Understanding the role of dietary phytochemicals and vitamin B12 in host-microbe interactions to support host gut integrity and health

by

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ABSTRACT

Diet is a strong modifier of gut host-microbe interactions that alter host physiology and immunity against pathogenic bacteria. Some dietary components are required to support host defences that maintain gut homeostasis and symbiosis, whereas others can be detrimental, leading to changes in microbial communities and impaired intestinal barrier function and immunity. In this thesis, a mouse model of infectious *Citrobacter rodentium* was used to challenge the effects of phytochemical and vitamin B12 consumption on intestinal ecology and integrity to promote health.

The fiber and phytochemical content in the seed coat of peas (*Pisum sativum*) has been associated with beneficial health outcomes, including weight and cardiovascular health; however, the indirect effects of peas by host-microbe interactions remain poorly understood. To determine the effect of pea phytochemicals and fiber on host-microbe interactions and intestinal health, the seed coat of two cultivars of peas rich and poor in proanthocyanidins were fed to mice as raw or acid hydrolyzed fractions. In accordance with a previous study, the acid hydrolyzed anthocyanidin fraction reduced weight gain in mice fed a high fat diet. Supplementation of both pea seed coat fractions altered the microbial communities and encouraged pathogen colonization by day three post-infection; however, the proanthocyanidin containing diet had a more robust antimicrobial affect and consistently led to higher pathogen loads as determined by fecal enumeration. Acid hydrolysis processing to both pea fractions reduced the effects on the microbiota and ability of *C. rodentium* to colonize the gut. In addition, pea phytochemicals increased mucin accumulation in the intestinal lumen, and this may have contributed to the improved ability of *C. rodentium* to colonize the gut. This study shows how pea phytochemicals

directly contributes to microbial ecology and provides insight into how their antimicrobial and mucin accumulating activities affect the gut environment and pathogen colonization resistance.

The effect of mucin accumulation in the gastrointestinal lumen in response to phytochemicals has previously been associated with beneficial health outcomes. Since our study shows that increased mucin corresponded with higher levels of *C. rodentium* colonization, we set out to determine the contributions of mucin to gut ecology and the dietary phytochemicals that stimulate their effects in the gut. Germ-free mice fed the proanthocyanidin-rich containing fraction stimulated mucin accumulation in the feces, indicating that phytochemicals directly impact the mucus layer independently of the microbiota. Supplementing both the red-osier dogwood extract, a hydrolysable tannin, and our non-hydrolysable proanthocyanidin-rich pea fraction led to greater mucin levels in the feces of conventional mice compared to control. The increase in mucin corresponded to an enrichment in *Lachnospiraceae* and *Clostridium leptum* species and a reduction in *Romboutsia* species in the colon. This study provides insight into how dietary phytochemicals impact specific members of the Firmicutes population and shows that a common compound is likely directing the increased fecal mucin phenotype independently of the gut microbiota.

Vitamin B12 is a known modulator of the microbial ecosystem. To determine how B12 impacts the gastrointestinal microbiota, we supplemented it in drinking water at 100 times the amount found in diet and challenged mice with *C. rodentium*. Survival and early colonization models show that mice supplemented B12 were more susceptible to pathogen colonization and virulence. Cecal meta-transcriptomics revealed that the activities of the Firmicutes population was altered by B12 supplementation and this contributed to a more virulent *C. rodentium* population as confirmed by reduced glucosidase activity and increased virulence genes. In

addition, host interleukin-12p40 cytokine levels were higher from B12 supplementation prior to infection and was determined to be dependent on the microbiota.

Collectively, this thesis adds to our understanding of diet-microbe-host interactions that impact intestinal integrity as to improve nutritional strategies and therapies to combat infectious disease and improve health.

PREFACE

This thesis is an original work by Andrew Forgie.

Chapter 1 has been published as Forgie AJ, Fouhse JM, Willing BP. Diet-microbe-host interactions that affect gut mucosal integrity and infection resistance. Front Immunol. 2019;10:1802. AF planned and wrote the review with support from JF on the fiber section. JF edited the review. BW supervised, edited, and approved the review.

The study in Chapter 2 has been published as Forgie AJ, Gao Y, Ju T, Pepin DM, Yang K, Gänzle MG, et al. Pea polyphenolics and hydrolysis processing alter microbial community structure and early pathogen colonization in mice. J Nutr Biochem [Internet]. 2019 May 8;67:101–10. Initial studies were designed and conducted by YG and BW. AF designed, conducted follow up experiments, collected and analyzed the data, and wrote the manuscript. DP and TJ helped with experiments and data collection. All authors edited and approved for publication. BW supervised the study.

The study in Chapter 3 is in preparation for publishing as Forgie AJ, Ju T, Tollenaar S, Willing BP. 2021. Short Communication: Phytochemical-induced mucin accumulation in the gastrointestinal lumen is independent of the microbiota. AF designed, conducted the experiment, collected and analyzed the data, and wrote the manuscript. All authors edited and approved for publication. BW supervised the study.

