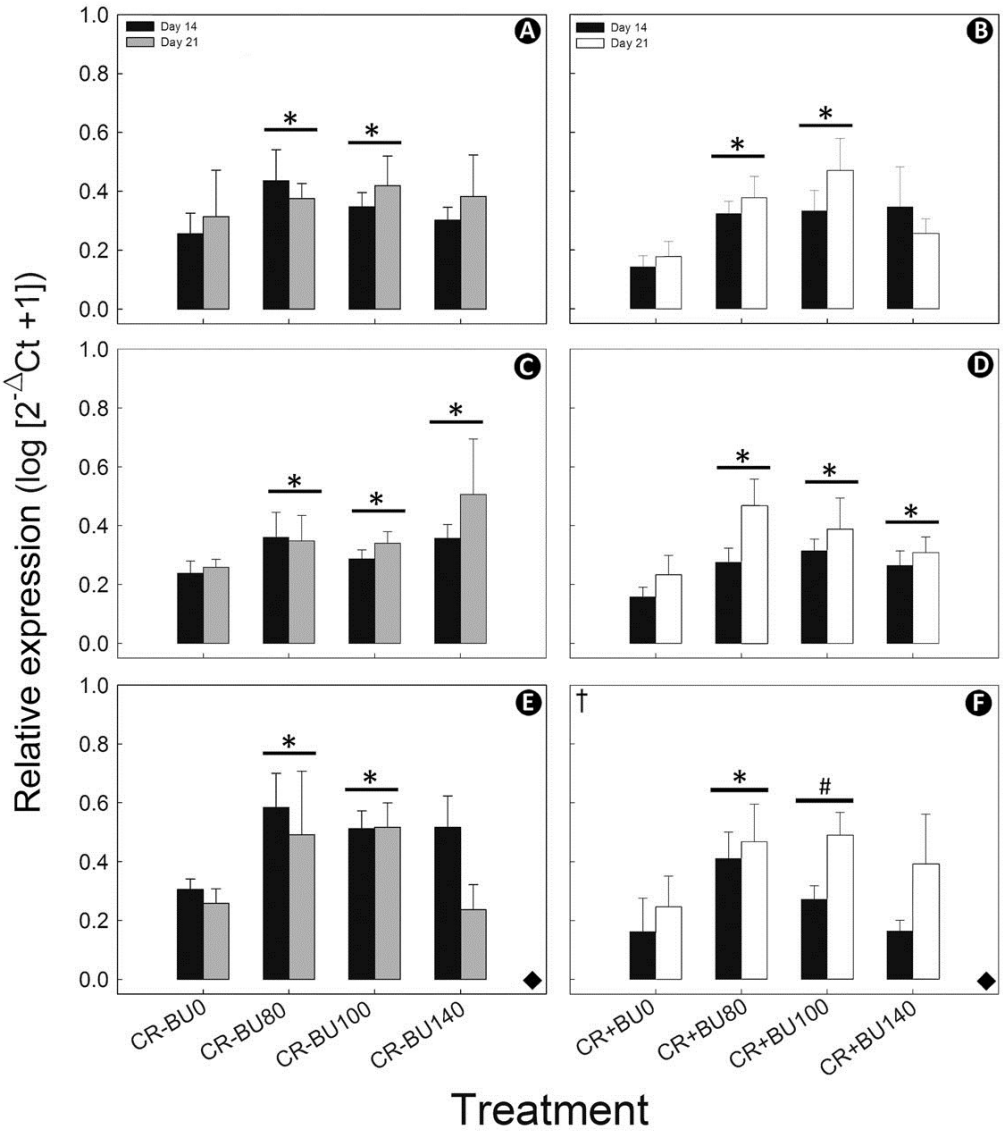

Figure 2.13 Relative mRNA gene expression of regulatory cytokines and mucus producing genes in colonic tissue harvested from mice gavaged with PBS (CR-) or inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at concentrations of 80 mM (BU80), 100 mM (BU100), and 140 mM (BU140). **(A-B)** *IL-10*. **(C-D)** *TGFβ*. **(E-F)** *Muc2*. Vertical lines associated with histogram bars represent standard errors of the mean (n=4). $^{\#}P\leq0.100$ and $^{*}P\leq0.050$ relative to the BU0 treatment. $^{\blacklozenge}P=0.053$ represents the difference in overall *Muc2* expression due to *C. rodentium* infection. †Statistical value represents difference determined by excluding a butyrate treatment, and comparing CR+BU0 to only two of the three other treatments.

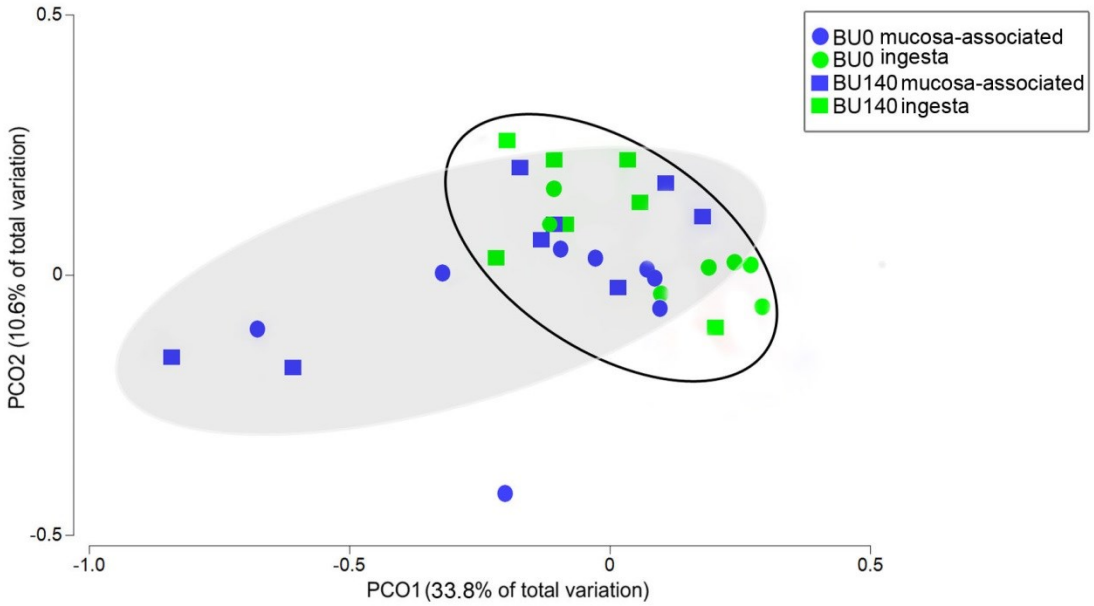

Figure 2.14 Principle coordinates analysis of bacterial communities within the distal colonic ingesta and associated with mucosa in the distal colon from mice gavaged with PBS, and rectally administered PBS (BU0) or 140 mM butyrate (BU140). Shaded ellipsoid identifies clustering between mucosa-associated communities; open ellipsoid identifies clustering between communities in the ingesta. Communities are compared based on differences in phylogenetic distances between taxonomies per treatment.

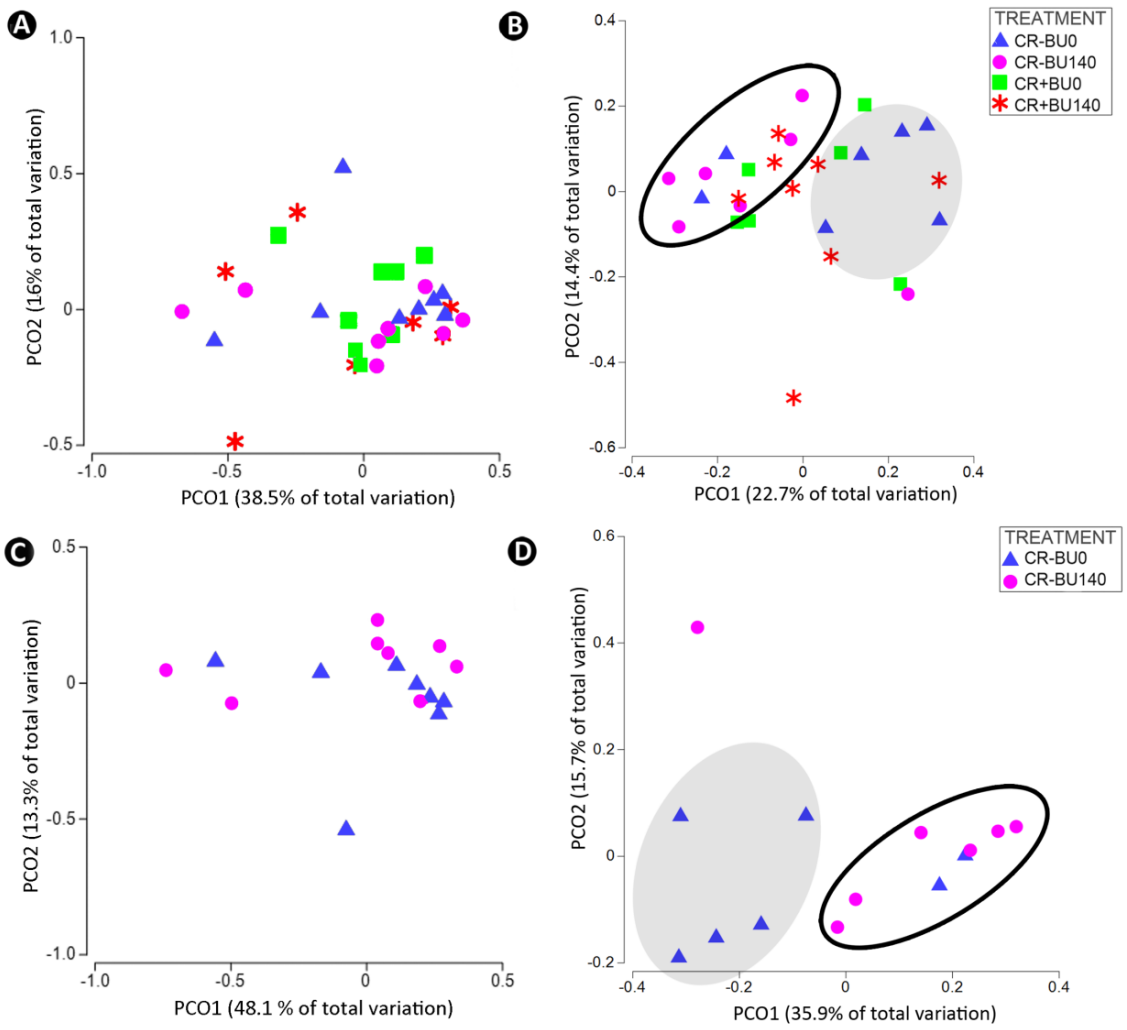

Figure 2.15 Effects of rectal administration of PBS (BU0) or 140 mM butyrate (BU140) on bacterial community structures in the distal colon of mice (mucosa-associated and within ingesta) gavaged with PBS (CR-) or inoculated with *C. rodentium* (CR+) as determined by weighted Uni-Frac analysis. Axes identify percent variation among treatments, and ellipsoids are used to highlight clustering of communities by treatment. (A-B) Mice with (CR+) and without (CR-) enteritis. (C-D) Mice without enteritis (CR-). **(A)** Mucosa-associated. **(B)** Ingesta; the shaded ellipsoid highlights clustering of communities from CR- and BU0 treatment mice, and the open ellipsoid highlights clustering of communities from CR- and BU140 treatment mice; a butyrate effect was observed for the CR- treatments (P=0.071 with 753 random permutations). **(C)** Mucosa-associated. **(D)** Ingesta; the shaded ellipsoid highlights clustering of communities from CR- and BU0 treatment mice, and the open ellipsoid highlights clustering of communities

from CR- and BU140 treatment mice; a butyrate effect was observed (P= 0.071 with 762 random permutations).

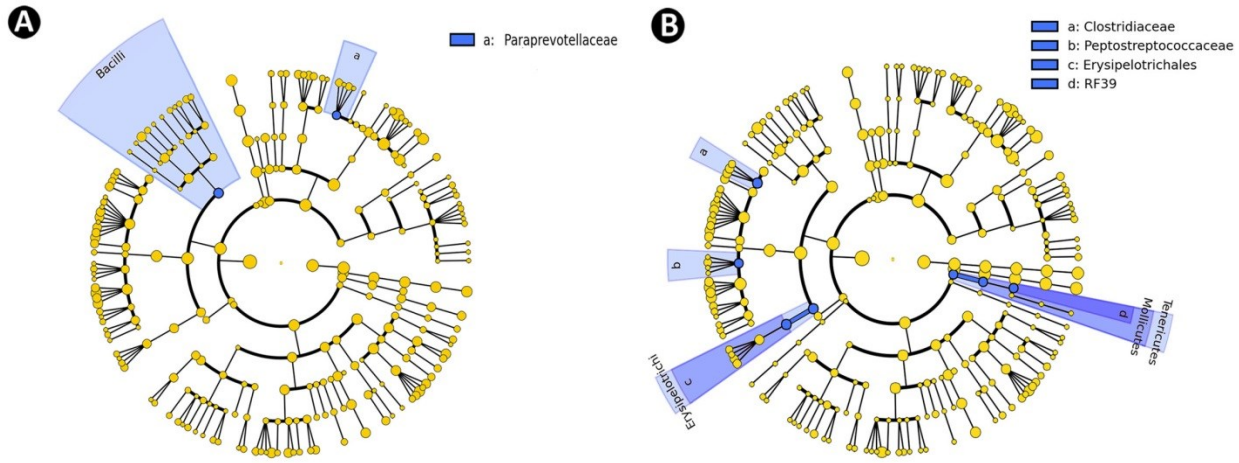

Figure 2.16 Cladograms of bacteria associated with mucosa and within ingesta in the distal colons of mice gavaged with PBS (CR-) and inoculated with *C. rodentium* (CR+) and rectally administered PBS (BU0) or 140 mM butyrate (BU140) as determined by the linear discriminant analysis effect size (LEfSe) method (LDA value >2.000). The abundance of bacterial taxa highlighted in blue display how BU0 differed (P≤0.050) from the BU140 treatment. **(A)** Mucosa-associated**. (B)** Ingesta. Data used to construct phylogenetic trees used summarized taxonomic values per treatment averaged over four replications.

Figure 2.17 Bacterial changes observed in the mucosa-associated and ingesta bacterial communities within the distal colon of mice inoculated with *C. rodentium* at peak (Day 14 p.i.) and late infection (Day 21 p.i.), and the changes observed due to the overall butyrate effect. Green boxes indicated a significant (P≤0.100) increase in the abundance of specified taxa supplemented with butyrate (BU140) when compared to butyrate control taxa (BU0) with each enteritis treatment group. Red boxes indicated a significant (P≤0.100) decrease in the abundance of specified taxa supplemented with butyrate (BU140) when compared to butyrate control taxa (BU0) with each enteritis treatment group. *p*: Phylum; *c*: Class; *o*: Order; *f*: Family; *g*: Genus. Butyrate effect represents significant taxa increased or decreased in response to butyrate administration averaged over time points.

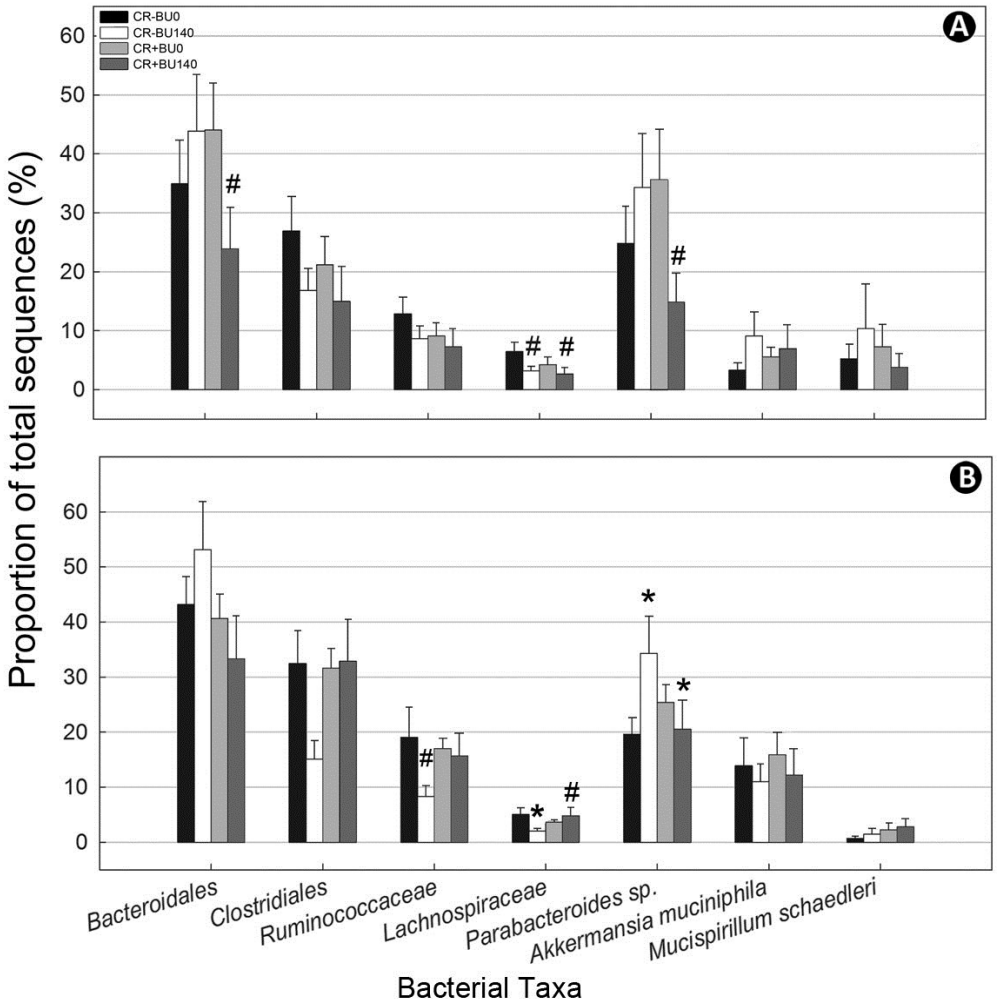

Figure 2.18 Abundance of bacteria within the distal colon of mice inoculated with PBS (CR-) or *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at a concentration of 140 mM (BU140). **(A)** Mucosa-associated. **(B)** Ingesta. Vertical lines associated with histogram bars represent standard errors of the mean (n=4/treatment). #P≤0.100 and *P≤0.050 when comparing butyrate treatment effect within CR- mice (BU0 to BU140) and CR+ mice (BU0 to BU140).