The study in Chapter 4 was designed by AJ Forgie, DM Pepin, BP Willing. The manuscript is in preparation as Forgie AJ, Pepin DM, Ju T, Tollenaar S, Sergi CM, Gruenheid S, and Willing BP. 2021. Excessive oral intake of cobalamins alters microbe-host-gut interactions that stimulate *Citrobacter rodentium* growth and virulence in mice. SG and BW collaborated on the original design of the experiment. DP and BW designed and conducted pilot and initial

studies. AF designed and conducted subsequent experiments, collected and analyzed the data, and wrote the manuscript. TJ and ST helped with the animal trials. CS conducted the histopathology scoring of intestinal tissues. All authors edited and approved for publication. BW supervised the study.

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1.1 Introduction

Infectious enteric diseases are a major cause of morbidity and mortality worldwide and are of particular concern in hospital settings and developing countries. According to the World Health Organization, infectious enteric diseases are one of the top ten causes of death leading to over two billion cases and one million deaths worldwide in 2010 (Kirk et al., 2015). Host resistance towards invading pathogens requires tight regulation of the gastrointestinal environment, maintained through a synergistic relationship between the host immune system and microbiome. Disruption to a host's intestinal homeostasis, including insults from diet, stress, antibiotic and drug treatment, allergies, cancer, and related illnesses can leave the host vulnerable to enteric pathogens (Stecher, 2015). It is well understood that diet can play a major role on health by positively and negatively shaping gastrointestinal ecology (Byndloss et al., 2018; R. K. Singh et al., 2017), and therefore should be a major focus in mitigating the severity of infection.

Although humans have successfully reduced pathogen exposure through effective sanitation practices, the adoption of a "Western diet", over-sanitation and lack of physical exercise are hypothesized to have contributed to the rise in autoimmune disorders (Manzel et al., 2014). The "Western diet" is characterized by the excessive consumption of fats, proteins, refined sugar and low intake of dietary fiber. Other dietary patterns such as the Mediterranean, Vegetarian-based, Japanese-based, and Ketogenic type diets can positively regulate immune responsiveness to reduce immune activity and support health (Soldati et al., 2018). However, human epidemiology studies on diet tend to exclude important interindividual variations that

govern the gastrointestinal microbiota and may explain the diverse claims to which foods are known as ‘protective’ and ‘harmful’ (Ananthakrishnan, 2015). Establishing a mechanistic link between individual diet components using host-microbe interactions will aid to provide evidence driven recommendations to help control an overactive immune response.

An overactive immune system is associated with autoimmune disorders such as inflammatory bowel disease (IBD) that affects host immune activity and leads to increased incidence of infection (Hong et al., 2017; H. Singh et al., 2017). Likewise, “westernized diets” have shown to enhance *Escherichia coli* colonization and associated inflammation in mice by altering the host mucus layer, increasing intestinal permeability, and impairing immune function (Martinez-Medina et al., 2014). Dietary fiber and other microbiota-accessible carbohydrates (MACs) are a key component missing from the “westernized diet” that when re-introduced provides a beneficial balance to host health and microbiome (Hryckowian et al., 2018a). Fiber is exhaustively studied as a microbial fermentation substrate that produces short chain fatty acids (SCFA(s)) with known benefits to host intestinal homeostasis and health (Makki et al., 2018b). However, we fear that this focus on the beneficial effects of fiber associated SCFA production has led researchers to overlook other common dietary components that may positively or negatively influence the host gastrointestinal environment and health.

Diet intervention should be considered a valuable tool to manipulate the host-microbe axis to help sustain intestinal homeostasis and infection resistance. Dietary components such as carbohydrates, lipids, proteins, phytochemicals, minerals and vitamins all have unique structural and chemical (physicochemical) properties that influence host pathogen resistance directly and indirectly through the microbiome. Bridging the gap between diet, host, and microbiome as they relate to immunity and disease resistance is a multifaceted field that requires an understanding of

their combined effects on intestinal homeostasis (Figure 1.1). This review explores the role of common dietary components on host-microbe interactions that modulate host resistance and tolerance towards common infectious diseases. We highlight the opportunity to improve outcomes, yet recognize the current knowledge limits the ability to provide concrete dietary advice. This is partially limited by the fact that diet focused infection resistance research is scarce and difficult to translate to humans.

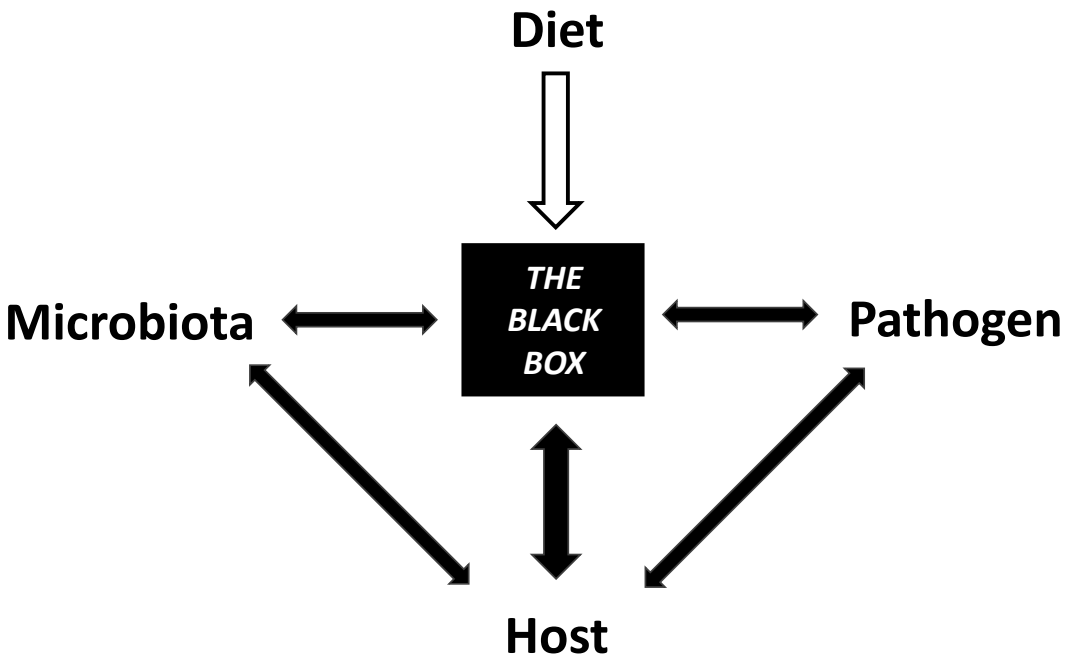


Figure 1.1: Diet contributes to a black box of intertwined mechanisms between the microbiota, host and pathogen that have yet to be elucidated.

1.2 GALT and microbiome regulate host defenses

The gut associated lymphoid tissue (GALT) plays a crucial role in regulating intestinal homeostasis and is composed of lymph nodes, lamina propria, and epithelial cells that together provide the host with a protective barrier and immune defence against invading pathogens (Forchielli & Walker, 2005). On the other hand, the microbiota provides a physical presence that can directly prevent pathogen colonization by competing for attachment sites or nutrient resources. Indirectly, the microbiota helps to improve host resistance by modulating intestinal integrity through the mucus layer, tight junction proteins, and antimicrobial peptides (AMPs: cathelicidins, C-type lectins and defensins) (Anhê, Varin, et al., 2015; Dudek-Wicher et al., 2018). Mucins secreted by goblet cells provide the first line of defense by forming a physical barrier composed of highly glycosylated and interlinked proteins between luminal bacteria and host epithelial cells (Dupont et al., 2014). The loose layer of the mucosa provides lubricant and is metabolized by mucin-degrading (mucolytic) bacteria (Sicard et al., 2017), whereas the adherent layer, when properly formed, secures a balance of host AMPs and immune factors that maintain intestinal homeostasis (Antoni et al., 2013).

Disruptions to the balanced microbial ecosystem greatly increase a host's vulnerability to infection (Willing, Russell, et al., 2011). In particular, antibiotic exposure can cause major shifts in microbial communities leading to mucus layer thinning, predisposing and exacerbating infections, as shown with antibiotic accompanied *Citrobacter rodentium* infections in mice (M. Wlodarska et al., 2011). Antibiotic-induced microbiota imbalances are well documented to alter the production of AMPs, tight junction proteins, and immune factors that normally contribute to intestinal homeostasis and infection resistance (Cash, 2006; Menendez et al., 2013). Secretory immunoglobulin A (SIgA) antibodies are abundant immune factors of the intestinal lumen that

protect epithelial cells from enteric pathogens and toxins by blocking their access to epithelial receptors and entrapping them in mucus to promote clearance (Mantis et al., 2011). Although SIgA targets and disrupts pathogens and antigens, commensal microbes such as *Bacteroides fragilis* alter their surface proteins to attract SIgA to enhance mucosal colonization (G. P. Donaldson et al., 2018). Intestinal epithelial cells (IECs) produce reactive oxygen species (ROS)(Knaus et al., 2017) and Resistin-like molecules (e.g. RELMβ)(Pine et al., 2018) that hinder commensal and pathogenic bacteria colonization, further maintaining intestinal homeostasis. IECs apical surface fucosylation is another useful host strategy that controls commensal microbes and inhibits pathogens. Secreted fucose is metabolized by bacteria to produce bioactive metabolites, reduce virulence factors, and enrich beneficial gut microbes to strengthen colonization exclusion (Pickard & Chervonsky, 2015). Alternatively, fucose can be fermented by commensal microbes into 1,2-propanediol and utilized by *Salmonella* during inflammation to drive their fitness in the colon (Faber et al., 2017).

The host has significant control over microbial communities of the small and large intestine; however, this relationship is complex and is managed in part through gastric acid secretions, intestinal motility, bile secretions, oxygen gradients, and regulation of pattern recognition receptors (PPRs), such as Toll-like receptors (TLRs) (Byndloss et al., 2018). The host recognizes commensal bacteria through activation of TLRs and relays an appropriate response in accordance to the specific microbial derived ligands (e.g. peptidoglycan, lipoprotein, lipopolysaccharide (LPS), and flagellin) (Hug et al., 2018). Innate lymphoid cells (ILCs) have been identified as key immune regulatory cells of the GALT controlling pathogen resistance, inflammation, and metabolic homeostasis (Klose & Artis, 2016). ILCs concentrate within mucosal surfaces and relay signals sent between the microbiota, epithelia, immune cells, and

metabolites in the intestine to maintain epithelial barrier function. Transcriptomic analysis of 15 ILC subtypes revealed their regulatory functions depend on the presence of the microbiome, nutrients, and xenobiotics (Gury-BenAri et al., 2016). Ultimately, it is the combined relationship between the gut microbiota, host, and diet that help improve or worsen a host's ability to tolerate and resist pathogenic bacteria (Figure 1.2). The remainder of this review will focus on specific dietary components and how they stimulate some of these and other host-microbe interactions resulting in impaired or improved host disease resistance.