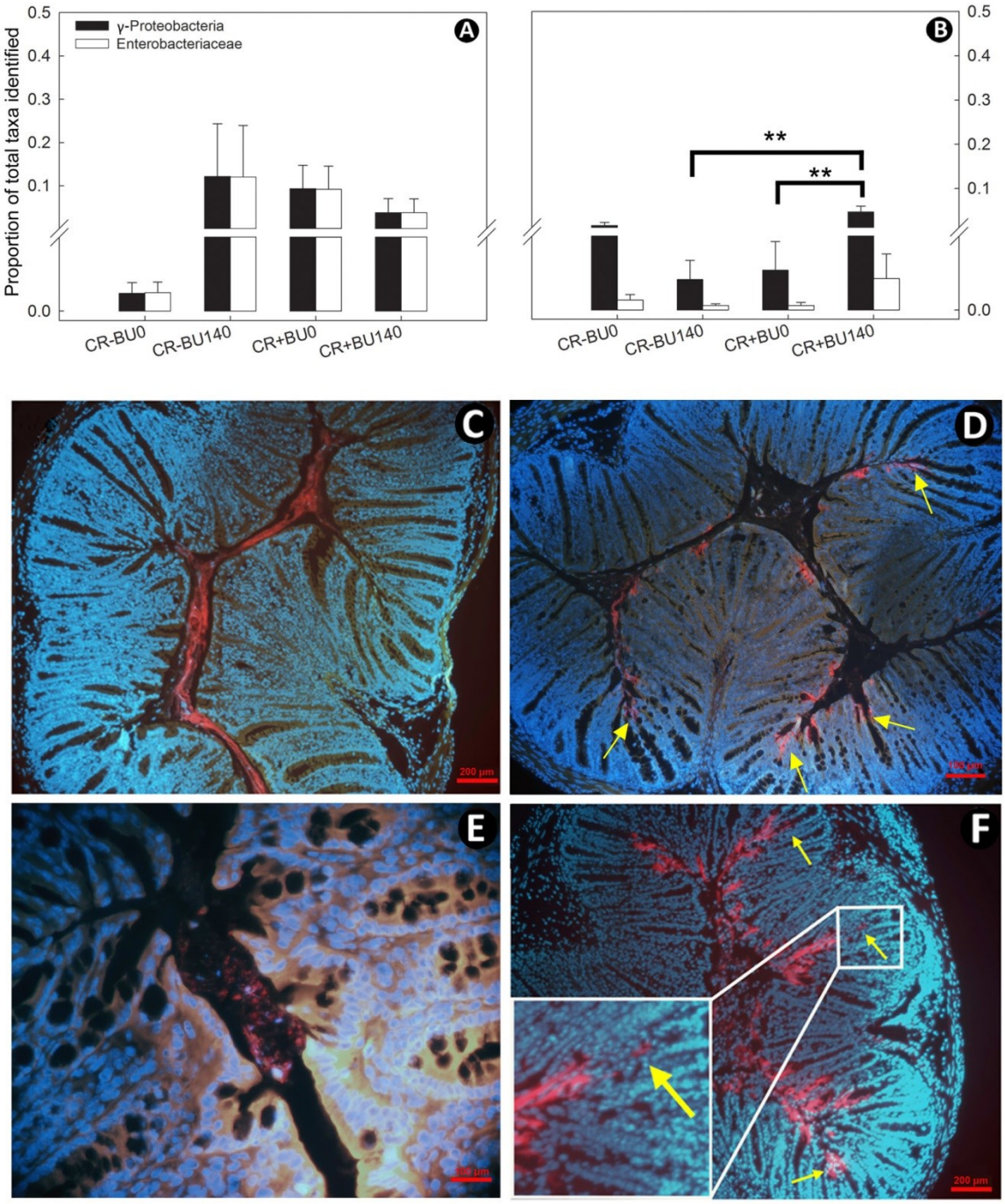

Figure 2.19 γ-*Proteobacteria* within the mucosa and crypts in the distal colons of mice inoculated with *C. rodentium* (CR+), and rectally administered PBS (BU0) or butyrate at a concentration of 140 mM (BU140). **(A)** Abundance of *Enterobacteriaceae* and γ-*Proteobacteria* associated with the mucosa on Day 14 p.i. in mice inoculated with PBS and *C. rodentium* **(B)** Abundance of *Enterobacteriaceae* and γ-Proteobacteria associated

with mucosa on Day 21 p.i. in mice inoculated with PBS and *C. rodentium*. Vertical lines associated with histogram bars represent standard errors of the mean (n=4 per treatment). $^{**}P \leq 0.010$ for treatments linked by the horizontal lines. (C-F) Fluorescent micrographs of distal colonic tissue hybridized with γ-*Proteobacteria* probe (red). **(C)** CR+ BU0 at day 14 p.i. (scale bar is 200 μm). **(D)** CR+ BU140 at day 14 p.i. (scale bar is 200 μm). **(E)** CR+ BU0 at day 21 p.i. (scale bar is 100 μm). **(F)** CR+ BU140 at day 21 p.i. (scale bar is 200 μm). The arrows indicate subluminal *Proteobacteria*.

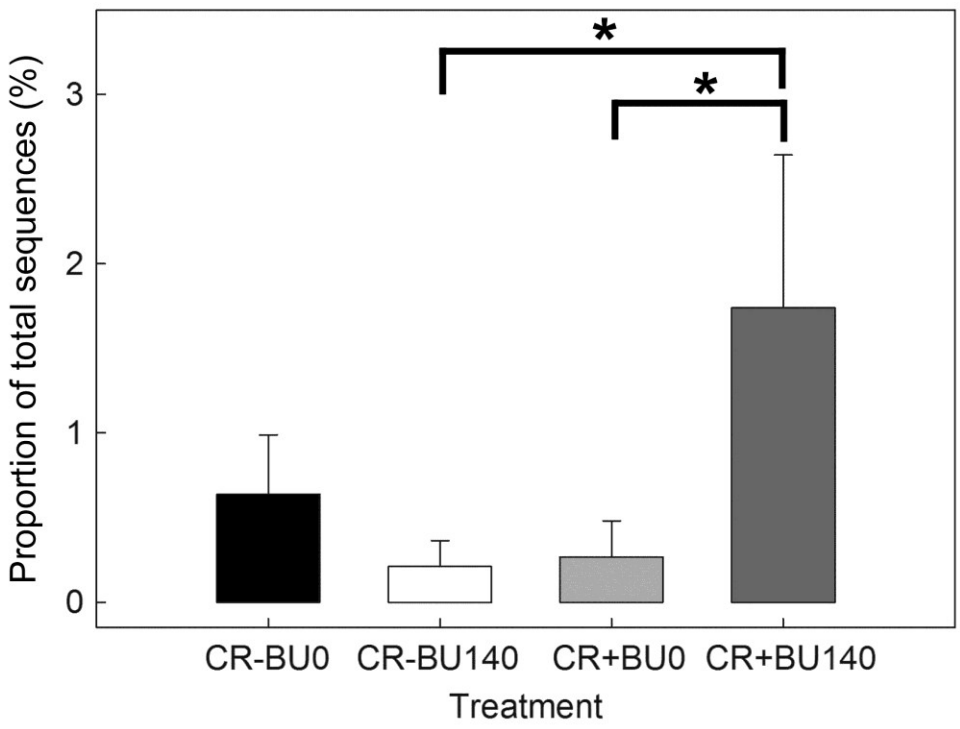

Figure 2.20 Abundance of *Pseudomonadales* species identified in the ingesta collected from the distal colons of mice gavaged with PBS (CR-) and inoculated with *C. rodentium* (CR+) and administered PBS (BU0), 80 mM (BU80), 100 mM (BU100) and 140 mM (BU140) butyrate. Vertical lines associated with histogram bars represent standard errors of the mean (n=8). $^{*}P \leq 0.050$ for treatments linked by the horizontal lines.

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Chapter 3: Resistant starch and wheat bran consumption reduce enteric inflammation by increasing short-chain fatty acid concentrations in the colon and altering the enteric bacterial community structure in mice

3.1 Introduction

The intestine is highly influential to host health and contributes greatly to the balance and regulation of the host intestinal immune system and microbiome. The association between the quality of foods consumed and the severity of intestinal diseases has become a topic of scientific interest, and diets rich in dietary fibres (DFs) are becoming increasingly popular, and can be considered as potential natural therapeutics to reduce intestinal inflammation (Breton et al., 2015; Wedlake et al., 2014). Dietary fibres are defined as carbohydrates with three or more polymerized saccharide units that resist digestion in the small intestine by host-derived intestinal enzymes, and are only susceptible to fermentation by bacteria in the colon (Health Canada, 2010). Certain enteric bacterial species possess specialized metabolic enzymes that ferment specific forms of DF, and the products of fermentation contribute to the bacterial diversity observed within the intestinal microbiome (Bach Knudsen, 2015; Birt et al., 2013; Nielsen et al., 2015). By-products of bacterial fermentation are mainly composed of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Englyst et al., 2007; Topping and Clifton, 2001) and can also include gases. Butyrate is a primary energy source for colonocytes, and it is also involved in mucus production and the regulation of intestinal immune function (Velázquez et al., 1997). Other functions of SCFA within the intestine include maintaining homeostatic intestinal pH and mucosal osmolarity (Topping and Clifton, 2001), and influencing mucin production and secretion (Gaudier et al., 2004), and these events are considered important for both cellular and microbial functions.

Resistant starch is a highly soluble and readily fermented carbohydrate that produces high quantities of butyrate following bacterial fermentation (Bird et al., 2010). Resistant starch is primarily comprised of amylose and branched-amylopectin carbohydrate units, and exists in many complex structures that differ in the accessibility of amylose and amylopectin to enzymatic digestion (Englyst et al., 2007). The digestion of RS by

intestinal bacteria can affect the structure of the microbiota within the large intestine, and RS fermentation often leads to an increase in *Bifidobacteria* spp., *Parabacteroides* spp., *Ruminococcus* spp., and *Eubacterium* spp. in the colon (Haenen et al., 2013; Martinez et al., 2010). The microbial fermentation of RS increases intestinal SCFA concentrations, which lower the intestinal pH to control bacterial growth, as well as increase water and ion uptake to counter the losses of these experienced during diarrheic events in the colon (Topping et al., 2003). Wheat bran is a DF that is not as easily fermented when compared to RS, but can lead to an increase in SCFA production in the intestine (den Besten et al., 2013). Wheat bran is a complex molecule composed mainly of insoluble non-starch polysaccharides (NSPs), and these include arabinoxylan, cellulose, and β-glucan fractions that comprise ≈46% of the total fibre content found in WB (Eswaran et al., 2013; Maes and Delcour, 2002). The complex structure and low percentage of soluble fibre present in WB contributes to slower WB fermentation rates by intestinal bacteria, and as such is important for the increase in fecal bulking and stool frequency (Kumar et al., 2012). Importantly, the fermentation of WB also improves intestinal lipid profiles that can lower the risk for the development of intestinal cancers (Bach Knudsen, 2015; Birt et al., 2013; Reddy et al., 2000). Similar to RS digestion, bacterial fermentation of WB can affect the presence of bacterial communities within the microbiota. The bacterial digestion of WB increases the abundance of *Clostridia* cluster XIVa, including butyrogenic bacteria such as *Faecalibacterium praznutzii* and *Roseburia intestinalis* (Nielsen et al., 2014). Although RS and WB both alter the intestinal microbiota and affect intestinal function by increasing water uptake and fecal bulking respectively, these DFs appear to influence the intestinal environment and immune response in different ways. Thus, the impacts of both RS and WB on the enteric microbiota and host were examined to elucidate key aspects of the relationship between diet, host responses, and inflammation.

Citrobacter rodentium is a Gram negative bacterium that incites epithelial cell hyperplasia and inflammation within the murine colonic mucosa and is commonly used to study intestinal inflammation in mice (Jiminez et al., 2015). Colitis incited by *C. rodentium* is a self-limiting process, and mice infected with the bacterium usually resolve disease ≈7 to 14 days post inoculation (p.i.) (Wiles et al., 2006), although disease can be prolonged up to 3 to 4 weeks p.i. (Collins et al., 2014). Infections with *C. rodentium*

induce a pro-inflammatory immune response that is characterized by an initial Th17-mediated immune response, followed by a Th1 immune response towards recovery (Costa et al., 2011; Luperchio and Schauer, 2001). This bacterium is commonly used as an inducer of acute inflammation in the murine intestine (Jiminez et al., 2015), but few studies have examined the impacts of DF on the pathobiology of *C. rodentium*-induced intestinal inflammation. Generally, research investigating the therapeutic potential of DFs has focused on the alterations to the microbiota in models that do not exhibit acute inflammation (Kalmokoff et al., 2015; Kalmokoff et al., 2013; Tamura et al., 1999). A number of studies have investigated the effects of DFs on the host using inflamed enteric models using chemical incitants (Algieri et al., 2014; Hartog et al., 2015; Joo et al., 2013; Panasevich et al., 2015; Zarepoor et al., 2014), and very few studies have used a bacterial incitant to induce intestinal dysbiosis (Johnson-Henry et al., 2014). In the current study the effects of the DFs, RS and WB collectively on the intestinal microbiota and the host were examined, with an emphasis on understanding the host immune response following challenge with *C. rodentium* as a Th1/Th17 inflammation incitant. It was hypothesized that DFs will increase bacterial fermentation and SCFA concentrations in the mouse colon, and this will stimulate mucus secretion, alter luminal bacterial communities including selection for butyrogenic taxa, and modulate the intestinal immune response to ameliorate intestinal inflammation. It was also hypothesized that as RS will be more readily fermented than WB, and fermentation of RS will contribute to an increased amount of intestinal butyrate, and thereby differentially increase mucin secretion to reduce *C. rodentium* induced mucosal injury.

3.2 Materials and Methods

3.2.1 Experimental Design

The experiment was designed as a completely randomized design with three levels of diet (CN, RS and WB), two levels of immunological stress (± *C. rodentium*), and three levels of sample time (14, 21, and 28 days p.i.). Each replicate included 36 mice, and four replicates were conducted on separate occasions (144 animals in total). It is noteworthy that separate mice were used for the quantification of SCFA (n=18 mice/replicate).

3.2.2 Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LRDC) Animal Care Committee (Animal Use Protocol Review 1405), and the LRDC Biosafety and Biosecurity Committee before commencement of the research.

3.2.3 Mouse maintenance

Specific pathogen free C57BL/6J mice were obtained from Charles River Laboratories (Montreal, QC) at 3-weeks of age. For each of the four replicates, mice were group-housed with six mice per cage upon arrival, and were given 5 days to acclimate under a 10:14 hr dark:light cycle. After adaptation, mice were individually housed and permitted to eat and drink *ad libitum.* Cages were lined with sterile bedding and housing units, and along with the food and water were replaced and replenished weekly. Mice were monitored daily to confirm their health status (Council, 2009), and initial body weights were taken prior to the inoculation of either phosphate buffered saline (PBS; 0.01 M NaH_2PO4, 0.04 M Na_2HPO4, 0.07 M NaCl, Sigma-Aldrich; pH 7.4) or *C. rodentium*, and were measured again at euthanization to determine total weight gained. Feed was also weighed the day of inoculation and at euthanization to determine total food consumed during the course of infection.

3.2.4 Dietary fibre supplementation

Upon arrival at LRDC, mice were maintained on a conventional rodent chow. After the 5-day adaptation period, mice were switched to experimental diets for a period of 14 days prior to bacterial inoculation. Experimental diets included a modified AIN-93G purified rodent diet with sterile vitamin free casein (DYET#103455GI) as the control diet (i.e. CN diet); a modified AIN-93G sterile vitamin free casein enriched with 117 g/kg of Bob's Red Mill WB (DYET #103456GI) (i.e. WB diet); and a modified AIN-93G sterile vitamin free casein enriched with 125 g/kg of King Arthur Flour Hi-Maize® RS (DYET#103457GI) (i.e. RS diet). All diets were (Bethlehem, PA, USA) were color coded with dye (i.e. to ensure accurate administration), pelleted, vacuum sealed and irradiated and stored at -20°C prior to use.

3.2.5 *C. rodentium* inoculation

A green fluorescent protein-labelled *C. rodentium* DBS100 (ATCC 51459) was used. The bacterium was grown aerobically on Lysogeny Broth agar (LA) with 30 μg/mL chloramphenicol to select for only GFP-labelled *C. rodentium* at 37°C for 24 hr. The inoculum was prepared and administered as per the protocol described in Chapter 2.2.5. Cell densities were adjusted to 2 x 10^9 CFU/mL with PBS. Eighteen mice were gavage inoculated with *C. rodentium* cells (100 μL) using a 22G X 2.5 cm-long gavage needle with a 1.25 mm ball tip on two consecutive days. Similarly, 18 mice were gavage inoculated with PBS alone (100 μL). Animals were monitored for discomfort/pain for 4 hr after the enemas were administered.

3.2.6 Isolation of *C. rodentium* from collected feces

Fecal samples from mice were collected 3, 7, 10, 14, 21, and 28 days p.i. Isolation and collection were performed as described in Chapter 2.2.6. Briefly, fecal samples were homogenized in PBS, diluted in a 10 fold dilution series, and duplicate aliquots (100 μL) were spread on MacConkey agar (Becton, Dickinson and Company, Mississauga, ON) with 15 μg/mL chloramphenicol (Sigma Aldrich, Oakville, ON) (Chapter 2.2.6). After 24h incubation at 37°C, *C. rodentium* colonies were enumerated and confirmed with colony PCR looking for the EspB protein (Newman et al., 1999). Primers specific for the *espB* gene, (F:5'-GCTTCTGCGAA-GTCTGTCAA-3', R:5'-CAGTAAAGCGACTTAACAGATT-3') (Newman et al., 1999) were used with PCR conditions that commenced with one cycle of 15 sec at 95°C, followed by 35 cycles of 45 sec at 95°C, 1 min at 57°C, 1 min at 72°C, and a final cycle of 5 min at 72°C. The amplicon was 270 bp in size.

3.2.7 Animal euthanization and intestinal sample collection

On days 14, 21 and 28 p.i., arbitrarily selected mice from each treatment were anesthetised with isoflurane and euthanized by cervical dislocation under anesthetic. Sample collection occurred as described in Chapter 2.2.7. To visualize the intestine and collect samples, a mid-line laparotomy was used to exteriorize the intestine. A gross pathological assessment of the large intestine with photo-documentation was completed, and the length and width of the cecum and colon were measured. The cecum and colon

were longitudinally incised, and luminal contents were collected and stored at -20°C for DNA extraction and characterization of the microbiome. Sections of the distal colon were collected in RNAlater™ (Qiagen Inc., Toronto, ON) and immediately stored at -20°C for mRNA extraction. Sections from the proximal and distal colon were collected for DNA analysis, and samples were collected in Surgipath® 10% neutral buffered formalin (Leica Biosystems, Concord, ON) for histopathological staining and Methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for mucus analysis (Johansson and Hansson, 2012).

3.2.8 Histopathology

Formalin-fixed tissues were dehydrated in increasing concentrations of ethanol (80%, 95%, 100%) at RT and placed in Histo-Clear (Diamed Lab Supplies, Mississauga, ON) prior to embedding in paraffin at 60°C as described in Chapter 2.2.8. Sections (5 μm) were deparaffinized with xylene at RT, and stained with hematoxylin and eosin (H&E) dyes according to a standard protocol. Tissues were scored for mucosal damage by a veterinary pathologist (RREU) blinded to the treatments using an established scoring guide (Barthold et al., 1977) that ranked common characteristics of mucosal damage (i.e. epithelial cell injury and hyperplasia, goblet cell depletion, crypt height, mitotic activity, and inflammation) from 0 to 4, with 4 being pronounced damage and 0 representing minimal to no damage (Table A1). The maximum achievable score for tissue injury was 22.

3.2.9 Visualization of mucus

To visualize mucus, colonic tissue samples were fixed overnight in Methacarn at RT (Johansson and Hansson, 2012), prior to dehydration with ethanol and Histo-Clear (Diamed Lab Supplies, Mississauga, ON). Sections were deparaffinized and stained with Alcian Blue (pH 2.5; American MasterTech, Lodi, CA), followed by 0.5 % Periodic Acid (American MasterTech, Lodi, CA) at RT for 5 min, and Schiff's solution (American MasterTech, Lodi, CA) as described in sections 2.2.9.

3.2.10 SCFA analysis

To quantify SCFA, the cecum, proximal colon, and distal colon were collected from one mouse per treatment, per replicate. The total weight of the tissue including ingesta

was recorded, and after removal of the cecum, the colon was measured for length, and divided equally into halves as the proximal and distal colon. SCFA concentrations were determined as described in Chapter 2.2.10. Briefly, sections were homogenized in PBS at a 1:9 ratio (w/v). Meta-phosphoric acid (Sigma Aldrich, Oakville, ON) was added to the homogenate at a 1:4 ratio (v/v), and incubated at RT for 30 min. Samples were centrifuged at RT for 75 min at 16,100 x *g*, and the supernatants collected and stored at -20°C. Acetate, butyrate, and propionate concentrations were quantified with a gas chromatograph (Agilent Technologies, Model 6890N with 7683 Series Injector) according to established protocols (Cottyn and Boucque, 1968; Playne, 1985).