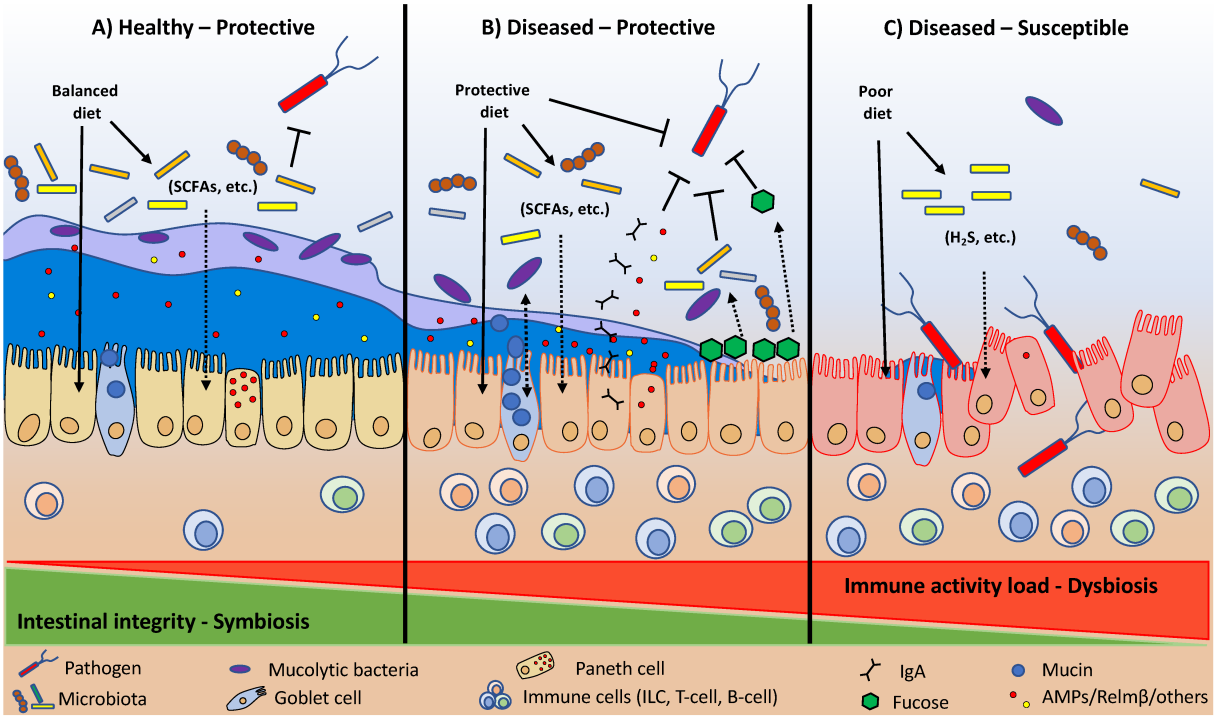


Figure 1.2: Diet and immune activity load (allergies, cancer, other illness, etc.) determine host intestinal integrity towards invading pathogens. Diet affects intestinal integrity directly by stimulating IECs, ILCs, and microbial communities, and indirectly through microbial fermentation by-products (SCFAs, H_2S, etc.). A healthy individual following a balanced diet to maintain symbiosis between host and microbial populations has enhanced intestinal integrity

with a thick inner and outer mucus layer that retains AMPs and other compounds to protect the host against pathogen colonization (A). A diseased host with heightened immune activity maintains symbiosis by consuming dietary components that protect and boost host innate defenses (IgA, AMPs, mucus, fucosylation) and adaptive immune responses to prevent pathogen colonization (B). Whereas diseased individuals with heightened immune activity consuming a poor diet are more susceptible to enteric infections due to impaired host defenses that cannot control the dysbiotic intestinal environment (C).

1.3 Carbohydrates

Dietary carbohydrates are often classified by their degree of polymerization into mono-, di-, oligo-, or poly- saccharides and composition of their monosaccharides: glucose, fructose, galactose, and xylose. Typically, carbohydrates are categorized as either digestible or indigestible (fiber). Binding and structural properties of carbohydrates dictate the glucosidase enzymes required to break bonds into their basic units for absorption (Goodman, 2010). The digestible carbohydrates escaping host small intestinal digestion, along with dietary fiber, become available as microbial energy substrates and are able to substantially alter the intestinal ecosystem and community structure (Desai et al., 2016).