3.2.11 Characterization of cecal and distal mucosal bacterial communities

Bacterial genomic DNA was extracted from distal and proximal colonic samples (mucosa-associated) using QIAGEN® DNeasy Blood and Tissue Extraction kits (Qiagen Inc.). Genomic DNA was also extracted from the cecal ingesta using the QIAamp® Fast DNA stool extraction kit (Qiagen Inc.). The entire cecum was collected, and the ingesta contents scraped out and used for analysis. Extracted DNA was processed using an Illumina protocol for creating 16S metagenomic sequencing libraries (Illumina, 2013). Briefly, extracted DNA was normalized to 5 ng/µL in 10 mM Tris (pH 8.5) and 2.5 µL was PCR amplified with 5 µL of each amplicon primer specific for the V3 and V4 region of the 16S gene (F:5'TCGTCGGCAGCGTCAGATG-TGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; R:5'GTCTCGTGGGCTCGGAGA-TGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') and 12.5 µL of 2x KAPA HotStart Ready mix for a final volume of 25µL (Illumina, 2013). A PCR clean-up using AMPure XP (Beckman Coulter, Inc.) beads on a magnetic stand was performed on the 500 bp products, and an indexing PCR reaction was used to add dual indices to each sample. Conditions included 5µL of DNA, 5 µL of each index primer (specific non-repeating pair per sample) and 25 µL of 2x KAPA Hifi HotStart Ready mix and Nuclease-free water (Qiagen Inc.) to a final volume of 50 µL per sample. A final PCR clean-up was performed on the 630 bp product. The resulting indexed DNA libraries were quantified and normalized to 4 nM, and 5 µL of each normalized library was pooled into one sample for sequencing using a MiSeq (Illumina, San Diego, CA). A PhiX

control was run in parallel with the normalized DNA libraries, and both were denatured and diluted to 4 pM prior to loading onto the MiSeq cartridge.

Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0) analysis was used to assemble forward reads (Caporaso et al., 2010b). Barcodes were extracted from each sample and joined for each library. Libraries were then split, and sequences were filtered to include high fidelity sequences using the parameters described in Chapter 2.2.11. These sequences (n=1,556,473) were checked for chimeras using USEARCH 6.1 software, and the resulting chimeras were filtered out prior to picking operational taxonomic units (OTU) from the Greengene reference database. In total, 1,392,105 OTUs were identified using a 97% similarity parameter, and the most common sequence was used to define the groups of similar OTUs. After, OTUs were aligned using the NAST algorithm (Caporaso et al., 2010a) to the Ribosomal Database Project classifier of 0.8 (sequences having at least 80% similarity to reference database sequences). Taxonomy was assigned to each sequence cluster using UCLUST (Edgar, 2010) and classified using the Greengenes reference database (McDonald et al., 2012). An OTU table was produced, and all samples were rarified so that 1,250 OTUs were randomly chosen from each sample and compared for analysis (OTU per biological sample ranged from 1,250-57,439). Diversity among species (β-diversity) was examined using Bray-Curtis, weighted and unweighted UniFrac analyses.

3.2.12 Quantification of gene expression

Cytokine expression profiles were generated from total RNA that was extracted from colonic tissue using an RNeasy® Mini kit (Qiagen Inc.). The following procedure was adapted from Chapter 2.2.13. Differential expression of 15 genes involved in the Th1, Th17, and Treg immune responses were measured, as well as mucus expression, bacterial recognition, and epithelial repair genes (Table A2). The primers used were uniquely designed using sequence information within the NCBI database. Reference genes from Chapter 2.2.13 were used to normalize measured C_t values (i.e. *Ppia*, *Hprt*, and *GusB)*. All primers were diluted to 10 nM, and HPLC purified. Quantitech SYBR Green Mastermix (Qiagen Inc.) was used as an indicator of double stranded DNA and product amplification. Individual PCR reactions consisted of: 1 µL of cDNA; 3 µL of Optima water (Fisher Scientific, Edmonton, AB); 0.5 µL of 10 µM forward primer (Table A2);

0.5 µL of 10 µM reverse primer (Table A2); and 5 µL of SYBR green. Reactions were run in triplicate per cDNA sample (i.e. treated as observations). Quantitative PCR reactions were run on an ABI 7900HT qPCR thermocycler (384-well block; Life Technologies, Burlington, ON), with an activation step of 95°C for 15 min, and 40 cycles of 94°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, followed by 1 cycle of 95°C for 15 sec, 55°C for 15 sec and 95°C for 15 sec. The mean of the three observations was calculated, and normalized gene expression was calculated using qbasePLUS (Biogazelle, Zwijnaarde, Belgium) based on geNorm and qBase quantification models (Hellemans et al., 2007; Vandesompele et al., 2002).

3.2.13 Statistical analysis

The majority of the statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). Continuous data was checked for normality, and analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Where applicable, collection time was treated as a repeated measure; the appropriate covariance structure was utilized according to the lowest Akaike's Information Criterion. In the event of a significant main effect, the least squares means test (LSM) was used to compare treatments within factors. Probability values ≤ 0.050 were considered to be statistically significant, whereas P values >0.050 and ≤ 0.100 were considered to be a significant trend. For bacterial community data, both SAS and Primer 7 were used to analyze data. In Primer 7, PERMANOVA and principal coordinates analyses (PCoA) were used to analyze the β-diversity, whereas, analyses of variance (MIXED procedure) with a protected LSM test were used to analyze α-diversity. Differences in the abundance of bacterial OTUs were also analyzed using analysis of variance (ANOVA) with a protected LSM test, and P values ≤ 0.100 were deemed statistically significant. A one-way ANOVA with a Wilcoxon two sample test for location differences, as well as the Kruskal-Wallace test was used to analyze significance between diet treatments for the histopathologic, gene expression, and LEfSe sequencing data.

3.3 Results

3.3.1 *C. rodentium* incited enteritis in mice

The presence of *C. rodentium* was not detectable in the feces of mice inoculated with PBS. In contrast, mice in all treatment groups inoculated with *C. rodentium* exhibited the highest (P=0.001) levels of shedding on days 3 to 10 p.i. (Figure 3.1). Mice consuming the WB and RS diets shed the highest amount of *C. rodentium* on day 7 p.i., while mice ingesting the CN diet shed the highest counts of *C. rodentium* on day 10 p.i. (Figure 3.1). Mice consuming the WB diet shed *C. rodentium* at higher quantities than mice ingesting the CN (P=0.004) and RS (P=0.005) diets. Densities of *C. rodentium* in feces steadily decreased for all diet treatments after day 10 p.i. At the later stages of infection (>14 days p.i.), a trend (P=0.168) of lower *C. rodentium* densities in the feces was observed in mice consuming the WB diet in comparison to the RS and CN diet treatment mice.

3.3.2 Mice consuming WB and RS are less affected by *C. rodentium* infection than those fed a low fibre diet

Mice without enteritis exhibited total average histopathological scores of ≤1.25 ± 0.63 in the distal colon, and ≤1.75 ± 0.48 in the proximal colon; furthermore, these mice did not show any clinical manifestations of intestinal disease or signs of infection (Figure 3.2, 3.3). Mice inoculated with *C. rodentium* exhibited substantially higher histopathologic scores (P≤0.001) relative to mice inoculated with PBS for all of the categories examined. Histopathologic evidence of acute inflammation incited by *C. rodentium* was observed in both the proximal (Figure 3.2) and distal (Figure 3.3) colon. The degree of cellular injury was highest on day 14 p.i. (i.e. peak infection) and day 21 p.i. (i.e. late infection), and decreased by day 28 p.i. (i.e. clearance) (Figure 3.4A-F).). Histopathologic scores of cellular injury in the distal colon were similar (P≥0.322) among the diet treatments for mice with enteritis (Figure 3.5A-G). However, in the proximal colon, mice fed the WB diet (P=0.029), and to a lesser degree those fed the RS diet (P=0.147) showed altered histological scores when compared to those fed the CN diet (Figure 3.4A-F). In this regard, mice fed the WB diet displayed lower measures of epithelial cell hyperplasia (P=0.035), goblet cell depletion (P=0.018), epithelial cell injury (P=0.027), mitotic activity (P=0.032), crypt height (P=0.024), and a lower total score of inflammation (P=0.029) CN diet treatment mice. Mice fed the RS diet displayed a trend of marginally

reduced (P=0.093) goblet cell depletion in comparison to mice consuming the CN diet. Mice consuming the RS diet also had longer (P=0.015) colon lengths in comparison to the CN and WB diet mice averaged over time (Figure 3.6). Overall, mice consuming diets high in DF displayed lower scores of inflammation in the proximal colon, in comparison to the distal colon.

3.3.3 Diets high in DFs do not affect overall consumption of food but do increase weight gain in mice without acute enteritis

Diet treatment had no effect (P=0.285) on the amount of food consumed in mice without enteritis (Figure 3.7A). However, mice with enteritis administered the WB diet consumed less food (P=0.030) than those fed the CN diet (Figure 3.7B). In all mice, the average final weights of mice steadily increased (P<0.001) over the 28-day experimental period (Figure 3.7C-D). Mice with enteritis gained less (P<0.001) weight overall in comparison to mice without enteritis. In mice without enteritis (Figure 3.7C), individuals consuming the RS diet gained weight more rapidly (P=0.017) than those consuming the CN diet.

3.3.4 Diets rich in RS and WB increase quantities of SCFA in the cecum and distal colon

Short-chain fatty acid concentrations were measured in the cecum, proximal colon, and distal colon, and quantities of total SCFA differed between these locations (Figure 3.8A-C). Generally, the highest quantities of total SCFA were observed in the cecum and distal colon. Concentrations of total SCFAs (P<0.001), and butyrate specifically (P=0.003) were higher in mice without enteritis than with enteritis in the distal colon (Figure 3.8C-D). In the cecum, enteritis had no effect on total SCFA concentrations (P=0.497) (Figure 3.8A). However, total SCFA (P=0.008) (Figure 3.8A), acetate (P=0.013) (Table 3.1), and butyrate (P<0.001) (Table 3.1) were all affected by the diet treatment. Butyrate concentrations in the cecum were higher for the WB diet than the CN (P<0.001) and RS (P=0.009) diet treatments (Table 3.1). In the proximal colon, mice fed the WB diet had higher (P=0.051) overall concentrations of SCFA when compared to those fed the RS and CN diets (Figure 3.8B; Table 3.1). In the distal colon, concentrations of total SCFA (P<0.001), acetate (P<0.001), propionate (P=0.002), and

butyrate (P=0.003) were substantially reduced in mice with enteritis at the peak, late, and clearance stages of infection (Figure 3.8C; Table 3.1). Furthermore, mice consuming the WB (P=0.005) and RS (P=0.053) diets showed higher SCFA concentrations when compared to those consuming the CN diet (Figure 3.8A-C). In mice without enteritis, butyrate concentrations in mice consuming the WB diet were higher (P=0.001) relative to the CN diet treatment in the distal colon (Figure 3.8D; Table 3.1).

3.3.5 Mucus accumulated in the intestinal lumen and goblet cells in the colon of mice inoculated with *C. rodentium* and consuming WB-enriched diets

Mucus accumulation in the distal colon of mice without enteritis did not differ between the diets (Figure 3.9). Mucus accumulation in goblet cells and to a lesser extent within the intestinal lumen of mice with enteritis was marginally increased for mice consuming the WB diet (Figure 3.10B, E, H). Increased mucus accumulation in the goblet cells was also observed mice consuming the RS diet (Figure 3.10C, F, I) relative to the CN diet at the clearance stage of infection. Mice with enteritis exhibited reduced expression of *Muc2* when compared to mice without enteritis (P<0.001) (Figure 3.11D, Fig 3.12D). At peak infection, it was observed that RS diet treatment mice exhibited the highest expression (P=0.043) of *Muc2* in the distal colon (Figure 3.11D) when compared to the CN diet, but by late infection, there was no difference (P=0.387) between the two treatments.

3.3.6 DF supplementation to mice with enteritis increases the expression of bacterial recognition genes

In mice without enteritis, there was no effect (P≥0.100) of diet on the expression of Th1 (*IL-1β, IFNγ, TNFα*) or Th17 (*IL-17A, IL-22 and IL-23*) associated cytokines, Treg (*IL-10, TGFβ*), mucus-associated (*Muc2, Tff3, Relmβ*) or bacterial recognition (*TLR2, TLR4, Myd88, RegIIIγ*) genes (Figure 3.12). In mice with enteritis, the Th1 (*IL-1β, TNFα*) and Th17 (*IL-17A*) associated cytokines did not show changes in gene expression in response to DF-enriched diets, but were highly expressed when compared to mice without enteritis (Figure 3.11A, B, E). At peak infection, mice consuming the RS and WB diets exhibited the highest expression (P=0.040) of *TLR4* relative to CN diet treatment mice (Figure 3.11C). For the RS diet treatment, a trend of increased (P=0.083)

TLR4 expression was observed in mice with enteritis relative to those consuming the WB diet. Mice consuming the RS diet also exhibited a trend of increased (P=0.083) expression of *IFNγ* during late infection, and increased (P=0.043) expression of the defence cytokine, *Relmβ* at peak infection when compared to mice consuming the WB diet (Figure 3.11F, J). Mice consuming the RS diet tended to exhibit elevated (P= 0.083) expression of Treg cytokines (*IL-10, TGFβ*), and this was evident during peak infection when compared to mice consuming the WB and CN diets, respectively (Figure 3.11G, H). The expression of *RegIIIγ* was highest (P=0.016) at peak infection in all mice, and those consuming the WB diet exhibited the lowest expression of this gene (P=0.021) when compared to mice consuming the CN diet (Figure 3.11I). Generally, mice consuming the WB diets displayed lower expression of bacterial recognition genes (*TLR4, RegIIIγ*), the defense cytokine gene, *Relmβ*, the Th1 regulatory cytokine gene, *INFγ*, and the regulatory cytokine, *IL-10*.

3.3.7 DF consumption alters the bacterial community structure in the large intestine

The bacterial communities associated with mucosa in the proximal and distal colon, and within ingesta in the cecum were distinct from one another. Both enteritis and diet affected community structure (Figure 3.13). The DF treatments were associated with unique community structures in the proximal colon (P=0.001), and within cecal ingesta (P=0.001) (Figure 3.13B-C). Although less conspicuous than in the proximal colon, diet also affected (P=0.024) the structure of the mucosa-associated community in the distal colon (Figure 3.13A). Within the distal colon, enteritis had a profound impact (P=0.006) on bacterial communities (Figure 3.13A). In contrast, *C. rodentium* infection did not alter community structure in the proximal colon or cecum (P>0.658). The consumption of the RS diet was associated with an increase (P=0.005) in the abundance *Roseburia* spp. in the distal colon of mice without enteritis, and an increase (P=0.034) in the abundance of *Ruminococcus* spp. in the distal colon of mice with enteritis (Figure 3.14A) in comparison to mice consuming the CN diet. The consumption of the WB diet was associated with an increase in the abundance of *Firmicutes* in the distal colon during infection compared to mice consuming the CN diet (Figure 3.15A), namely in *Lachnospiraceae* (P=0.016) and *Lactobacillales* (P=0.031) species. The abundance of *Proteobacteria* in the distal colon was lower (P=0.016) in mice with enteritis when

compared to mice without enteritis (Figure 3.15A). In mice consuming the RS diet, an increase (P=0.001) in the abundance of *Verrucomicrobia* and the mucus-associated species, *A. muciniphila* was observed in the proximal colon (Figure 3.14B). In addition, the abundance of *A. muciniphila* were increased (P=0.045) in the proximal colon of mice that consumed the WB diet, and most notably in mice with enteritis (Figure 10B). Furthermore, an increase (P<0.001) in the abundance of *Christensenellaceae* species was observed in the proximal colon of mice without enteritis that consumed the RS relative to the CN diet (Figure 3.14B). In the cecal ingesta, species in the *Bacteroidetes* phylum (Figure 3.15C), and specifically the *Bacteroidales* order were affected (P=0.066) by diet; mice ingesting the RS diet had the lowest (P=0.055) abundance of species within the *Bacteroidales* in comparison to mice consuming the CN diet. However, in mice without enteritis that consumed the RS diet, a conspicuous increase (P≤0.050) in the abundance of species in the *Actinobacteria* phylum such as *Anaeroplasma* sp. was observed (Figure 3.14C). The consumption of the WB diet was associated with a change (P≤0.050) in the abundance of bacteria within the *Actinobacteria* (i.e. *Coriobacteriia*) and *Bacteroidetes* (i.e. *Porphyromonadaceae* and *Paraprevotellaceae*) in comparison to mice consuming the RS and CN diets (Figure 3.14C). It is noteworthy that all species within the *Verrucomicrobia* phylum were identified as *A. muciniphila*, while all species identified within the *Deferribacteres* phylum were identified as *M. schaedleri*. As such, a general trend of reduced *Deferribacteres* and *M. schaedleri* abundance was observed in the cecal ingesta of mice with enteritis that consumed both the WB and RS diets; however, the mucus degrading *A. muciniphila* showed a trend of increased abundance in mice without enteritis that consumed either the WB or RS diets (Figure 3.15C). Finally, the abundance of butyrogenic species including *Dorea*, *Ruminococcus*, and *Roseburia* species was increased in the ceca of mice with and without enteritis that consumed diets enriched with WB or RS (Figure 3.16C).

3.4 Discussion

Understanding the mechanisms by which functional foods such as DF influence the functions within the intestine (i.e. the diet-microbiota-host interaction) is important to identify food components that can potentially improve host health. This investigation examined the effects of DFs on the mitigation of intestinal injury in mice with enteritis.

Mice fed diets enriched with RS and WB were subjected to pathogen challenge with *C. rodentium* to induce inflammation. These results demonstrated that DFs alter bacterial communities within the large intestine, increase total SCFA concentrations in the cecum and colon, reduce inflammation, and improve weight gain.

3.4.1 DF consumption modulates weight gain and food intake

Healthy mice consume more food and proportionally gain more weight in comparison to mice experiencing intestinal inflammation (Melgar et al., 2007). WB consumption triggers stimuli that promote satiation within the host (Brownawell et al., 2012), and I observed a consistent decrease in food intake by mice with and without enteritis consuming the WB-enriched diet. RS-enriched diets have been reported to increase levels of glucagon-like pepetide-1 and peptide YY, which are secreted hormones that reduce appetite and food intake in human and non-human animals (Zhou et al., 2015). These hormones act in response to ingested nutrients, and both RS and WB diets are triggers to their secretion (Shen et al., 2009; Zhou et al., 2015). Although it was observed that food consumption was reduced in mice fed DF-enriched diets during infection, the final weights of these mice did not differ among the diets. Many studies have promoted the conclusion that consumption of DF reduces food consumption and obesity in people (Cluny et al., 2015; Lafond et al., 2015; Ye et al., 2015). In the present study, mice with enteritis did not show a significant reduction in weight gain. A plausible explanation is that under conditions of enteric inflammation, mice generally gain less weight and have less efficient nutrient absorption due to cellular injury (Peuhkuri et al., 2010). It was observed that mice without acute enteritis that consumed a RS-enriched diet had higher final weights, and a longer colon; the increased body weight and length of the colon is consistent with an increase in the combined mass of the intestinal cells (Higgins, 2014) that translates to enhanced catabolism. Although DF intake can reduce feed intake, generally, the total amount of calories required for growth is not affected, and individuals can still maintain a healthy weight (Otles and Ozgoz, 2014). Therefore, my research suggests that the consumption of diets enriched for WB and RS reduces the amount of food required to maintain a healthy body weight in mice.