Increasing intake of digestible carbohydrates has been scrutinized for contributing to the worldwide obesity and diabetes epidemics. However, carbohydrates are essential energy substrates for the central nervous system and red blood cells, are required to maintain cellular energy balance after sustained increases in metabolic activity, and to restore energy levels and glycogen stores (Mergenthaler et al., 2013). Humans and animals are able to regulate blood glucose levels; however, excessive dietary carbohydrate consumption can worsen acute

hyperglycemia, particularly during times of an illness (Ingels et al., 2018; Thaiss et al., 2018b) and stress (Marfella et al., 2001; van der Kooij et al., 2018). A medical illness can enhance the negative effects of acute hyperglycemia, which include inhibition of neutrophil migration, phagocytosis, superoxide production, and microbial killing, compromising host innate immunity against bacterial and fungal infections (Jafar et al., 2016). Diets high in simple and refined carbohydrates are shown to negatively impact gastrointestinal microbial communities leading to intestinal barrier dysfunction and greater risk for enteric infection (Thaiss et al., 2018b). Whereas balanced diets containing resistant starch and fiber stimulate microbial fermentation leading to a stable diverse microbiome and production of beneficial SCFAs (Martens et al., 2018). Understanding both negative and positive effects of carbohydrate consumption on gastrointestinal immunity and microbial populations will provide vital insight towards dietary strategies to help maintain pathogen resistance.

Dietary trehalose, a food component used to improve a product's texture, flavour, glycemic index and shelf life, was introduced in the early 2000's and has since been proposed to have contributed to the global *Clostridioides difficile* epidemic (J. Collins et al., 2018). Trehalose is a disaccharide composed of two glucose molecules linked by a resistant α,α - 1,1-glucosidic bond found in plants, algae, fungi, yeast, bacteria, insects, and other invertebrates (Richards et al., 2002). Mammals and other vertebrates lack the ability to synthesize trehalose, and the dietary fate of trehalose depends on the capacity of the small intestinal trehalase enzyme to hydrolyze it into glucose (Argüelles, 2014). Trehalase deficiency is rare in humans but excessive consumption of trehalose can lead to negative intestinal imbalances similar to those associated with lactose and fructose intolerances. Researchers believe the increased use of trehalose in food production has naturally selected for *C. difficile* with the capacity to metabolize trehalose more

efficiently, thus increasing pathogen fitness and contributing to their hypervirulent outbreaks in the human population (J. Collins et al., 2018). To combat reoccurring *C. difficile* infections a fecal microbial transplant (FMT) from a healthy donor has become a helpful treatment option; however, the mechanism of remission remains unclear (Baktash et al., 2018). The success of FMTs to treat *C. difficile* infections highlights the importance of a 'healthy' gut microbiome to promote infection resistance. Additional research is needed to confirm the impact of specific carbohydrates and their malabsorption on immune and microbial networks in the gut as it relates to pathogen fitness. Interestingly, studies in mice comparing fiber-rich and fiber-deprived diets support the detrimental effect of a simple carbohydrate dominated diet and the importance of fiber on infection resistance (Desai et al., 2016; Hryckowian et al., 2018b).

1.4 Dietary fiber

Health benefits associated with foods rich in non-digestible dietary fiber depend on their type, source, and proportion of water soluble and insoluble carbohydrate components (Eswaran et al., 2013). Fruits, vegetables, and grains are excellent sources of numerous fiber types; however, not all fiber sources and types are created equal. The food source, glycosylated chain structures and their fermentability, along with other inherent components are key parameters for their functional quality within the gastrointestinal tract (Makki et al., 2018a). Non-digestible carbohydrates are composed of monosaccharide units (glucose, fructose, galactose, xylose, fucose, and sialic acid) found naturally in plants, algae, fungi, bacteria, and mammalian milk, or produced by chemical or enzymatic processes (Belorkar & Gupta, 2016; Mussatto & Mancilha, 2007). Short chain fructo-oligosaccharides (FOS) have received a great deal of attention due to their prebiotic effects (S. P. Singh et al., 2017) and fact that they occur naturally (mostly as

inulin) with different degrees of polymerization in foods (Belorkar & Gupta, 2016). The consumption of prebiotic fibers have helped with diarrhea and constipation (Beleli et al., 2015; Souza et al., 2018; Suares & Ford, 2011); however, not everyone benefits from their consumption, and can even lead to excessive gas production, bloating and discomfort (Souza et al., 2018; J. Yang et al., 2012). In cases of gastrointestinal discomfort, a diet low in fructans (FODMAP-restricted diet) or reducing dietary fiber is often effective but remains controversial, and individualized (Eswaran et al., 2017; Ho et al., 2012; S. S. C. Rao et al., 2015).

The effects of various non-digestible fiber on health and microbiota is thoroughly reviewed (Eswaran et al., 2013; Makki et al., 2018b; Mussatto & Mancilha, 2007). In general, dietary fiber can modify gastrointestinal function directly through fecal bulking and indirectly through the modification of microbial community structure, and by increasing microbial biomass and fermentation products (Eswaran et al., 2013). Fiber fermentation leads to beneficial SCFAs (mainly acetate, propionate, and butyrate) but also undesired gases such as carbon dioxide, hydrogen, and methane (Cummings & Macfarlane, 1991). Increased gas production, fecal bulking and delayed gastric emptying can lead to discomfort, bloating, and flatus in many individuals (Eswaran et al., 2013). Microbial fermentation products such as SCFAs interact with the intestinal epithelium to promote certain defense mechanisms. In particular, microbial production of butyrate provides an energy substrate to epithelial cells (Hamer et al., 2008), maintains the hypoxic environment (Litvak et al., 2018), and promotes improved barrier function through hypoxia inducible factor (HIF) (Caleb J. Kelly et al., 2015). Induction of HIF transcription factor subsequently stimulates downstream signalling to increase mucus production (Louis et al., 2006) and expression of AMPs (C. J. Kelly et al., 2013) ultimately helping to minimize facultative pathogen growth.

According to the Global Burden of Diseases, Injuries and Risk Factors Study of 2015 infectious diarrhea is a leading cause of death globally among all ages (1.3 million deaths); with a large proportion of those occurring in infants under 5 years of age (499,000 deaths) (Troeger et al., 2017). Providing children with MACs is an important strategy to mitigate infection burden by stabilizing the microbiota and by bolstering intestinal immunity. Infants that are exclusively breast fed have reduced risk of developing diarrheal disease (Popkin et al., 1990), partially due to the naturally occurring human milk oligosaccharides (HMOs) present in breast milk. HMOs are soluble complex carbohydrates that act as prebiotics, providing a substrate for the intestinal microbiota and can prevent pathogenic bacterial adhesion through a variety of mechanisms (Le Doare et al., 2018). *In vitro* studies determined that HMOs act as pathogen decoy receptors to prevent infections and their activities depend on the location and degree of fucosylation (Craft et al., 2018). Human breast milk contains a multitude of other bioactive factors, immunoglobulins, cytokines, chemokines, growth factors, hormones, and lactoferrin which all likely contribute to the improved disease resistance of breast fed infants and is reviewed elsewhere (Andreas et al., 2015). Human milk has shown the ability to directly inhibit the adherence of *Streptococcus pneumonia* and *Haemophilus influenza* to human mucosal cells *ex vivo* (Andersson et al., 1986). When HMOs were fractioned, it was found that the acidic fraction had greater anti-adhesive properties towards enteropathogenic *Escherichia coli* (EPEC)*, Vibrio cholera,* and *Salmonella fyris* compared to the neutral high and low molecular weight fractions (Coppa et al., 2006). Similarly, HMOs blocked EPEC adherence to epithelial cells *in vitro* and reduced EPEC colonization in newborn mice, further implying the essential role HMO play in the prevention of infectious disease in human infants (Manthey et al., 2014). Experimentally, it was shown that supplementing formula with HMO reduced the duration of diarrhea in rotavirus-infected pigs and

promoted interferon-γ (IFNγ) and interleukin (IL) -10 expression in the ileum, suggesting HMOs may also protect infants against rotavirus infection (M. Li et al., 2014). Therefore, research efforts have focused on HMO substitutes that can be added to formula fed to infants that are unable to breastfeed. Human and animal studies suggest supplementing formula with fermentable fiber (e.g. soy polysaccharides, fructo- & galacto- oligosaccharide) reduces infection-associated diarrhea burden by improving intestinal homeostasis (Correa-Matos et al., 2003) and increasing beneficial *Bifidobacterium* species (K. H. Brown et al., 1993; Giovannini et al., 2014; Vanderhoof et al., 1997).

Minimizing infectious diarrheal disease with dietary tools has become the focus of recent research efforts. The importance of non-digestible fermentable fiber or microbiota-accessible carbohydrates intake in adults has clearly been shown where a greater intake (comparing top vs. bottom quartiles) reduced risk of death from cardiovascular, infectious, and respiratory disease by 24-56% in men and 34-59% in women (Park et al., 2011). Galacto-oligosaccharides (GOS) have shown to increase bifidobacteria and beneficially modulate immune function when supplemented to elderly volunteers. Along with improving phagocytosis and natural killer cell activity, the GOS supplemented volunteers had an anti-inflammatory cytokine profiles with increased IL-10 and reduced IL-1B, IL-6, and tumour necrosis factor alpha (TNFα) (Vulevic et al., 2008). In a double blind placebo controlled trial, those supplemented with GOS had reduced diarrhea incidence, duration, and severity (Drakoularakou et al., 2010). *Clostridioides difficile* is the leading cause of health care-associated diarrheal infections, commonly affecting the elderly and antibiotic treated hospitalized patients (Leffler & Lamont, 2015). Significant evidence suggests that the inclusion of soluble fiber to the diet, specifically MACs that increase SCFA production, may be a useful strategy to enhance infection resistance (Verspreet et al., 2016). In a

mouse model, dietary inclusion of MACs or inulin alone was shown to suppress *C. difficile* infection; whereas diets devoid of MACs exacerbated the infection (Hryckowian et al., 2018b). The mechanisms by which MACs help to mitigate *C. difficile* infection is through the expansion of fiber fermenting microbiota (via competitive exclusion) and subsequent increases in their immune-stimulatory metabolites (promote host defenses), which limit a pathogen's fitness (Hryckowian et al., 2018c).