3.4.2 The consumption of WB is associated with a lowered severity of infection, while the ingestion of RS promotes protective cellular inflammation

Mice inoculated with *C. rodentium* have transmural crypt hyperplasic changes within the distal colon, and although tissue injury is profound, the infection is resolved 3 to 4 weeks post inoculation (Collins et al., 2014). I observed that mice consuming the WB-enriched diet shed more CFU/g in feces during early infection, but by peak and late infection, shed less CFU/g when compared to mice consuming the RS-enriched and CN diets. *Citrobacter rodentium* infection initially elicits a predominantly Th17 immune response, and this switches to a Th1 response as infection culminates (Costa et al., 2011). In mice consuming the WB-enriched diet, the expression of the Th17 cytokine, *IL-17A* was lower than mice consuming the RS-enriched and CN diets during peak infection, and by late infection, these mice exhibited the lowest expression of Th1 (*IL-1β*, *IFNγ*, *TNFα*) cytokines. Furthermore, the overall amount of *C. rodentium* shed in feces was the lowest in mice consuming the WB-enriched diet at late infection. This suggests that mice ingesting the WB-enriched diet were less affected by infection relative to mice consuming the RS-enriched and CN diets, and confirm a previous conclusion that fermentation of WB produces by-products that reduce intestinal inflammation and the host immune response (Macfarlane et al., 2006). Measures of tissue injury and cellular inflammation in the colon indicated that infection in the proximal colon was less severe as compared to the distal colon in the current study. As *C. rodentium* mainly causes damage to the distal colon (Collins et al., 2014), it was concluded that the ingestion of WB is ameliorative to inflammation in tissues that are less severely inflamed (i.e. in the proximal colon). Furthermore, it has been shown that DF administration does not influence the growth of invasive pathogens such as Enteropathogenic *Escherichia coli* and *C. rodentium* through elevated Treg (*IL-10*, *TGFβ*) cytokine expression (Johnson-Henry et al., 2014). It was also observed that genes indicative of bacterial infection, namely *TLR4* and *RegIIIγ* were expressed at lower amounts in mice consuming the WB-enriched diet compared to RS-enriched diet. *TLR4* is involved in increasing the pro-inflammatory immune response through the recognition of bacterial lipopolysaccharide (Ortega-Gonzalez et al., 2014), and *Relmβ* is involved in promoting protective epithelial cell proliferation during *C. rodentium* infection in mice (Bergstrom et al., 2015). I also

observed elevated expression of *Relmβ* in the distal colon of mice consuming the RS-enriched diet in concert with high scores of epithelial cell hyperplasia, suggesting that the heightened histopathologic scores observed in the distal colon were a result of protective cell growth rather than destructive cell growth (Bergstrom et al., 2015). Furthermore, in mice gavaged with PBS alone, those consuming the RS-enriched diet exhibited elevated levels of *Relmβ* as well as an increase epithelial cell hyperplasia in the distal colon compared to mice consuming the WB-enriched diet. Overall, the consumption of DFs, namely WB, was beneficial in reducing the severity of *C. rodentium* infection in the proximal colon, and this was confirmed by the lowered expression of genes that are hallmarks of inflammation. In the distal colon where infection is more severe, DF was less effective at reducing pathologic changes, however, the RS-enriched diet stimulated innate factors (i.e. *Relmβ*) that were observed in association with enhanced host immune responses and increased cell proliferation to reduce tissue damage.

3.4.3 DF consumption increases intestinal SCFA that are associated with the enhanced growth of butyrogenic and mucus-associated bacteria

Intestinal SCFAs are important in the maintenance of colonic function and intestinal homeostasis. Accurate measurements of colonic SCFAs can be challenging as they are rapidly metabolized by epithelial cells in the large intestine (Cummings, 1981). Fermentation rates are highest in the cecum and proximal colon (Hamer et al., 2008), yet this data shows that the proximal colon had the lowest quantities of SCFA of the three sites examined. This suggests that SCFAs may be absorbed at a faster rate in the proximal colon than in the distal colon and cecum (Macfarlane et al., 1992; Topping and Clifton, 2001). In the proximal colon, it was observed that no differences in SCFA concentrations between mice with and without acute enteritis occurred. In contrast, SCFA concentrations were higher in the distal colon of mice without enteritis. Amongst these mice, SCFA concentrations in the distal colon were elevated in mice that consumed DF-enriched diets, and those ingesting the WB-enriched diet specifically showed the highest concentrations of SCFA. My results suggest that DF consumption is most effective at increasing SCFA concentrations and contributing to host health in the non-inflamed or moderately inflamed colon (Topping and Clifton, 2001).

An increase in enteric concentrations of SCFA is purported to confer a variety of health benefits, and individually, each SCFA may elicit positive effects on the intestine (Al-Lahham et al., 2010; Andoh et al., 1999; Bach Knudsen, 2015). Butyrate in particular has been associated with increasing intestinal mucus (Gaudier et al., 2009; Wrzosek et al., 2013, Chapter 2), and although studies are conflicting, many suggest a positive correlation between butyrate presence and mucin secretion. Although changes in *Muc2* expression in response to diet treatment were not observed, it was observed that mice consuming the RS-enriched diet exhibited more mucus within goblet cells. Of the diet treatments that were examined, the WB-enriched diet continuously produced the highest concentrations of total SCFA, including butyrate. Previous studies have revealed that β-glucan can contribute to enhanced mucus secretion in the intestine (Morel et al., 2005). Furthermore, others have shown that diets enriched for long-chain arabinoxylans and inulin increase the concentration of SCFA in the colons of mice, and concomitantly increase host-derived mucins and butyrogenic bacteria (Van den Abbeele et al., 2011). Although RS fermentation is known to increase intestinal butyrate concentrations (Bird et al., 2010), arabinoxylan fractions found in WB can also increase butyrogenic bacteria, and contribute to higher butyrate concentrations within the colon than would be produced via fermentation of RS alone (Nielsen et al., 2014). In this regard, the highest concentrations of butyrate were observed in feces from human subjects that consumed wheat arabinoxylan over a 3-week period (Gråsten et al., 2003).

An increase in the abundance of mucus-associated bacteria in the colon of mice was consistently observed in the current study, especially in mice with enteritis. The abundance of bacterial species possessing known associations with mucus can be an indicator of high mucus content within the intestine. *Mucispirillum shaedleri* is recognized as mucus-dwelling bacterium (Berry et al., 2012; Robertson et al., 2005), however the degree and mechanisms by which *M. shaedleri* degrades mucus, and the importance of mucus as an ecological niche for this bacterium remains to be elucidated. Alternatively, *A. muciniphila* has been widely reported as a mucin degrader, and it utilizes gastric mucins as its main energy source in healthy individuals (Belzer and de Vos, 2012; Derrien et al., 2011). It was also observed that the abundance of the mucus-dwelling bacterium, *M. schaedleri* in the proximal and distal colon (mucosa-associated)

and cecum (ingesta) was decreased in healthy mice consuming DFs. Conversely, *A. muciniphila* abundance was increased in the colon of mice administered DFs, and this was observed in all mice regardless of their inflammation status. This suggests that during infection, the increase in *A. muciniphila* abundance decreases the mucus available for colonization by *M. schaedleri* (i.e. due to increased mucus degradation by *A. muciniphila*). I did not detect profound elevation of *Muc2* expression in the distal colon as a function of diet treatment. It is plausible that other intestinal mucins other than MUC2 may have increased in the intestines of mice consuming WB and RS diets, such as MUC4 or MUC5B (Morel et al., 2005). The composition, architecture, and microbiology of mucus is complex (Ambort et al., 2011; Johansson et al., 2011), and the impact of DFs on the biochemical and microbiological characteristics of enteric mucus warrants examination.

3.4.4 DF consumption affects microbial community structure and increases diversity in healthy mice

The consumption of DFs altered community structure in the distal colon, proximal colon, and cecum. I observed that enteritis affected communities in the distal colon to a greater degree than did diet treatments. However, mice consuming the RS-enriched diet exhibited an increase in the abundance of *Firmicutes*, which has been reported in mice infected with *C. rodentium* (Hoffmann et al., 2009). A number of butyrogenic bacteria (e.g. members of the *Lachnospiraceae* and *Ruminococcaceae* families within the *Clostridiales* order) reside in the colon (Antharam et al., 2013), and an increase in the abundance of butyrate-producing bacteria including *Roseburia* species within the *Clostridia* XIVa group in mice that ingested DF-enriched diets was observed. Intestinal pH may stimulate the growth of butyrogenic bacteria (Walker et al., 2005). The lower pHs (≈pH 5.5) that occur in the cecum and proximal colon due to SCFA production has been shown to enhance the growth of bacteria in *Clostridia* cluster IV and *Clostridia* cluster XIVa (Louis and Flint, 2009). Thus, the increases in SCFA concentrations that occurred may have been due to the increased abundance of butyrogenic bacteria in the colon and cecum (Hamer et al., 2008). It was also observed that the proximal colon had a greater diversity of mucosa-associated bacteria in comparison to the distal colon. In the proximal colon of mice without enteritis, consumption of the RS-enriched diet was

associated with an increased abundance of *Christensenella* spp. In contrast, the abundance of *A. muciniphila* was increased in mice with enteritis that consumed the RS-enriched diet. Both these species have been shown to improve barrier function (Reunanen et al., 2015), as well as weight maintenance and health in people (Everard et al., 2013; Goodrich et al., 2014). In the cecum of mice without acute enteritis that consumed the WB-enriched diet, the abundance of *Paraprevotella* spp., *Clostridium* spp., and *Actinobacter* spp. increased, which is similar to observations reported in people (Liu et al., 2015). Thus, this data indicates that mice fed diets enriched with DFs exhibit enhanced growth of bacteria that thrive in low pH environments and produce butyrate as a metabolic by-product of DF fermentation. Although an association between SCFA concentration and the intensity of inflammation in the distal colon was not observed, the consumption of DF did alter the structure of the bacterial community, and in particular contributed to the increase in mucus-associated species in areas of less intense inflammation such as the proximal colon. Furthermore, an increase in the abundance of mucus-associated species may be associated with the regulation of host growth and weight gain, as well as contributing the barrier function and maintenance to enhance host health (Dao et al., 2016; Everard et al., 2013; Reunanen et al., 2015). Intestinal fermentation is exceptionally complex biochemically and ecologically, and varies spatially and temporally within the large intestine. Branched-chain fatty acid fermentation in the distal colon increases ammonia and nitrogen substrates that can also affect bacterial community composition (Kalmokoff et al., 2015), yet the impacts of nitrogen metabolism resulting from fermentation on intestinal health have not been extensively studied.

3.4.5 Conclusions

Mice challenged with *C. rodentium* developed acute enteritis characterized by enhanced epithelial cell hyperplasia, goblet cell depletion, increased crypt height, and stimulation of the host immune response denoted by increases in Th1/Th17 and Treg cytokines. In the inflamed colon, the consumption of the RS-enriched diet enhanced innate immunity by the RELMβ-stimulated increase in cell repair, and in the moderately inflamed intestine, the consumption of the WB-enriched diet reduced the severity of inflammation by inhibiting the growth of *C. rodentium* during peak and late infection.

Although I hypothesized that the RS-enriched diet would increase butyrate concentrations leading to an increase in MUC2 secretion to enhance host health, I saw that mice fed the WB-enriched diet exhibited the highest concentrations of total SCFAs, including butyrate. Concomitantly, butyrate concentrations within the colon were increased in mice ingesting both the RS- and WB-enriched diets, however, consumption of the WB-enriched diet was associated with the increase in total SCFA concentrations that stimulated the growth of butyrogenic bacteria. Whether butyrogenic bacteria increased the production of SCFA in the intestine, or the high concentrations of SCFA produced from DF fermentation selected for butyrogenic taxa remains to be elucidated. It is noteworthy that the DF-enriched diets were associated with an increase in the abundance of many bacteria purported to be health promoting within the intestine. Furthermore, many of the species that were identified to confer a health benefit were also associated with intestinal mucus. Accumulating evidence indicates that mucus secretion plays an important role in mediating inflammation, and it was observed that the ingestion of RS- and WB-enriched diets increased *Muc2* expression and mucus accumulation in the goblet cells during late infection in mice with enteritis, respectively. Further, mucus accumulation in mice without enteritis fed DF-enriched diets was primarily restricted to goblet cells, and was rarely observed accumulating in the lumen. Mice administered diets enriched for RS also produced high quantities of butyrate, and this diet was associated with an increase in mucus-associated bacteria in the proximal colon in mice with and without enteritis. This suggests that the RS diet may influence mucus secretion by increasing intestinal butyrate concentrations in support of my hypothesis. This is underscored by the observation that inflammation was not ameliorated to the same extent as in mice consuming the WB-enriched diet. Overall, it was observed that the fermentation of DFs in the large intestine enhanced intestinal SCFA that were associated with the amelioration of inflammation. Evidence also suggests that DFs are important in non-inflamed intestines to stimulate the host immune response and modulate the microbiota to improve intestinal and overall host health.

3.5 Tables and Figures

Table 3.1 Short-chain fatty acid concentrations (mM) in the distal colon, proximal colon and cecum of mice gavaged with PBS (white) and inoculated with *C. rodentium* (grey) measured 14 and 21 days after administration. Mice consumed a CN diet, or diets enriched for WB or RS. *P≤0.050 when compared to CN diet, ** P≤0.010 when compared to CN diet.

Diet	**CN**		**WB**		**RS**	
Distal Colon	**14**	**21**	**14**	**21**	**14**	**21**
Acetate	23.14 ± 7.86	23.46 ± 4.78	29.57 ± 15.26	39.59 ± 6.92	26.36 ± 11.35	25.54 ± 6.66
Propionate	6.23 ± 1.99	5.33 ± 1.10	4.21 ± 2.07	7.17 ± 1.42	7.94 ± 4.03	6.15 ± 2.07
Butyrate	1.55 ± 0.48	1.73 ± 0.80	**4.57 ± 2.32****	**4.60 ± 0.35****	3.40 ± 1.77	3.43 ± 1.33
Acetate	13.43 ± 8.97	10.56 ± 5.74	2.22 ±1.37	15.21 ± 4.59	5.18 ± 1.98	13.62 ± 4.98
Propionate	3.12 ± 2.29	2.68 ±1.89	0.34 ± 0.23	3.29 ± 0.56	1.04 ± 0.52	4.10 ± 1.41
Butyrate	1.26 ± 0.65	0.79 ± 0.70	0.26 ± 0.13	2.48 ± 0.76	0.42 ± 0.20	2.18 ± 0.78
Proximal Colon	**14**	**21**	**14**	**21**	**14**	**21**
Acetate	7.67 ± 3.86	12.56 ± 3.50	20.71 ± 9.28	14.75 ± 3.82	9.90 ± 4.95	14.85 ± 7.06
Propionate	2.25 ± 0.92	3.20 ± 1.14	3.05 ± 1.07	2.48 ± 0.46	3.20 ± 1.36	3.84 ± 1.84
Butyrate	0.44 ± 0.19	1.15 ± 0.65	**3.00 ± 2.16***	**1.93 ± 0.29***	1.86 ± 0.90	2.08 ± 0.71
Acetate	8.40 ± 4.60	12.59 ± 5.31	10.00 ± 2.58	18.50 ± 2.55	12.91 ± 3.72	5.91 ± 3.21
Propionate	2.02 ± 0.99	3.18 ± 1.43	1.58 ± 0.70	4.30 ± 1.22	2.87 ± 0.94	1.25 ± 0.72
Butyrate	1.15 ± 0.53	1.13 ± 0.58	1.12 ± 0.31*	3.42 ± 1.12*	1.32 ± 0.50	0.53 ± 0.30
Cecum	**14**	**21**	**14**	**21**	**14**	**21**
Acetate	22.46 ± 2.84	22.05 ± 3.48	31.78 ± 2.88*	32.68 ± 6.54*	25.71 ± 3.47	28.92 ± 3.95
Propionate	5.80 ± 0.96	5.13 ± 1.36	5.58 ± 0.74	6.01 ± 1.06	7.60 ± 1.19	7.18 ± 1.57
Butyrate	2.75 ± 1.10	2.90 ± 1.31	**6.99 ± 1.60***	**8.58 ± 2.49***	**5.93 ± 1.36***	**5.46 ± 1.20***
Acetate	29.25 ± 4.73	20.69 ± 5.68	32.83 ± 1.61	32.04 ± 5.29	25.42 ± 3.12	23.48 ± 3.41
Propionate	6.51 ± 1.28	4.86 ± 1.54	7.34 ± 0.97	7.99 ± 2.24	6.50 ± 0.94	6.78 ± 1.26
Butyrate	3.94 ± 1.03	2.35 ± 0.80	**7.80 ± 1.24***	**9.49 ± 2.14***	4.36 ± 1.12	4.31 ± 0.85

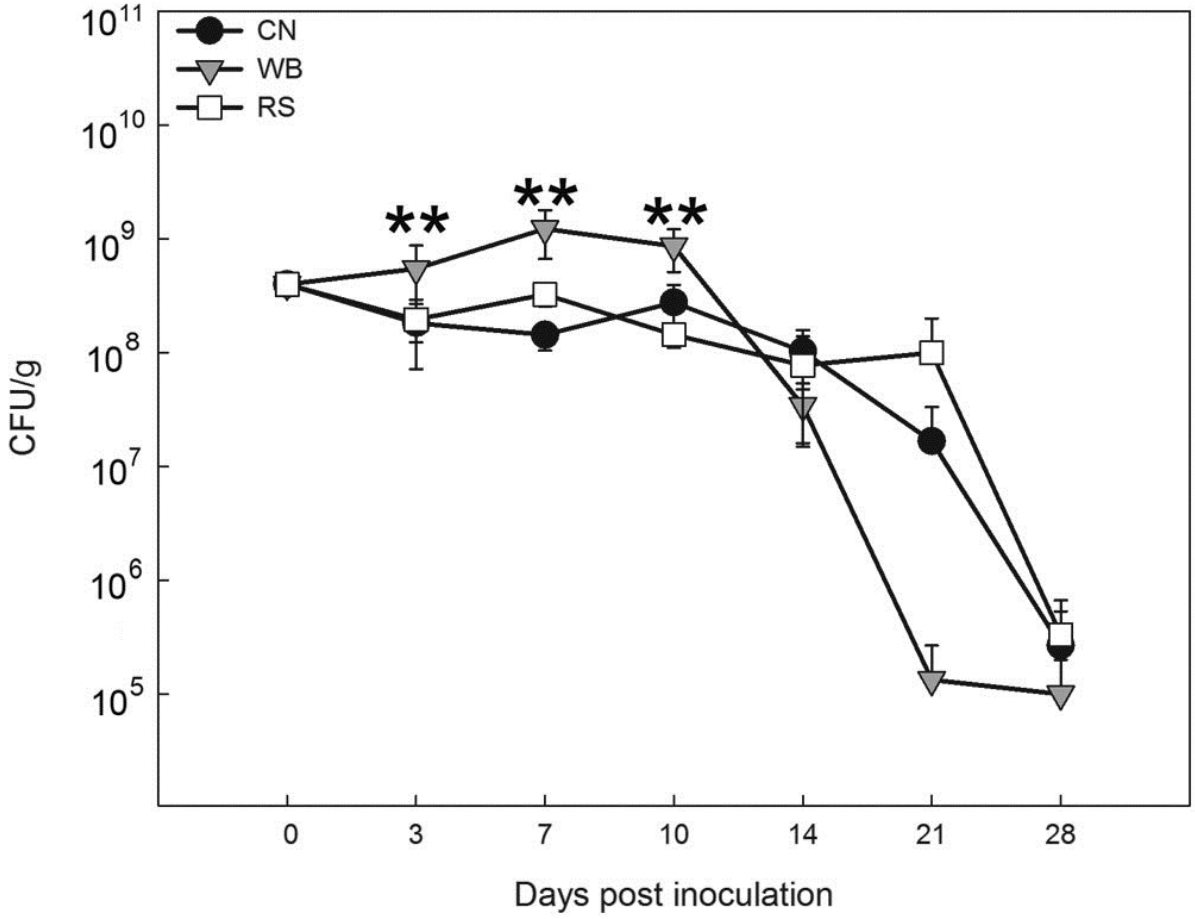

Figure 3.1 Densities of *C. rodentium* (CFU/g) in feces from mice inoculated with the bacterium over a 28 day period consuming a CN diet, or diets enriched for WB or RS. Vertical lines associated with markers represent the standard error of the mean (n=3); where error bars are not visible, variation is minimal and obscured by the marker.
**Different ($P \leq 0.010$) from the CN treatment.