β-glucans are one type of fermentable fiber that is frequently studied due to its common occurrence in the cell walls of yeast, fungi, and cereals such as barley and oats. Aside from acting as a microbial fermentation substrate, β-glucans are also of great interest for their direct effect on host immune activities and functions that alter immunity towards infections. In humans, the immune modulating property is due to the binding of β-glucans with host receptor dectin-1 (G. D. Brown & Gordon, 2001), which contributes to macrophages activation, and induce phagocytosis (Yun et al., 2003). Studies in mice found that oat derived β-glucans supplemented at 3 mg every other day stimulated a systemic immune response that reduced fecal oocyst shedding of *Eimeria vermiformis* by 39.6% post-challenge by increasing specific antibodies against the parasite (Yun et al., 2003). Oral administration of β-glucan from a fungal source (*Sclerotinia sclerotiorum* at 80 mg/kg every 2 or 3 days) was shown to directly stimulate proliferative responses of Peyer's patches to both T and B-cell mitogens, suggesting β-glucans may also stimulate a mucosal immune activation (Hashimoto et al., 1991). Intraperitoneal injection of β-glucans has also shown to work as a potent adjuvant to enhance host resistance to both bacterial(Yun et al., 2003) and parasitic (Leishmania) infections (Abid Obaid et al., 1989). The use of immunostimulants derived from naturally occurring polysaccharides (e.g. β-glucan or chitosan) has become somewhat commonplace in the aquaculture industry as an alternative

strategy for disease prevention. Inclusion of oligo-β-glucans (100-200 mg/kg) to striped catfish has shown to improve growth performance and reduce mortality post *Edwardsiella ictaluri* challenge via heightened phagocytic and lysozyme activity (Nguyen et al., 2017). The inclusion of dietary β-glucans (200 mg/kg) in poultry has also been used effectively to reduce the severity of necrotic enteritis when challenged with *Eimeria* and *C. perfringens* (X. Tian et al., 2016) and inhibited growth depression when challenged with *Salmonella* Enteritidis (Shao et al., 2016) by increasing specific antibody levels. In both cases, inclusion of dietary β-glucans reduced pathogen colonization (*C. perfringens* and *S.* Enteritidis).

Generally, increasing fiber will change the microbiome and improve gastrointestinal heath. As stated previously, the benefits associated from consuming food sources or supplements high in fiber is individualized and should be carefully monitored for side-effects.

1.5 Fats

Fats are an essential dietary macronutrient that have been criticized and are commonly avoided in developed countries with the objective of reducing weight, cholesterol levels, and cardiovascular disease risk. Fat avoidance and subsequent reliance on simple carbohydrates for caloric intake with reduced energy expenditure is believed to have contributed to the unintended rise of obesity worldwide (A. G. Liu et al., 2017). In healthy individuals most fats are emulsified and absorbed in the small intestine; however, in excess and during intestinal stress fats can travel towards the colon as a substrate for the microbiota (Agans et al., 2018). Human and animal studies have shown that intestinal microbes have the capacity to alter host homeostasis through a variety of metabolites, including carcinogenic and cytotoxic secondary bile acids (Ridlon et al., 2016). The effects of the microbiota on host homeostasis is through alteration to hepatic lipid and

bile metabolism, reverse cholesterol transport, energy expenditure, and insulin sensitivity in peripheral tissue (Ghazalpour et al., 2016). In this respect, dietary lipids are capable of directly affecting the host and microbiome, while indirectly altering host homeostasis through the microbiome and their metabolites.

The direct effect of microbial fat metabolism on intestinal health has yet to be established but studies have shown that dietary lipid profiles can alter the outcome of enteric infections. Fat consumption with regards to infection have been thoroughly reviewed elsewhere (Quin & Gibson, 2019), and provides a bases to establish the connection between microbe and host on enteric pathogen resistance. A study comparing dietary saturated (SFA, milk), monounsaturated (MUFA, olive oil) and polyunsaturated (PUFA, omega-6 corn oil) fatty acids uncovered distinct lipid mediated immune responses in mice after an acute *C. rodentium* challenge (DeCoffe et al., 2016). SFA and MUFA dominated diets induced protective T-regulatory cells, IL-10, IL-33 and SCFAs that helped mitigate inflammation during enteric infection (DeCoffe et al., 2016). Interestingly, in a dextran sodium sulfate (DSS) model, IL-10 knockout mice fed a diet containing milk SFAs, but not lard fat SFAs, resulted in a pro-inflammatory T_H1 immune response associated with a bloom of *Bilophila wadsworthia* and its metabolites, hydrogen sulphide and secondary bile acids (Devkota et al., 2012). Diets high in medium-chain SFAs like coconut oil have antifungal action towards *Candida albicans* (Gunsalus et al., 2016) and antibacterial properties against enteric pathogens (Shilling et al., 2013). Moreover, the addition of fish oil, high in omega-3 (n-3) fatty acids to a SFA dominated diet activated intestinal alkaline phosphatase (IAP), an enzyme that detoxifies proinflammatory lipopolysaccharide (LPS) endotoxins from gram-negative bacteria that accumulates during infection; whereas supplementing n-3 to an n-6 rich diet did not enhance IAP activity (DeCoffe et al., 2016). When