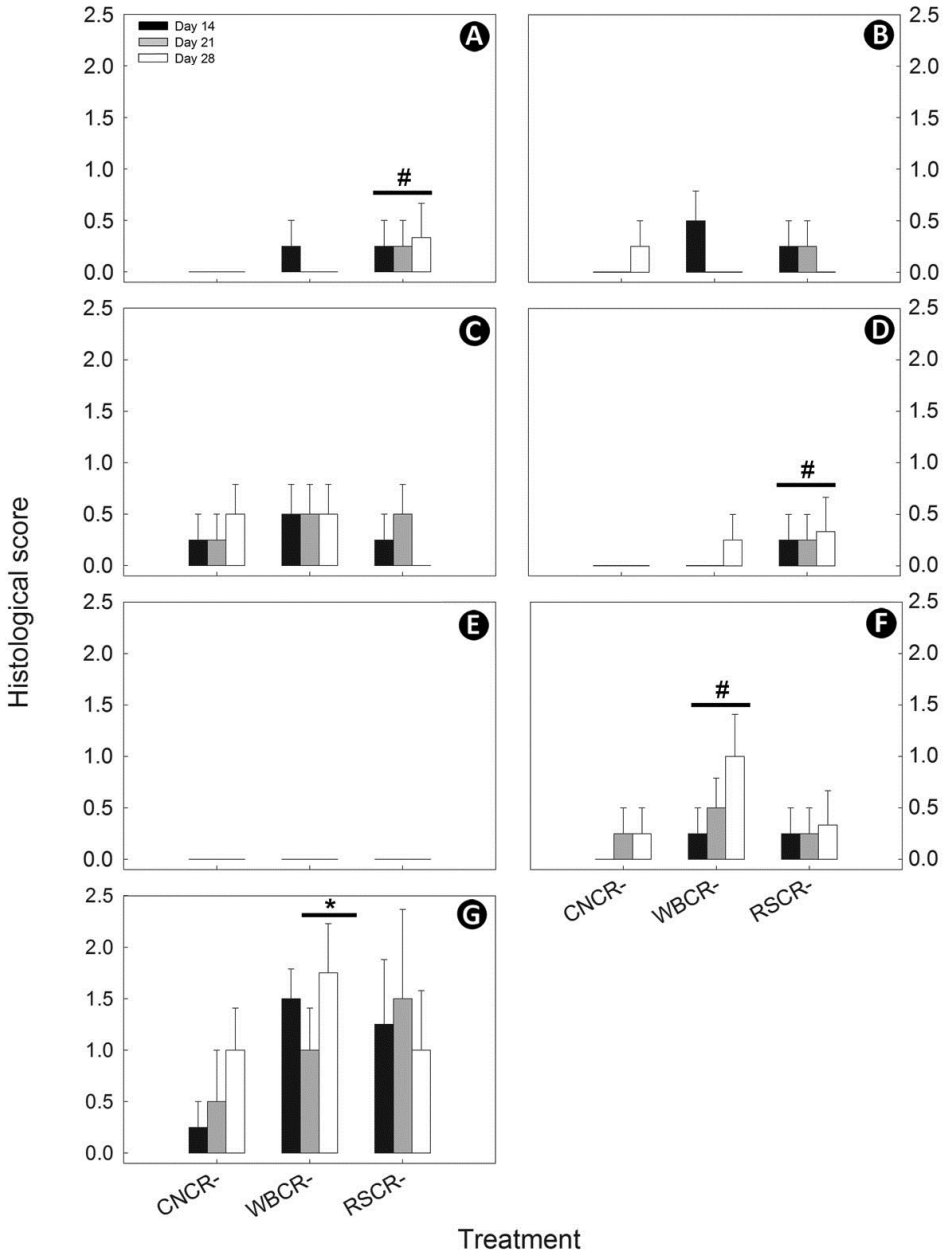

Figure 3.2 Histological scores measured from the proximal colon of mice gavaged with PBS (CR-) consuming a CN diet, or diets enriched for WB or RS. **(A)** Epithelial cell hyperplasia. **(B)** Crypt height. **(C)** Epithelial cell injury. **(D)** Inflammation. **(E)** Goblet cell depletion. **(F)** Mitotic acitvity. **(G)** Total average histological scores. Vertical lines associatedwith histogram bars represent the standard error of the mean (n=4). $^{\#}$Different ($P \leq 0.100$) from the CN diet treatment. *Different ($P \leq 0.050$) from the CN diet treatment.

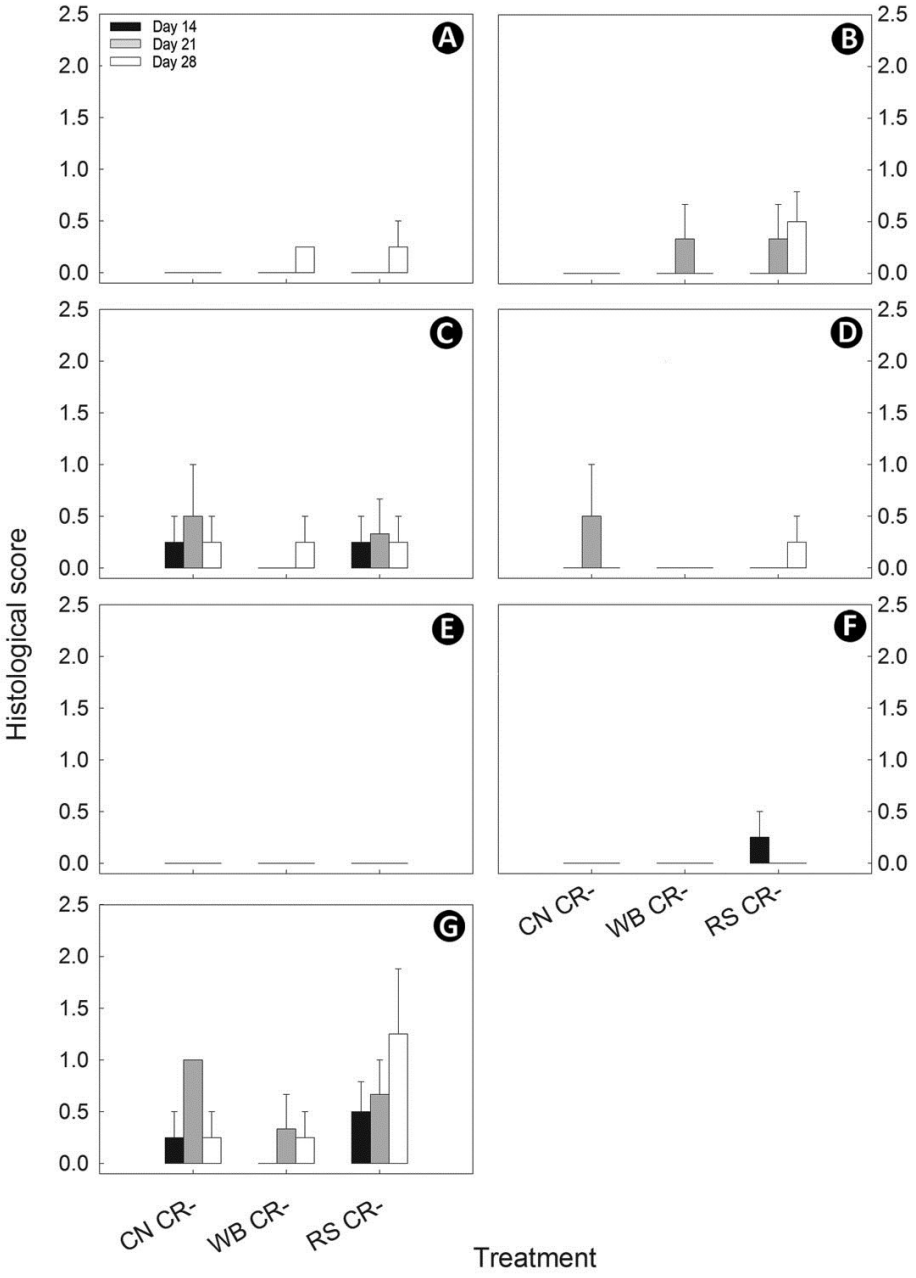

Figure 3.3 Histological scores measured in the distal colons of mice gavaged with PBS (CR-) consuming diets CN diets, or diets enriched with WB or RS. **(A)** Crypt height. **(B)** Epithelial cell hyperplasia. **(C)** Epithelial cell injury. **(D)** Inflammation. **(E)** Goblet cell depletion. **(F)** Mitotic activity. **(G)** Total average histological scores. Vertical lines associated with histogram bars represent the standard error of the mean (n=4), no variation was measured where vertical line is not present. $^{\#}$Different ($P \leq 0.100$) from the CN diet treatment. *Different ($P \leq 0.050$) from the CN diet treatment.

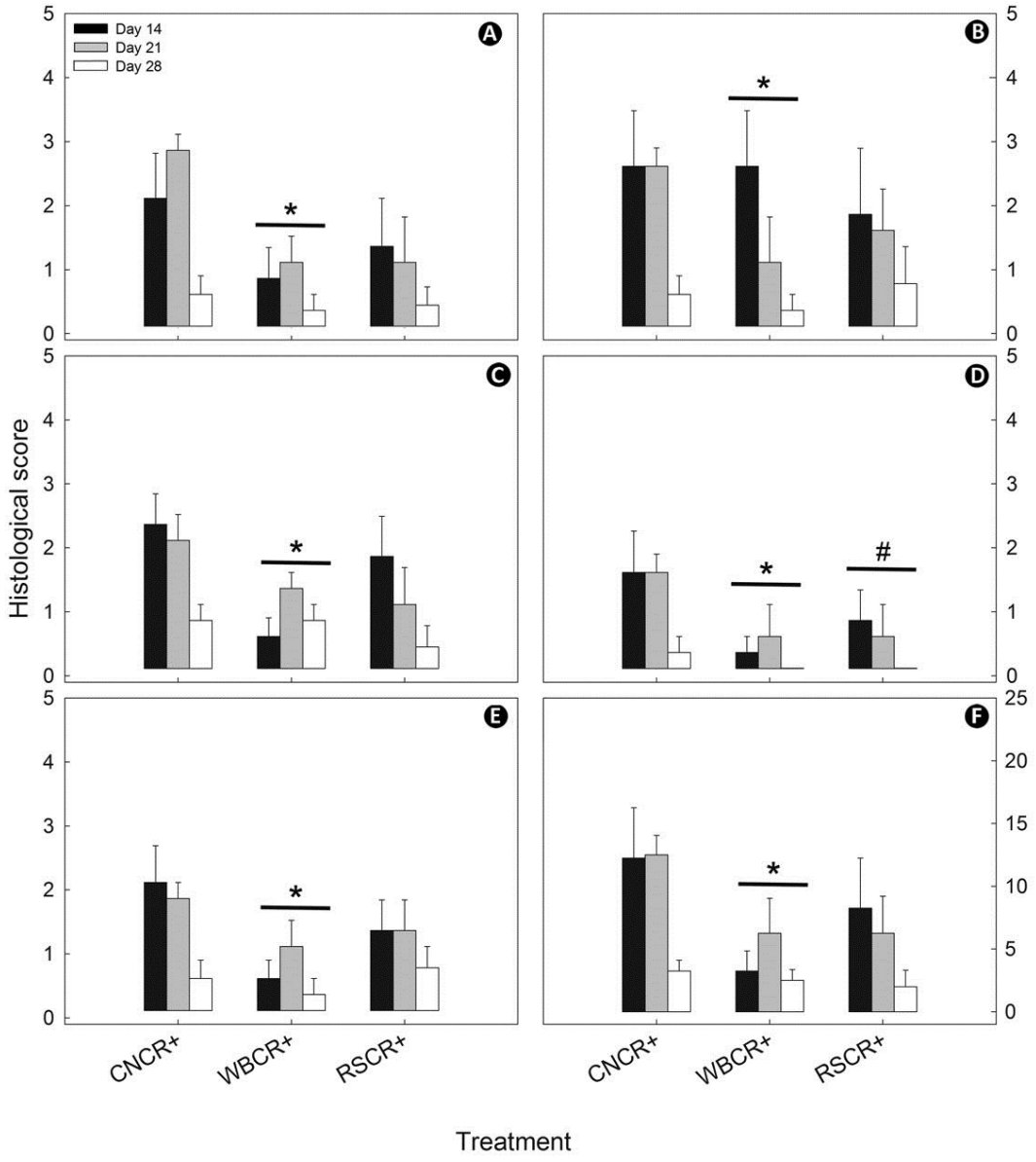

Figure 3.4 Histological scores measured in the proximal colons of mice inoculated with *C. rodentium* (CR+) consuming a CN diet, or diets enriched for WB or RS. **(A)** Epithelial cell hyperplasia. **(B)** Crypt height. **(C)** Epithelial cell injury. **(D)** Goblet cell depletion. **(E)** Mitotic cell activity. **(F)** Total average histological scores. Vertical lines associated with histogram bars represent the standard error of the mean (n=4). $^{\#}$Different ($P \leq 0.100$) from the CN diet treatment. *Different ($P \leq 0.050$) from the CN diet treatment.

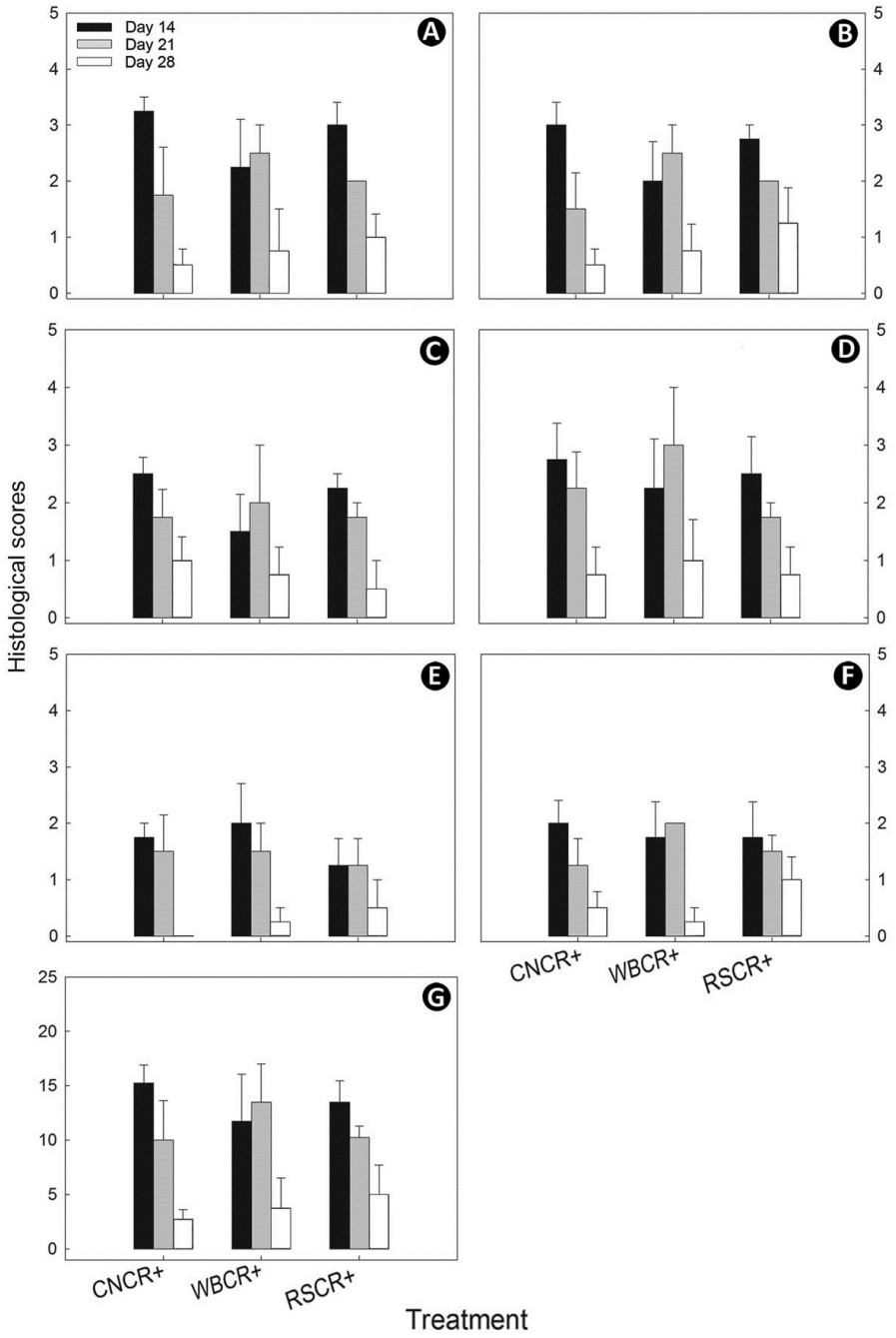

Figure 3.5 Histological scores measured in the distal colons of mice inoculated with *C. rodentium* (CR+) consuming diets CN diets, or diets enriched with WB or RS. **(A)** Crypt height. **(B)** Epithelial cell hyperplasia. **(C)** Epithelial cell injury. **(D)** Inflammation. **(E)** Goblet cell depletion. **(F)** Mitotic activity. **(G)** Total average histological scores. Vertical lines associated with histogram bars represent the standard error of the mean (n=4), no variation was measured where vertical line is not present.

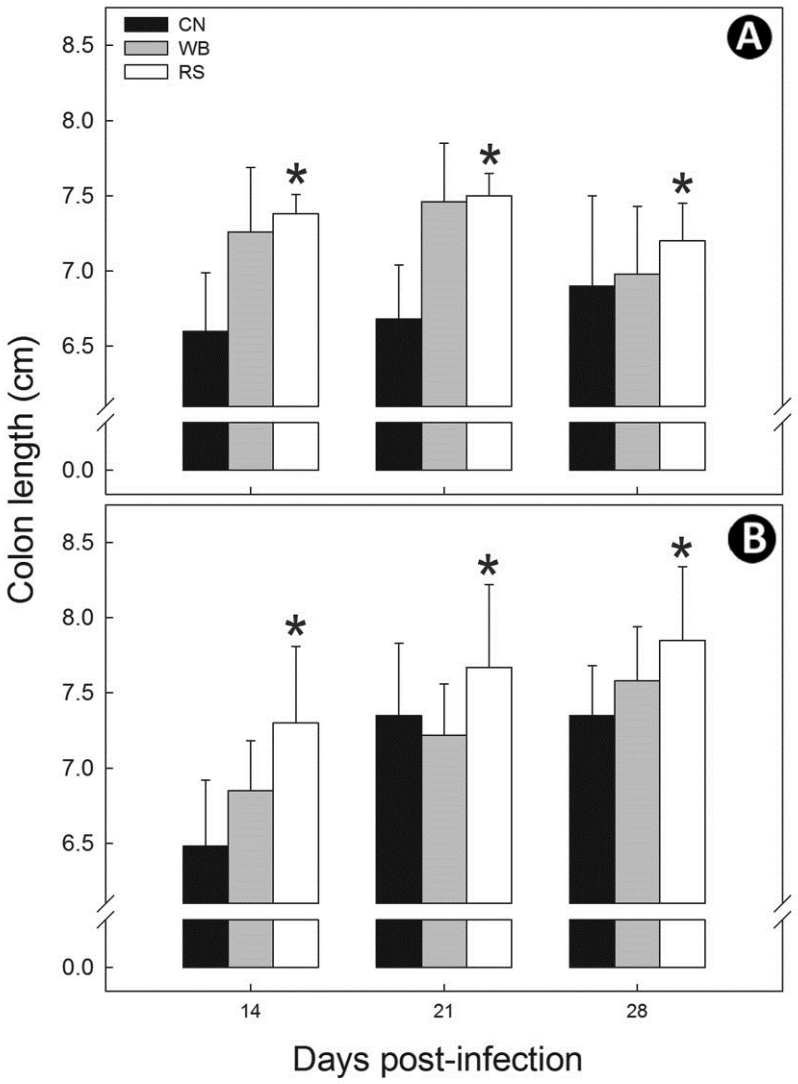

Figure 3.6 Average colon length (cm) in mice gavaged with PBS and inoculated with *C. rodentium* consuming a CN diet, or diets enriched for WB or RS over a 28 day period. **(A)** Mice gavaged with PBS. **(B)** Mice inoculated with *C. rodentium*. *Different (P≤0.050) from the CN diet treatment.

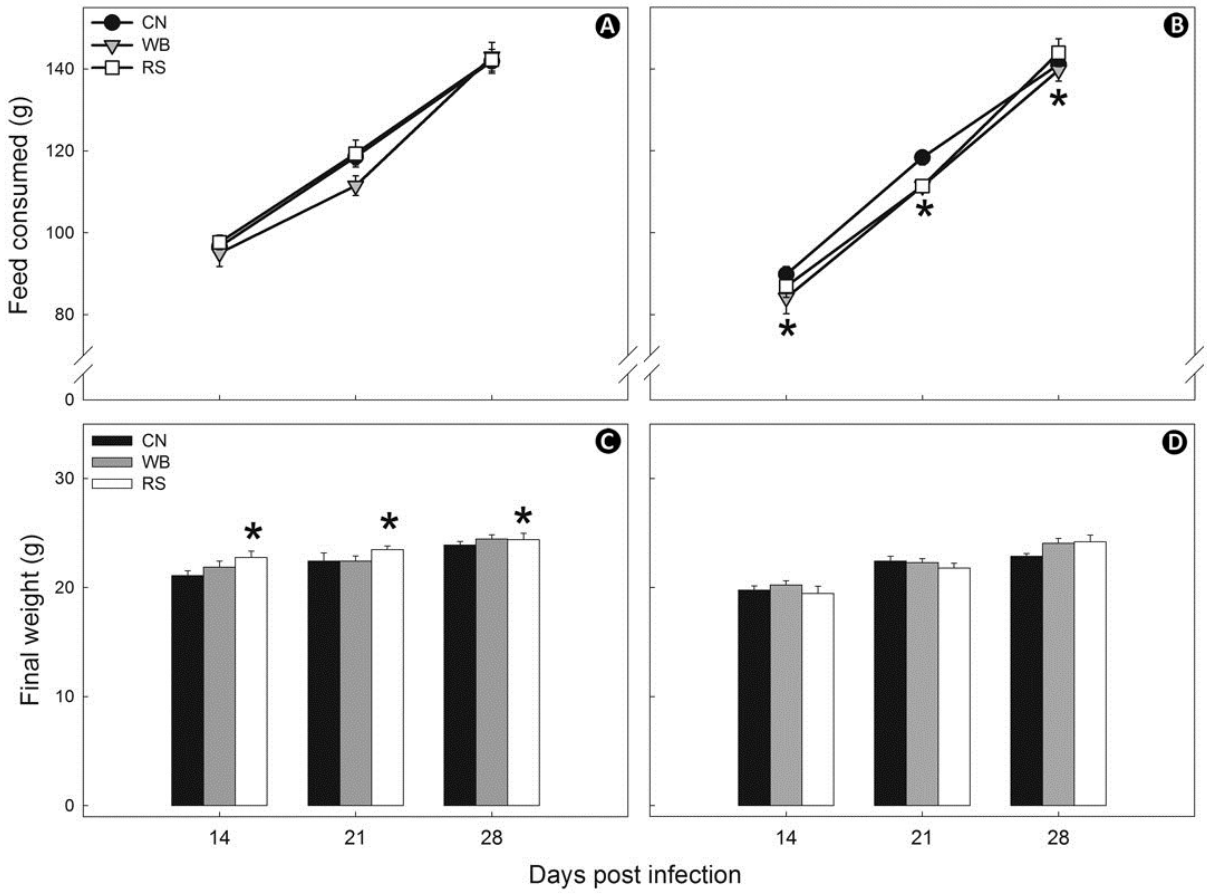

Figure 3.7 Feed consumption and final weight values in mice gavaged with PBS and inoculated with *C. rodentium* consuming a CN diet, or diets enriched for WB or RS. **(A)** Feed consumption in mice without enteritis. **(B)** Feed consumption in mice with enteritis. **(C)** Body weights of mice without enteritis. **(D)** Body weights of mice with enteritis. Vertical lines associaed with histogram bars represent the standard error of the mean (n=4), error bars are present for all samples, and in some cases are hidden by the markers. *Different ($P \leq 0.050$) from the CN diet treatment.

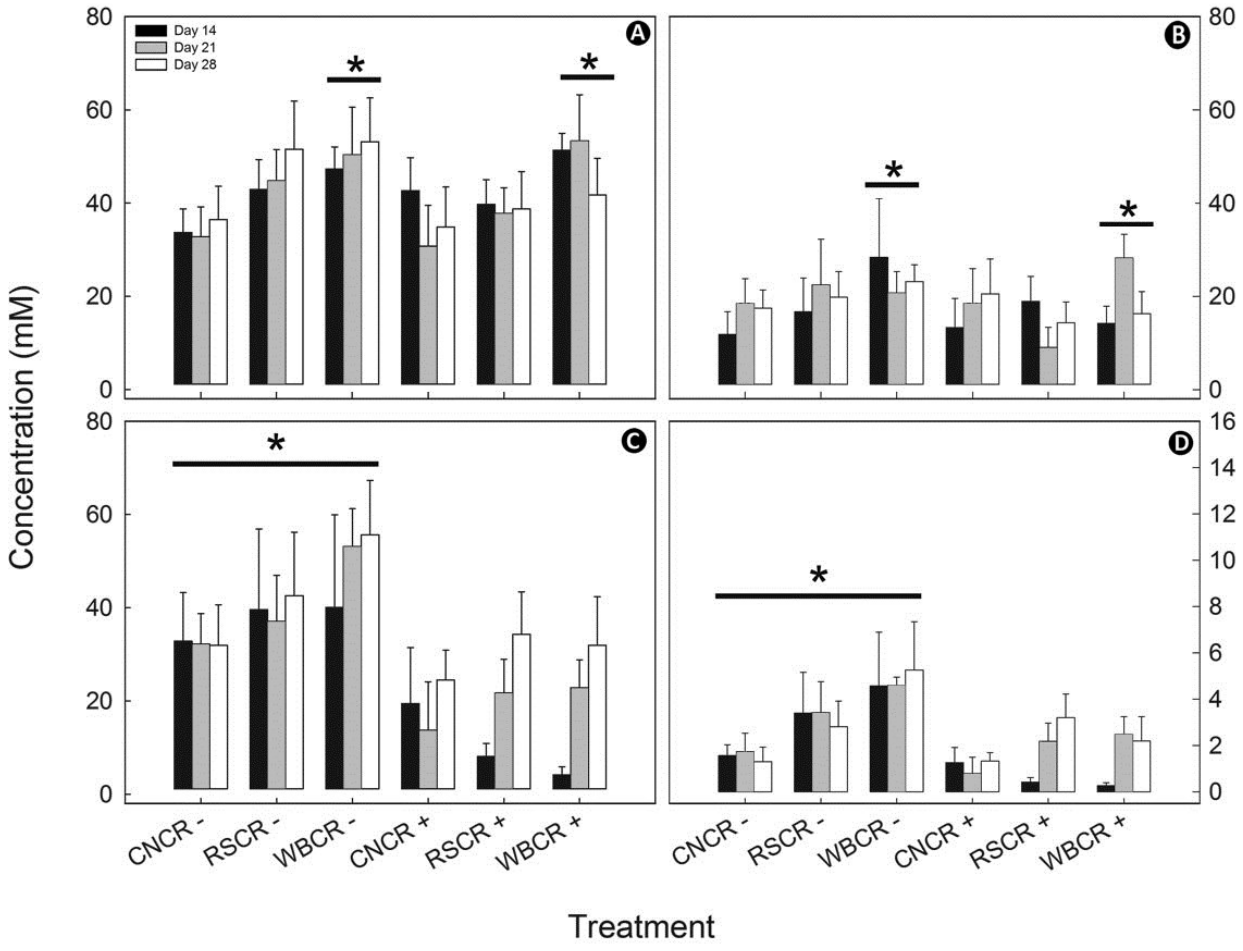

Figure 3.8 Total short-chain fatty acid (SCFA) and butyrate concentrations in the cecum, proximal colon and distal colons of mice gavaged with PBS (CR-) and inoculated with *C. rodentium* (CR+) and consuming CN diets, or diets enriched for WB or RS. **(A)** Total SCFA concentrations in the cecum. **(B)** Total SCFA concentrations in the proximal colon. **(C)** Total SCFA concentrations in the distal colon. **(D)** Butyrate concentrations in the distal colon. Vertical lines associated with histogram bars represent the standard error of the mean (n=4). *Different ($P \leq 0.050$) from the CN diet treatment or the *C. rodentium* treatment.

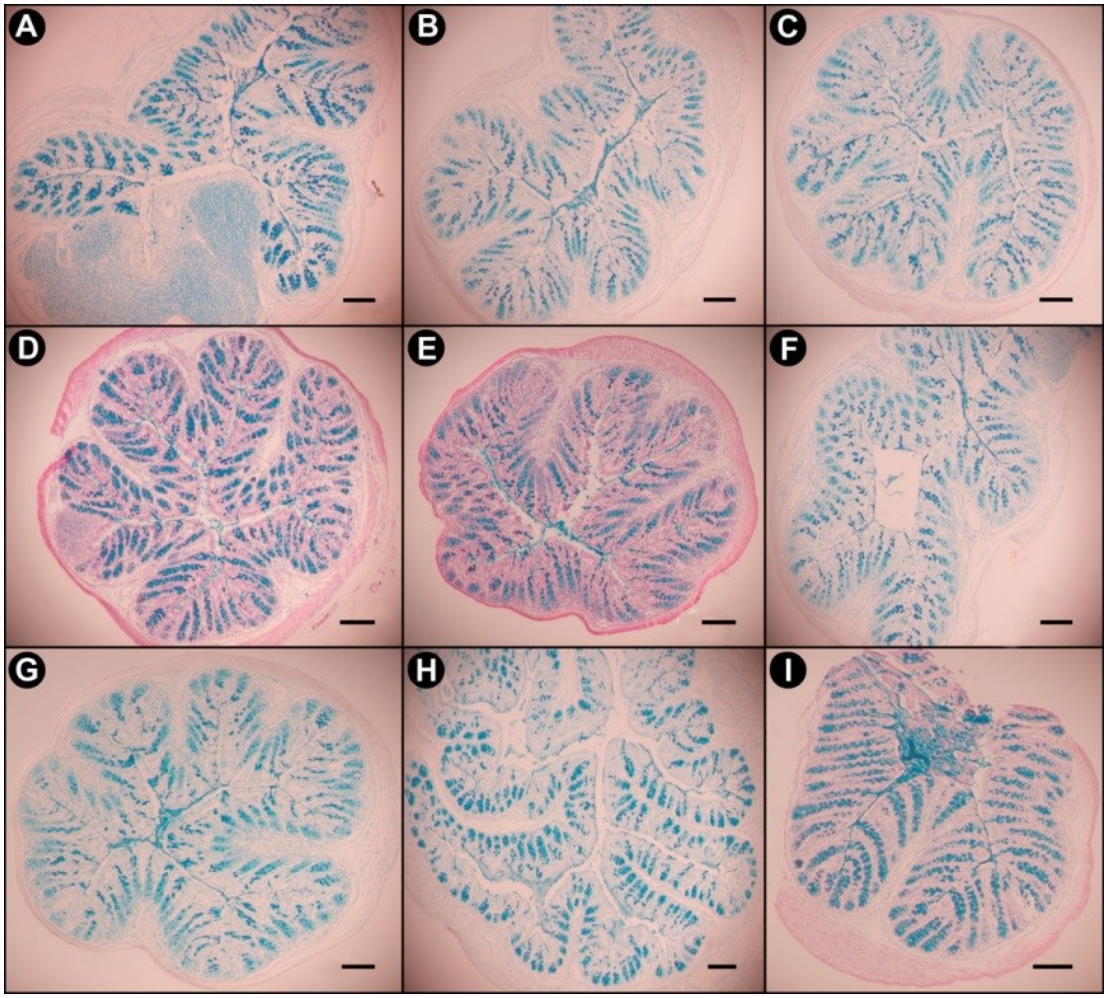

Figure 3.9. Mucus in the distal colons of mice gavaged with PBS (CR-) consuming a CN diet, or diets enriched for WB or RS. **(A)** CN treatment on day 14 p.i. **(B)** WB treatment on day 14 p.i. **(C)** RS treatment on day 14 p.i. **(D)** CN treatment on day 21 p.i. **(E)** WB treatment on day 21 p.i. **(F)** RS treatment on day 21 p.i. **(G)** CN treatment on day 28 p.i. **(H)** WB treatment on day 28 p.i. **(I)** RS treatment on day 28 p.i. Blue stain identifies mucus staining within the colonic sections. Bar, 100µm.

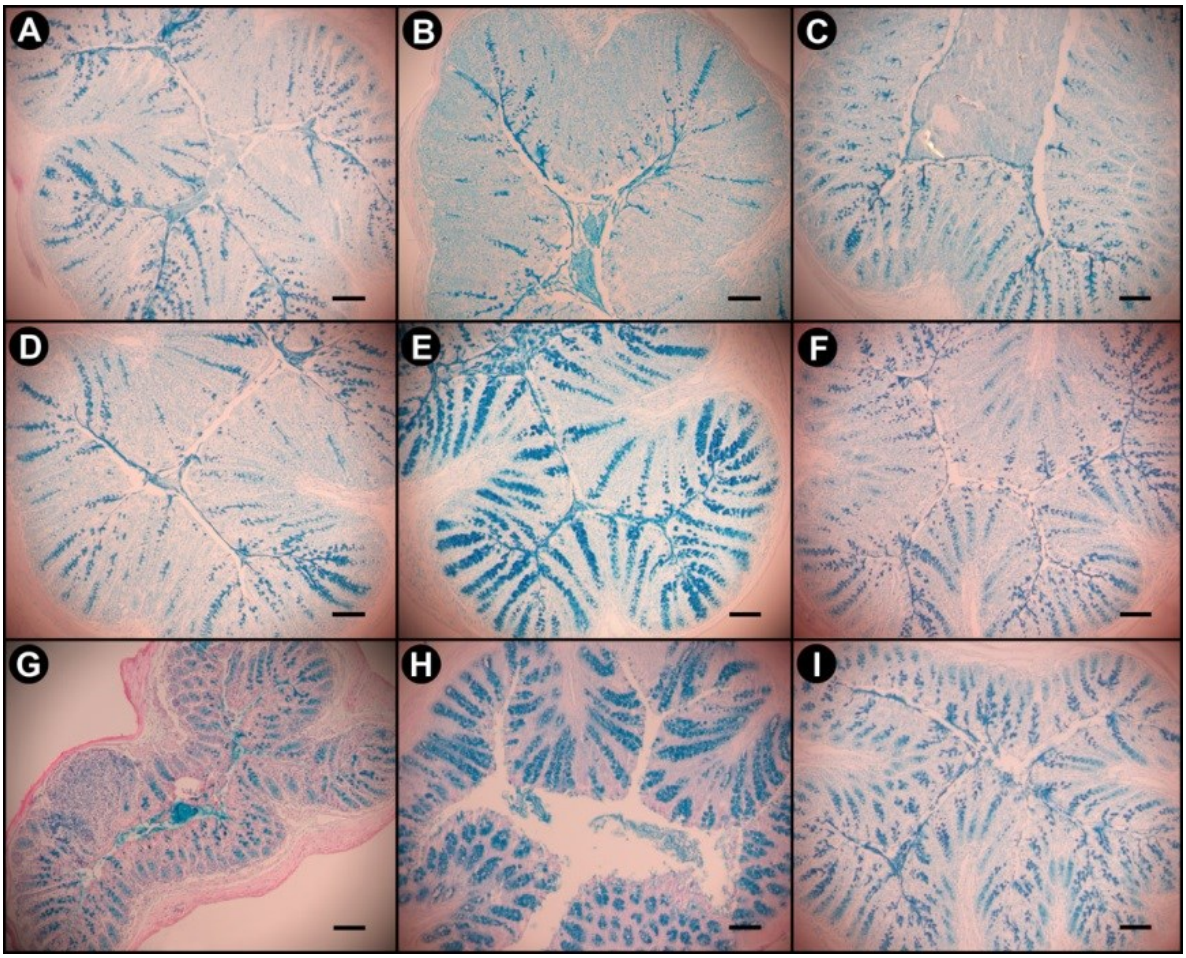

Figure 3.10 Mucus in the distal colons of mice inoculated with *C. rodentium* (CR+) consuming a CN diet, or diets enriched for WB or RS. **(A)** CN treatment on day 14 p.i. **(B)** WB treatment on day 14 p.i. **(C)** RS treatment on day 14 p.i. **(D)** CN treatment on day 21 p.i. **(E)** WB treatment on day 21 p.i. **(F)** RS treatment on day 21 p.i. **(G)** CN treatment on day 28 p.i. **(H)** WB treatment on day 28 p.i. **(I)** RS treatment on day 28 p.i. Blue stain identifies mucus staining within the colonic sections. Bar, 100µm.

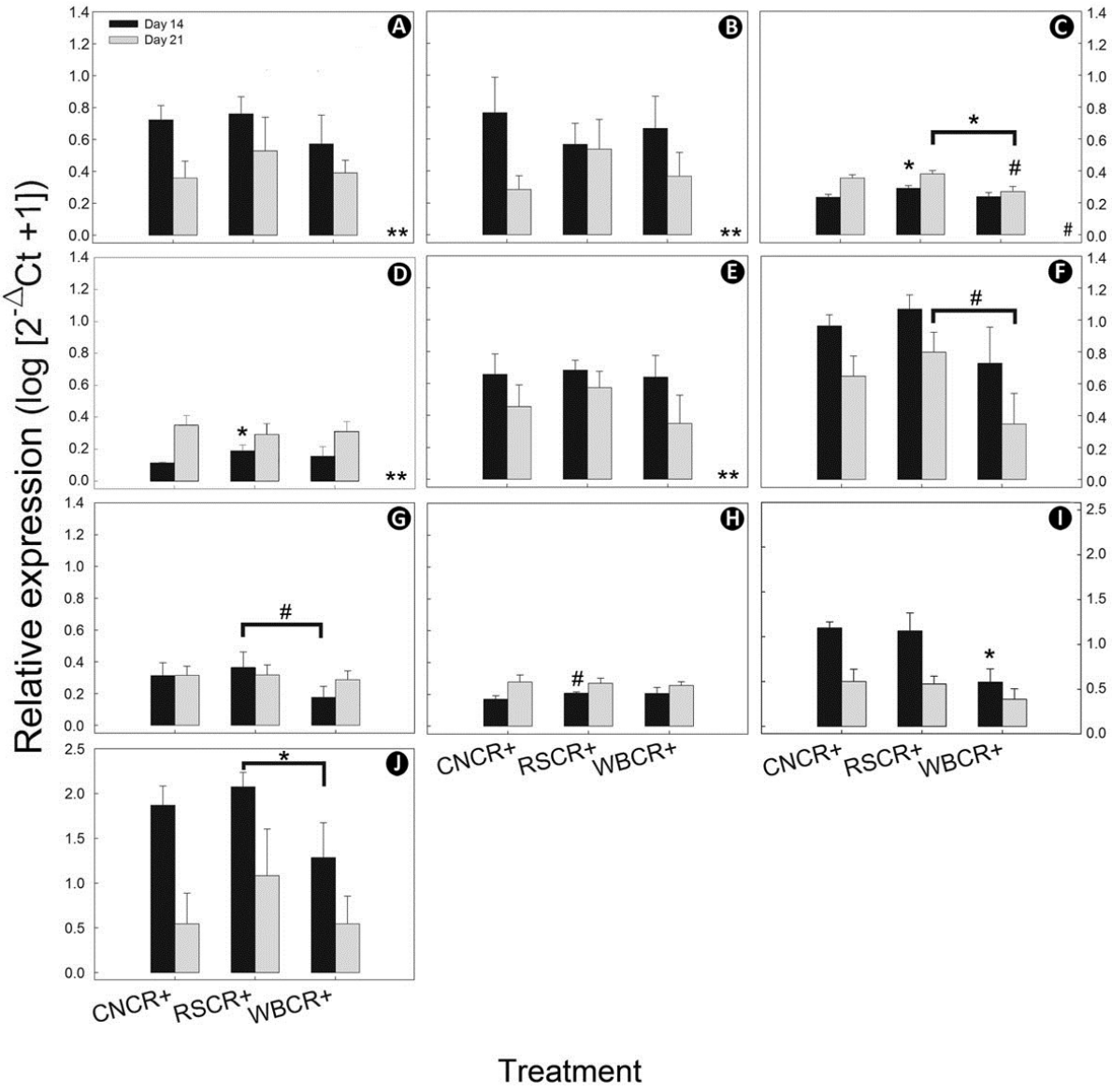

Figure 3.11 Expression of genes involved in the Th1, Th17, and Treg immune response as well as bacterial recognition and cellular repair genes from the distal colon of mice inoculated with *C. rodentium* (CR+) consuming a CN diet, or a diets enriched with WB or RS. **(A)** *IL-17A.* **(B)** *IL-1β.* **(C)** *TLR4.* **(D)** *Muc2.* **(E)** *TNFα.* **(F)** *IFNγ.* **(G)** *IL-10.* **(H)** *TGFβ.* **(I)** *RegIIIγ.* **(J)** *Relmβ*. Veritcal lines assoicatd with histogram bars represent the standard error of the mean (n=3). $^{*\ \#}$ with a horizontal line indicates pairwise differences (P≤ 0.050, P≤ 0.100). **, $^{\#}$ Found in bottom corner represent different (P≤ 0.010, P≤ 0.100) overall expression when compared to non-inflamed gene expression (Figure 3.12).

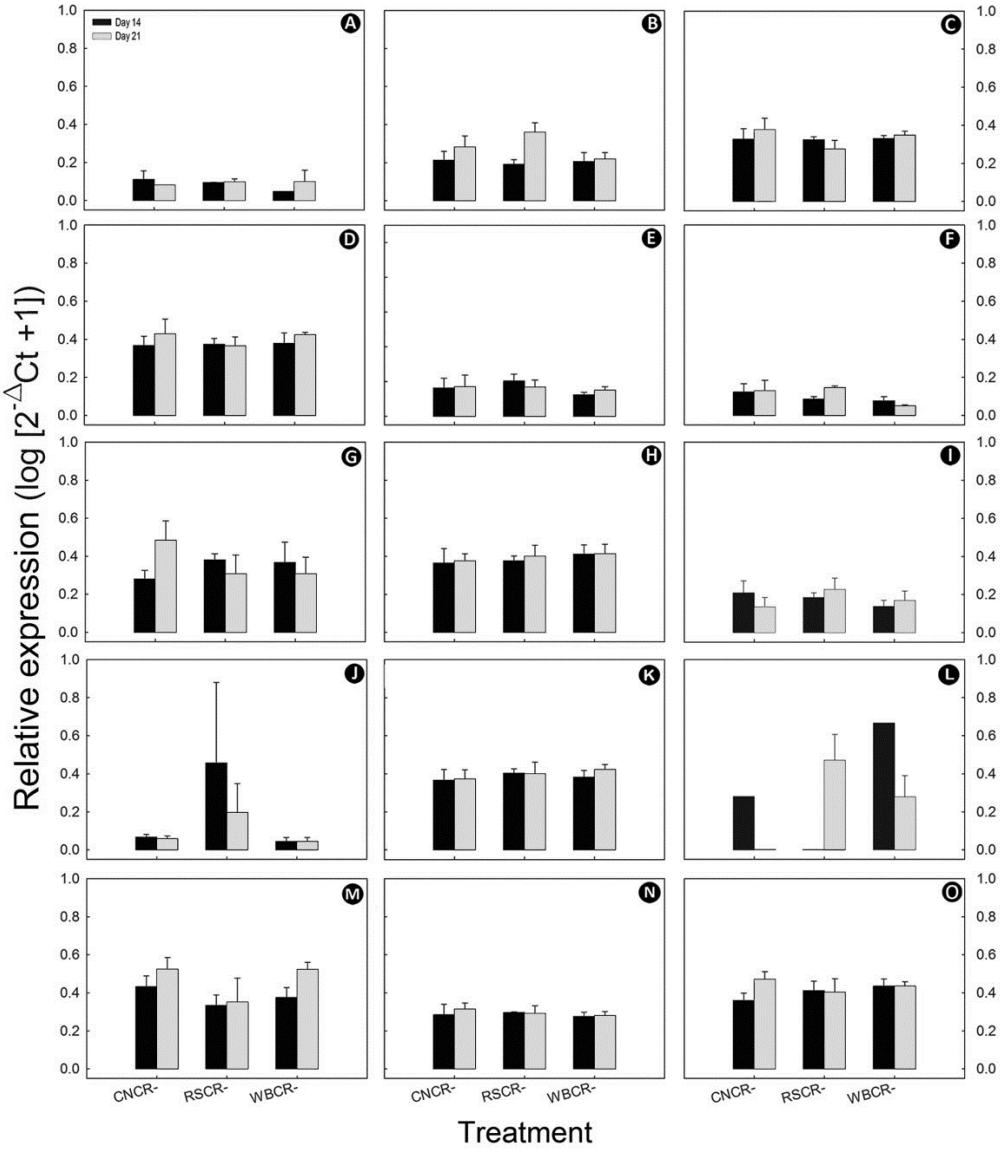

Figure 3.12 Expression of genes involved in Th1, Th17, Treg, and bacterial recognition and cellular repair from the distal colon of mice gavaged with PBS (CR-) consuming CN diets, or diets enriched for WB or RS. **(A)** *IL-17A*. **(B)** *IL-1B*. **(C)** *TLR4*. **(D)** *Muc2*. **(E)** *TNFα*. **(F)** *IFNγ*. **(G)** *IL-10*. **(H)** *TGFβ*. **(I)** *RegIIIγ*. **(J)** *Relmβ*. **(K)** *Myd88*. **(L)** *IL-22*. **(M)** *IL-23A*. **(N)** *TLR2*. **(O)** *Tff3*. Veritcal lines associated with histogram bars represent the standard error of the mean (n=3), no variation was measured where vertical line is not present. No statistical differences were observed relative to the CNCR- treatment.

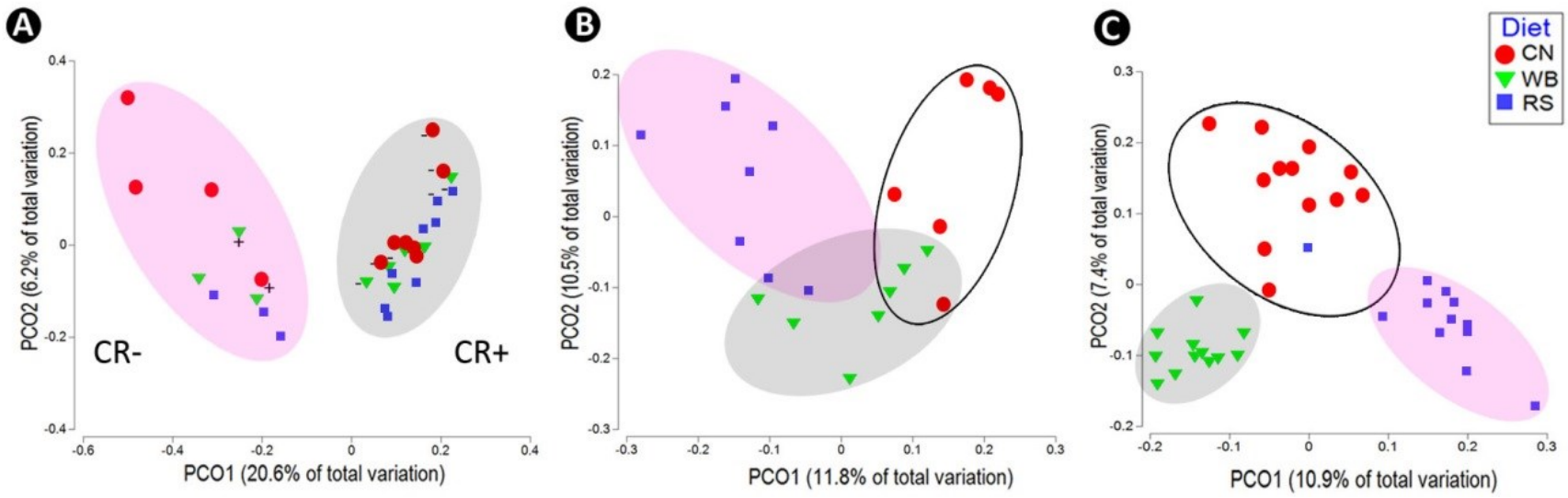

Figure 3.13 Unweighted Uni-Frac comparisons of the bacterial communities in the intestines of mice gavaged with PBS (CR-) and inoculated with *C. rodentium* (CR+) consuming a CN diet, or diets enriched WB or RS. **(A)** Distal colon. Ellipsoids identify clusters of communities by infection treatment. **(B)** Proximal colon. Ellipsoids identify clusters of communities by diet treatment. **(C)** Cecum. Ellipsoids identify clusters of communities by diet treatment.

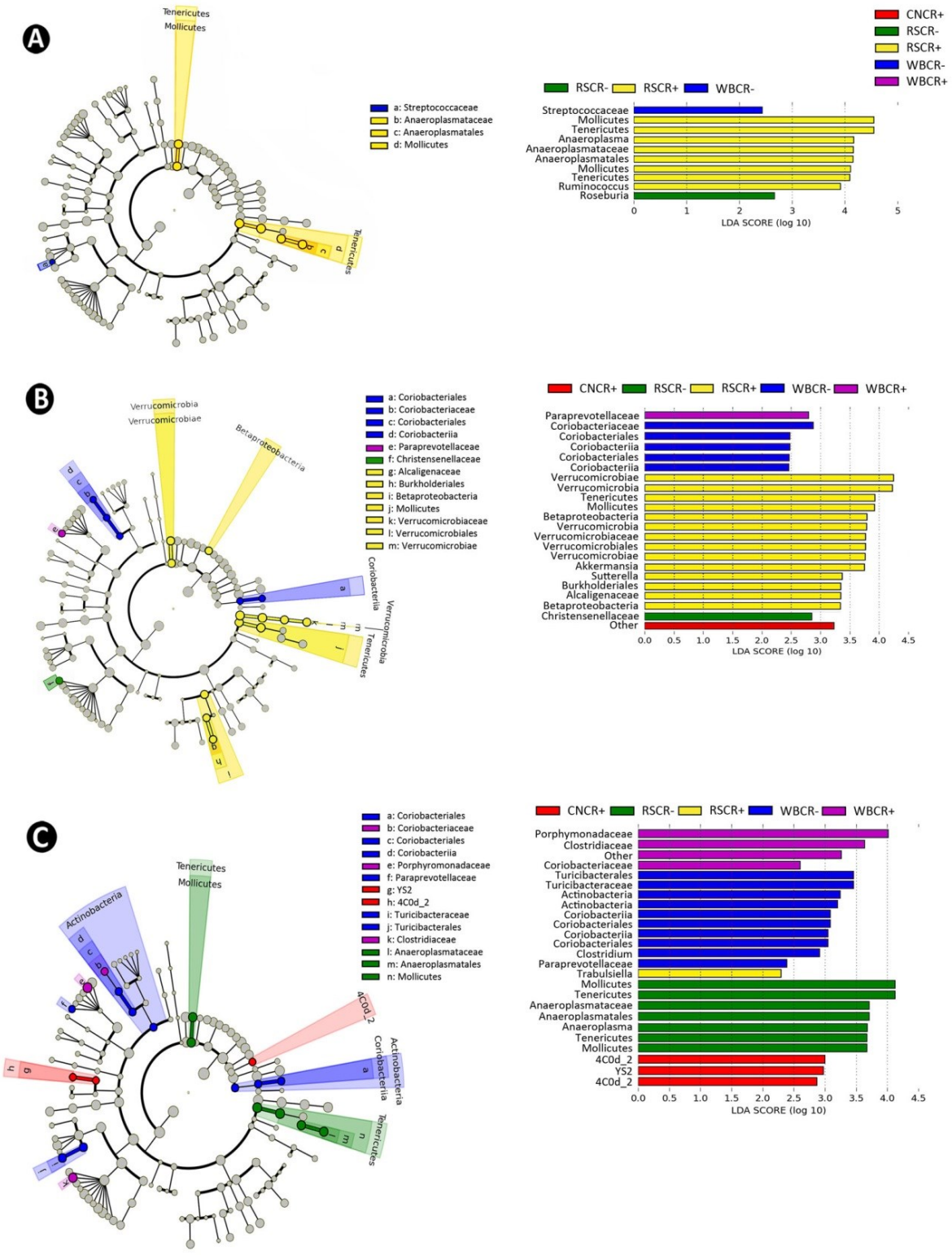

Figure 3.14 Cladograms identifying bacterial taxa that are differentially abundant and biologically consistent in mice gavaged with PBS (CR-) and inoculated with *C. rodentium* (CR+), comparing the differences between mice consuming a CN diet, or diets

enriched for WB or RS. **(A)** Distal colon mucosa-associated communities. **(B)** Proximal colon mucosa associated communities. **(C)** Cecal ingesta communities. Highlighted taxa represent significantly ($P \leq 0.050$) impactful communities within each treatment.

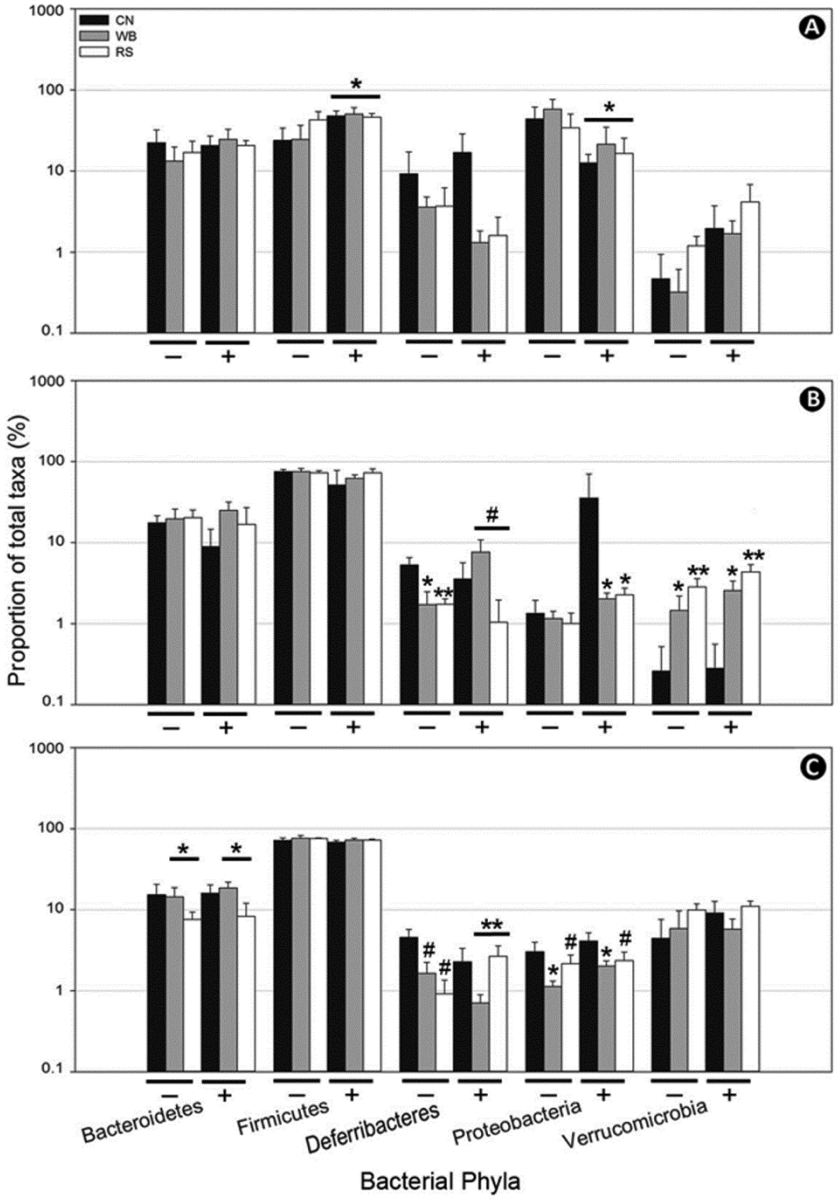

Figure 3.15 Proportion of bacterial taxa in the common phyla in mice gavaged with PBS (-) and inoculated with *C. rodentium* (+) and consuming a CN diet, or diets enriched for WB or RS. **(A)** Bacterial abundance in the distal colon mucosa-associated tissue. **(B)** Bacterial abundance in the proximal colon mucosa-associated tissue **(C)** Bacterial abundance in the cecal ingsta. Vertical lines associatedwith histogram bars represent the standard error of the mean (n=6). *Different ($P \leq 0.050$) relative to the CN treatment. #Different ($P \leq 0.100$) relative to the CN treatment. Horizontal bar represents more than one diet treatment being statistically different from the control diet, and in the distal

colon all diets in mice with enteritis being different from all diets in mice without enteritis.

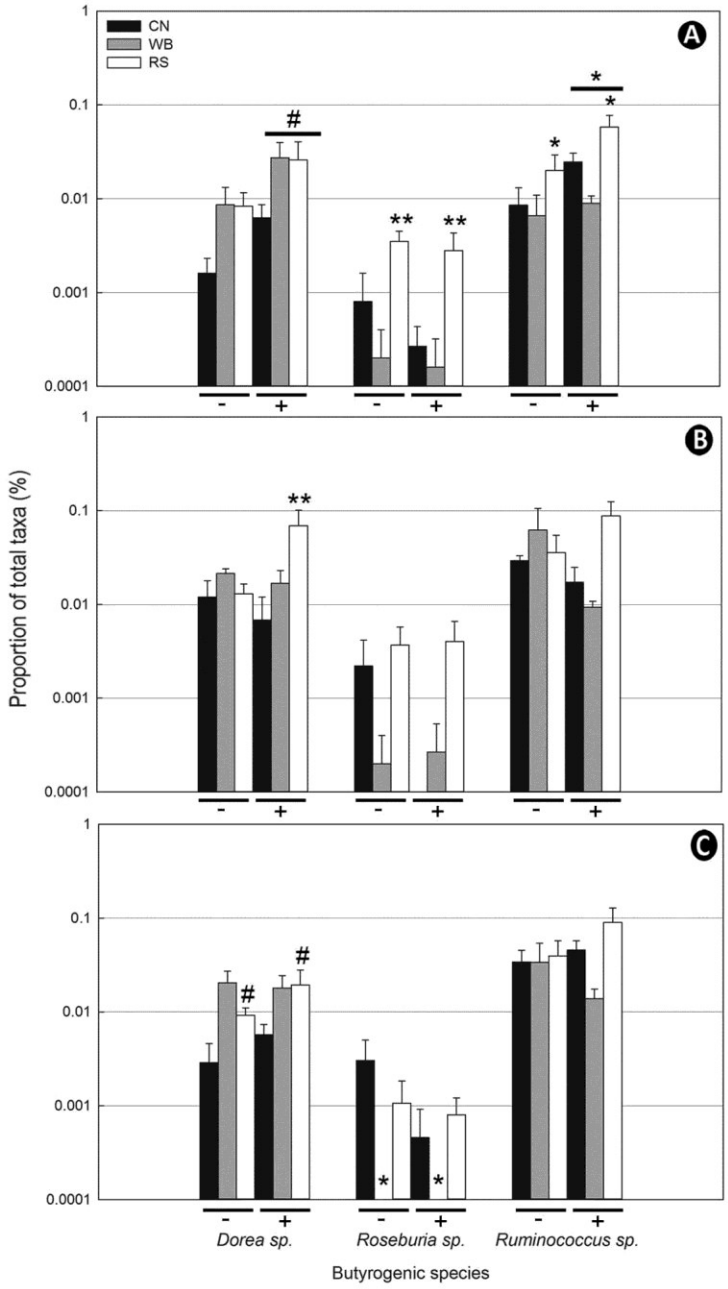

Figure 3.16 Proportional abundance of butyrogenic species in mice gavaged with PBS and inoculated with *C. rodentium* averaged over time. **(A)** Distal mucosa-associated. **(B)** Proximal mucosa-associated. **(C)** Cecal ingesta. *Different ($P \leq 0.05$) relative to the CN treatment. #Different ($P \leq 0.10$) relative to the CN treatment. **Different ($P \leq 0.010$) relative to the CN treatment.

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Chapter 4: General Discussion

Foods have a major role in maintaining 'good' health in Canadians, and food products such as DFs are important for maintaining intestinal health. Dietary fibres include RS and WB, and are known to provide beneficial metabolites to the intestine following the bacterial fermentation of complex carbohydrates (Bach Knudsen, 2015). One of the most influential fermentation by-products in the intestine is butyrate, as butyrate is readily metabolized into ATP as the main energy source for colonic epithelial cells (Topping and Clifton, 2001). Moreover, butyrate has also been associated with: modulating pro-inflammatory cytokine gene expression (Zimmerman et al., 2012); promoting ketone body synthesis (Guilloteau et al., 2010); effecting epithelial barrier function by regulating the production of tight junction proteins (Lewis et al., 2010); and enhancing the secretion of intestinal mucus (Brownlee et al., 2003). Previous research has examined SCFA produced from RS and WB fermentation, and the effect of butyrate on intestinal health and the host microbiome remains inconclusive. In particular, the impact of butyrate on mucus production and secretion using *in vitro* and *in vivo* pathogen-challenge models is inconsistent (Burger-van Paassen et al., 2009; Gaudier et al., 2009; Hamer et al., 2010). There are few studies that examine the beneficial effect of DFs and butyrate using animal models of acute enteritis induced by pathogenic bacteria. Therefore the aim of my study was to determine whether butyrate or the fermentation of RS and WB (i.e. SCFA production) could: 1) reduce tissue inflammation, 2) modulate pro-inflammatory gene expression, 3) promote epithelial repair and regeneration, 4) influence the production and section of mucus and 5) promote the growth of specific microbial communities that effect SCFA production and mucus secretion in the intestine using the *C. rodentium*-challenge model in C57BL/6 mice.

In Chapter 2, the effect of butyrate on intestinal health and the intestinal microbiome in mice was investigated. It was shown that within the inflamed intestine, mice administered butyrate at high concentrations had lower histological scores of inflammation, and it was also shown that high concentrations of butyrate expedited the clearance of *C. rodentium.* This was observed by the increased expression of Th1 and Th17 cytokines, enhanced tissue repair, and improved intestinal mucus secretion that was

dependent on butyrate concentrations. Infection with *C. rodentium* resulted in a robust expression of Th1and Th17 associated cytokines, which are necessary for pathogen clearance (Costa et al., 2011; Weaver et al., 2013). Similar to other studies, I observed elevated levels of epithelial cell hyperplasia and proliferation, in addition to increased expression of *TNFα, IL-1β, IL-17A* and *IL-22* in the distal colon of mice administered high concentrations of butyrate (Costa et al., 2011; Luperchio and Schauer, 2001). A butyrate dependent increase in regulatory cytokines (*IL-10* and *TGFβ*) was also observed, indicating that butyrate supplementation promoted the clearance of *C. rodentium* induced inflammation, and assisted in recovery to intestinal homeostasis and quiescence of an inflamed gut. As well as an increase in cell proliferation, it was also observed that an increase in the expression of genes involved in tissue repair and restitution, including *Tff3*, *Relmβ*, and *Myd88* occurred. Notably, TFF3 and RELMβ are proteins secreted from goblet cells that facilitate epithelial cell regeneration (Nair et al., 2008), and together maintain homeostasis by initiating repair during periods of inflammation (Kim and Ho, 2010; Kindon et al., 1995; Krimi et al., 2008). MYD88 is involved in the enterocyte caspase-1 inflammasome signaling pathway, and it is a required component for intestinal tissue repair (Saleh and Trinchieri, 2011). Moreover, there was also an increase in *Muc2* gene expression, and an increase in mucin present within goblet cells and the intestinal lumen in butyrate administered mice. In as such, this research demonstrated that butyrate administration reduced intestinal inflammation, enhanced tissue repair and improved clearance of *C. rodentium* in mice.

In Chapter 2, I also demonstrated an increase in the presence of bacteria associated with intestinal mucus. As an example, I observed a trend of increased *A. muciniphila* growth during periods of inflammation, and notably this bacterium has been linked to the increased expression of host mucus and immune modulating genes within the intestine (Everard et al., 2013). This observation suggests that certain bacteria may induce mucin production and secretion during periods of colitis. An observed increase in the abundance of mucus-associated species such as *A. muciniphila* further support my study hypothesis that butyrate improves host health through enhanced mucus secretion by modulating intestinal bacteria populations. Butyrate administration enhanced the growth of bacteria that produce butyrate within the intestine as well. The addition of high concentrations of

butyrate to colons in *C. rodentium*-challenged mice resulted in the selection of *Lachnospiraceae*, as well as other butyrogenic mucus-associated species namely *Coprococcus* spp., *Roseburia* spp., and *Butyrivibrio* spp., (Meehan and Beiko, 2014). Furthermore, studies have shown that *C. rodentium* reduces the diversity and abundance of various intestinal bacterial communities (Hoffmann et al., 2009). In this study, select *Firmicutes* bacterial populations were increased, despite the reduction in total number of *Firmicutes* bacteria. These observations suggest that butyrate administration to inflamed colons stimulates the growth of bacterial species that produce butyrate and stimulate mucus production in the intestine.

To summarize Chapter 2, the activation of coordinated physiological responses following treatment with high concentrations of butyrate reduced *C. rodentium* induced intestinal inflammation. This occurred through a coordinated Th1/Th17 host immune response that cleared infection. Butyrate administration also increased regulatory T-cell response cytokines, and facilitated the increase of mucus secretion. Importantly, mucus not only provides a physical barrier to limit pathogen invasion (Petersson et al., 2011); but mucus-associated proteins are also involved in epithelial cell repair and restoration. The administration of butyrate in high concentrations also promoted the growth of butyrogenic bacteria within the intestine, and increased the presence of bacterial species involved with mucus colonization and mucus metabolism, a process that could lead to the improvement of host intestinal health. Collectively, my research provides evidence that butyrate supplementation at high concentrations is beneficial to host intestinal health, and may be useful as a therapeutic agent to reduce intestinal inflammation.

In Chapter 3, I examined the effects of diets containing RS or WB on tissue inflammation, the expression of cytokines, mucus production, and changes to species populations within the microbiome. It was found that feeding diets rich in WB reduced the amount of *C. rodentium* shed in feces during late stage of infection, and had a greater impact in reducing the severity of disease than mice fed CN diets or diets rich in RS. I also observed that the consumption of DF reduced measures of inflammation in the proximal colon and mice fed WB had consistently lower scores of inflammation in this region. Although histological scores of inflammation within the distal colon were not

statistically different, mice fed diets containing WB showed a biological trend of lower histological scores of inflammation as compared to mice fed CN diets. Diets rich in oligosaccharides can reduce pathogen attachment and colonization in the intestine, and since WB contains arabinoxylan oligosaccharides (Francois et al., 2014), it is possible that these oligosaccharides were responsible for lower histological scores in *C. rodentium*-challenged mice. Mice fed diets fortified with WB also demonstrated reduced expression of pro-inflammatory Th1 (*IL-1β*) and Th17 (*IL-17A*) cytokines and genes involved in host innate defense (*TLR4*), and mucosal barrier function (*RegIIIγ*) in mice with enteritis (Figure 3.11). These observations provide evidence that infection was reduced in mice fed diets enriched for WB as compared to mice fed CN diets. In mice fed RS fortified diets, the expression of *TLR4,* a protein involved in recognition of bacterial lipopolysaccharides was elevated, and *Relmβ,* a protein involved in cellular repair was significantly increased. Collectively the histological scores suggested that DFs were more effective at reducing inflammation in the proximal colon than in the distal colon. Importantly, I did not measure mRNA gene expression of proteins involved in inflammation and cellular repair within the proximal colon, and as such extrapolation of cellular and immunological events from the distal colon to proximal colon are presumptive and must be considered with caution.

In Chapter 3, I also observed that DF fermentation increased SCFA production in the murine cecum, proximal colon and distal colon. In mice treated with either PBS or challenged with *C. rodentium* and fed WB or RS-rich diets, total SCFA concentrations were significantly increased in comparison to mice fed the CN diet. There were notable differences in SCFA production within the intestinal segments as well. The proximal colon is known to have the highest rates of SCFA absorption in the large intestine (Wong et al., 2006), and interestingly, the lowest concentrations of SCFA were observed in the proximal colon. The rapid absorption of SCFA may assist in ameliorating tissue injury and inflammation, as histological scores were generally lower in the proximal colon (Blottiere et al., 2003; Butzner et al., 1996; Tedelind et al., 2007; Topping and Clifton, 2001). In mice challenged with *C. rodentium*, the distal colon showed the greatest difference in total concentrations of SCFA while there were no changes in SCFA concentrations present in the cecum and proximal due to infection.

In the previous chapter (Chapter 2), it was demonstrated that mice treated with butyrate exhibited a significant increase in mucus secretion and *Muc2* expression. In Chapter 3 and in contrast, I observed that mice challenged with *C. rodentium* and fed WB fortified diets had modest increases in the presence of mucus within goblet cells and the intestinal lumen. Furthermore, bacterial populations in the intestinal microbiome and along the large intestine were altered dependent on the type of DF fed to mice. The growth of microbial communities differed based on both the anatomical location of the intestine (i.e. cecum, proximal colon, distal colon) and the chemical form of the DF consumed. I believe that increases in the various SCFA following DF fermentation could have induced differential changes in microbial communities. Although the intestinal pH was not measured in this experiment, it is possible that increases in intestinal SCFA subsequently reduce intestinal pH, a condition that is favorable for the growth of butyrogenic bacteria within the intestine (Walker et al., 2005). As examples of changes in bacterial population within the gut, I observed increases in the diversity of *Clostridiales* species in the cecum and proximal colons of mice fed RS and WB diets. In contrast, mice fed diets rich in RS increased the growth of *Ruminoccocus* spp. and *Roseburia* spp. in mice with and without enteritis. The proximal colon also exhibited an increase in the abundance of mucus-associated bacteria such as *A. muciniphila* in mice fed RS diets. This observation may correlate to an increase in mucus production of the proximal colon of mice as the growth of *A. muciniphila* is dependent on a rich supply of intestinal mucus (Derrien et al., 2011; Morel et al., 2005). Furthermore mice consuming RS diets also increased the populations of bacterial species that are associated with 'good' health in people. As an example, *Christensenella minuta* increased in mice fed RS, and this bacterium is associated with maintaining a lean body mass index in people (Dao et al., 2016; Goodrich et al., 2014; Rajilic-Stojanovic et al., 2013). Overall, it is apparent that the consumption of DF influences the growth of bacteria that may be beneficial to host health.

To summarize the findings from Chapter 3, my data suggests that the consumption of DF improves intestinal health, and these benefits are more prominent in the non-inflamed and mildly inflamed intestine. Mice fed diets containing WB and RS had increased concentrations of intestinal SCFA, and diets rich in WB produced more butyrate

following bacterial fermentation and induced significant growth of mucus-associated bacterial species. I believe these changes were linked to the general trend of reduced intestinal injury in *C. rodentium* infected mice. It was found that DF altered bacterial populations within the intestinal microbiome. In particular, within segments of the intestine with high SCFA concentrations, changes to bacterial species diversity and abundance were more conspicuous. Therefore the consumption of RS and WB in individuals with non-inflamed intestines may perhaps be better in the maintenance of physiological and immunologic homeostasis and a healthy intestine. In this study, diets enriched with RS and WB regulated weight gain, increased the production of intestinal SCFA, and promoted the growth of bacterial species that are associated with maintaining a healthy intestine. Furthermore, mice fed diets enriched for RS and WB exhibited signs of improved health. In conclusion, the observations from Chapters 2 and 3 suggest that the administration of butyrate and the consumption of diets rich in RS and WB are beneficial to help maintain a healthy intestine by influencing both intestinal function and bacterial populations within the intestinal microbiome.

To expand my knowledge of the functions of SCFA during infection within the colon, there are a number of other research areas that can be explored. The first research area would investigate the difference between secreted mucus, and mucus that accumulates in the lumen due to epithelial cell turnover and mucosal sloughing. At present, the general mechanisms of mucus secretion are still poorly understood, although it has been suggested that goblet cells induce autophagy and cytolytic pathways that cause the release of mucus at the epithelial surface (Wlodarska et al., 2014). A second study could examine the mechanisms involved in mucus secretion that allow secretion to occur without disrupting the tightly-adhered glycocalyx. The architectural structure of the epithelial surface suggests that mucus is only released at specific locations along the epithelium; a phenomena important to ensure the structural integrity of the tightly-adhered glycocalyx. A third study would investigate processes involved in the absorption of SCFA within intestinal cells. Although, it is well known that SCFA are rapidly metabolized within cells, it was determined that it is difficult to differentiate between SCFA that are absorbed and metabolized by the enterocytes, and SCFA metabolized by bacterial communities. A fourth study would discern whether the fermentation of

different forms of DF that produce varying amounts SCFA throughout the intestine contributes to the growth of intestinal bacterial populations, or whether the bacterial populations contribute to increased total amounts of SCFA within the intestine. Collectively, enhancing the understanding of the cellular, microbial, and systemic effects (i.e. metabolism in other tissues) of SCFA would further enable researchers to use SCFA as potential therapeutic agents. Identifying the effects of butyrate, RS, and WB on ameliorating intestinal inflammation was an important aspect of the research. A fifth study would augment my understanding of the disease process, and additional measurements within inflamed tissue could be performed. These include examining the expression of additional genes involved in inflammation, SCFA absorption, and mucus secretion pathways. As examples: 1) NO synthase expression could confirm TNFα function and the activation of macrophages involved in tissue inflammation; 2) analyzing the expression of *MCT1*, and G-coupled protein receptors (i.e. *GPR43* and *GPR41*) would aid in the study of the activity of SCFA absorption; 3) and finally the expression NLRP6 could be used to determine goblet cell autophagy activity and mucin secretion. A sixth study, would examine serum cholesterol and triglyceride levels, as this would provide more information on the nutritional benefits of SCFA following butyrate treatment and the fermentation of diets rich RS and WB. Finally, a continuation of this present study could examine the effects of RS and WB on mucus production in $MUC2^{-/-}$ knockout mic models. This would determine whether other mucins are involved in mucus production in butyrate and DF treated mice challenged with *C. rodentium*.

In conclusion, these investigations demonstrate that butyrate, RS and WB reduce intestinal inflammation in mice and help maintain a healthy intestine. Importantly, this research provides evidence that these products may be effective compounds to attenuate intestinal disease. Many individuals suffer from intestinal diseases, and although antibiotics and immunosuppressive drugs therapies can be beneficial, the mechanisms involved in the induction and progression of these intestinal maladies still remain unknown. Many intestinal diseases do not have single etiology, and are not induced by a single activator of disease (Jiminez et al., 2015). Moreover, the manifestation of intestinal disease is often associated with a combination of dysregulation of the pro-inflammatory immune responses, changes to the host microbiome and the effects of environmental

factors such as diet (Jiminez et al., 2015). My research demonstrated that SCFA, and in particular butyrate, can reduce intestinal inflammation, and improve intestinal health and the general health of the host. Moreover, the research suggests that SCFA could be added to the armamentarium of treatments of intestinal disease. Indeed, SCFA could be an ancillary treatment in combination with classical treatments for intestinal inflammation, namely antimicrobial and immunosuppressive drugs, and at present, this research suggests that diets rich in RS and WB could be used to reduce intestinal inflammation. This is underscored by the activity of DF and the fermentation by-product butyrate to effect: immune responses; intestinal inflammation; cell repair; mucus secretion; and growth of bacterial populations in the *C. rodentium*-challenged animal model. In conclusion, I believe this research provides evidence supporting the health claims that DF in food can be beneficial for improving intestinal health in people.

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Appendices

Appendix 1: The histological scoring parameters for mouse colonic tissue following treatment with *C. rodentium*.
The maximum score for damage is 22.

Histological Category	0	1	2	3	4
Epithelial cell wall hyperplasia	None	Mild increase, mucosal crypt columns 2x the normal amount off cells, focal areas of hyperplasia	Moderate increase, mucosal crypt columns contain 3x the normal amount of cells, crowding of cells in base of crypts	Marked increase, mucosal crypt columns contain 4x the normal amount of cells, crowding of cells in base of crypts	Severe increase, villous distortion, mucosal crypts contain more than 5x the normal amount of cells, focal dysplasia of epithelium surface
Crypt height	Normal	Mild increase, 25% increase crypt height	Moderate increase, 50% crypt height	Marked increase, 100% increase crypt height	Severe increase, >100% increase crypt height
Epithelial cell injury	None	Superficial; mild <10 surface epithelial cells shedding	Moderate, focal erosions, 11-20 surface epithelial cells shedding	Marked, multi-focal erosions of surface epithelial cells	Severe, multifocal erosions with or without deep crypt necrosis
Inflammation	None	Rare numbers of neutrophils and or mononuclear cells present within the lamina propria	Small numbers of neutrophils and or mononuclear cells present within the lamina propria	Large numbers of neutrophils and or mononuclear cells within the lamina propria that on occasion focal to multifocally extend into submucosa, muscularis and serosa	Large numbers of neutrophils and or mononuclear cells within extensive areas of the lamina propria, submucosa, muscularis and serosa
Goblet Cell Depletion	Normal	Low depletion of goblet cells, smaller size of mucin droplets	Evident depletion of goblet cells, marked decrease of the size of mucin droplets	Absent	Not applicable
Mitotic Activity	Normal	Mild, small increase in mitotic activity in deep crypt epithelial cells	Moderate, prominent increase in mitotic activity in the basal half of crypt epithelial cells	Marked, prominent increase in mitotic activity that extends the entire length of crypt epithelial cells	Not applicable

Appendix 2: Gene targets and primer sequences used to analyse the gene expression of cDNA harvested from murine colonic tissue for mRNA analysis in both studies.
†Primer sequences developed from PCR arrays specific for butyrate induced inflammation. *Primers developed for this study using the NCBI database.

Primer	Sequence (5' to 3')
TNFα*	F:GATCGGTCCCCAAAGGGATG R: GCTCCTCCACTTGGTGGTTT
TGFβ*	F: GTCCAAACTAAGGCTCGCCA R: CATAGTAGTCCGCTTCGGGC
Reg3γ*	F: GATGCCCCATCTTCACGTAG R: ACAAGGCATAGCAATAGGAGC
Myd88*	F: ATCGCTGTTCTTGAACCCTC R: CCAAGTACTCGAAGCCCATC
Muc2*	F: AAAGACCACAACAGGGCCAA R: GGTCCTGGTGGTCTCCAAAG
IL-10*	F: ACAGCCGGGAAGACAATAAC R: GGCAACCCAAGTAACCCTTA
IL-4*	F: AGCAACGAAGAACACCACAG R: TCGAAAAGCCCGAAAGAGTC
IL-17A*	F: GCAGCGATCATCCCTCAAAG R: ACGTGGAACGGTTGAGGTAG
IL-1β*	F: GTGTCTTTCCCGTGGACCTT R: GGAGCCTGTAGTGCAGTTGT
IL-22*	F: TGACACTTGTGCGATCTCTGA R: CTTGCACCGGGTGTTGACG
IFNγ (1)	F: ACGGCACAGTCATTGAAAGC R: TCTGGCTCTGCAGGATTTTCA
TLR2*	F: GCTCCTGCGAACTCCTATCC R: CAGCAGACTCCAGACACCAG
TLR4	F:5'GGCAACTTGGACCTGAGGAG R:5'TTCCTTCTGCCCGGTAAGGT
TLR9*	F: CAGTTGCCGACTGGGTGTAT R: GAGTCTTGCGGCTCCCATAG
Tff3*	F: TCTGGCTAATGCTGTTGGTG R: ATACATTGGCTTGGAGACAGG
Prg3†	F: AGCATAGAAGCTGCGTTGGA R: GATCAGGGGCTGTTTCATGT
Relmβ*	F: TCTCAGTCGTCAAGAGCCTAA R: AAGCACATCCAGTGACAACC
Ltb4r1†	F: AAACCCTGTCCTTTTGATGGC R: AGAACAATGGGCAACAGAGA
IL-23A*	F:5'AATGTGCCCCGTATCCAGTG, R:5'GCAGGCTCCCCTTTGAAGAT

1. Ramos-Payán R, Aguilar-Medina M, Estrada-Parra S, González-y-Merchand JA, Favila-Castillo L, Monroy-Ostria A, and Estrada-Garcia ICE**.** Quantification of cytokine gene expression using an economical real-time polymerase chain reaction method based on SYBR® Green I. *Scand J Immunol* 57: 439-445, 200.