n-3 is added to an n-6 rich diet it reduced IAP activity and led to increased sepsis and mortality in mice after *C. rodentium* challenge (Ghosh et al., 2013). The anti-inflammatory response associated with n-3 supplementation on the high n-6 diet is thought to have worsened the infection outcome because inflammatory responses are necessary for pathogens clearance (Ghosh et al., 2013). Previously it has been observed that high levels of dietary n-6 PUFAs in fact reduce IAP activity leading to LPS endotoxemia in mice (Kaliannan et al., 2015). Transgenic *Fat-1* mice, which genetically retain a higher concentration of n-3 in their tissues, demonstrated elevated serum IL-10 and IAP activity (Kaliannan et al., 2015). In mice, safflower and canola oil based diets (high in n-6) heighten mucosal T_H1/T_H17 responses and inflammation, whereas a fish oil based diet has shown to have a protective anti-inflammatory effect following a *C. rodentium* infection (Hekmatdoost et al., 2013). Diets rich in n-3 PUFAs have proven protective against many extracellular pathogens (*Mycobacterium tuberculosis*, *Salmonella* Typhimurium, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *C. rodentium*, *Helicobacter hepaticus* and *H. pylori*, and *Listeria monocytogene*); however, potentially damaging effects were observed during intracellular viral infections (Husson et al., 2016; Jones & Roper, 2017). Dose and timing of n-3 PUFAs is critical for intestinal immune homeostasis. Sustained high doses alter microbial communities and host immune system towards an anti-inflammatory state that could exacerbate infections, especially when proinflammatory responses are essential for infection clearance (Husson et al., 2016). Interestingly, lipid composition affects host-microbial interactions even when administered via a non-enteral route. The inclusion of mixed lipids containing soybean oil, medium-chain triglycerides, olive oil, and fish oil in parenteral formula was shown to reduce intestinal

inflammation and alter microbial composition in a piglet model of infant total parenteral nutrition as compared to soybean oil alone (Lavallee et al., 2018).

1.6 Protein

Protein homeostasis is crucial for host health, physiology, and immune development that together foster a fast-acting immune response towards pathogens. The role of dietary protein and amino acids on host immune function related to diet malnutrition and pathogen interactions has been thoroughly reviewed (P. Li et al., 2007; Wenkai Ren et al., 2018). Amino acids play a major role in regulating immune cell activation, cellular redox homeostasis, lymphocyte proliferation, and production of cytokines, cytotoxins, and antibodies (P. Li et al., 2007). Protein deficiency is well known to impair immunity and infection resistance, especially during stress and illness due to protein malabsorption and protein consuming processes such as tissue repair (Jonker et al., 2012). Protein deficits have been shown to exacerbate parasitic *Cryptosporidium* infections in mice through disruption of baseline (primary) Th1-type mucosal immunity (Bartelt et al., 2016). Furthermore, protein-deprived diets decreased small intestinal macrophage proliferation and IL-10 production independently of the microbiota (Ochi et al., 2016).

In contrast, researchers propose that protein-rich diets can be just as harmful since they led to an increase in undigested proteins that encourage protein-fermenting bacteria and disease susceptibility (Ma et al., 2017). Resistant and undigested proteins can interfere with host functions directly as biologically active proteins (BAP) like trypsin and chymotrypsin inhibitors, and indirectly through microbial proteolytic fermentation by-products (H_2, CO_2, CH_4, H_2S, SCFA, branched chain amino acids (BCAA), nitrogenous compounds, phenols and indoles) with poorly understood health outcomes (Yao et al., 2016). It is important to note that dietary crude

protein can contain a high concentration of BAPs whose activities can be reduced upon hydrolysis digestion (heating, chemical, or enzymatic). A study replacing crude protein (wheat and casein) with purified amino acids to diets fed to weaned pigs reduced proteolytic fermentation before and after an enterotoxigenic *Escherichia coli* (ETEC) K88 challenge (Opapeju et al., 2009). Three days post-infection, ETEC K88 colonized the small intestine of pigs fed the crude protein diet whereas no colonization was observed in the small intestine of pigs receiving the purified amino acid diet. In this context, undigested protein or other components associated with crude protein diets promoted ETEC growth and colonization in the small intestine.

Furthermore, the source of proteins can impact microbial communities depending on the digestibility and total amino acids in the diet (Ma et al., 2017). For instance, animal proteins tend to be highly digestible in the proximal intestine compared to plant-based proteins (Windey et al., 2012). Processing proteins with heat can impact their digestibility, for example, rats fed thermolyzed (heated to 180°C for 1-2h) casein, soy, or egg white protein had reduced proximal intestinal digestibility, leading to a greater degree of protein fermentation in the cecum (Corpet et al., 1995). The number of aberrant crypts were measured after azoxymethane challenge to assess the carcinogenic promoting properties of casein, soy, and egg proteins. For the heat-treated proteins, the number of aberrant crypts increased with casein, remained unchanged with soy, and decreased with egg white compared to untreated protein diets. In agreement, a DSS mouse model study using multiple custom diets demonstrated that casein and soy proteins worsened DSS associated weight loss, whereas no effect was seen in mice fed the egg white protein diets (Llewellyn et al., 2018). In contrast, a human trial compared high- and low-fat diets with non-meat protein (legumes, nuts, grains, soy), red meat protein (beef) or white meat protein (chicken

and turkey) on the gut microbiome and found only a modest impact of protein source on the microbiome (Lang et al., 2018). For cardiovascular health, the plant-based proteins outperformed meat protein diets but white meat was no better than red meat for reducing disease risk (Bergeron et al., 2019). However, animal protein dominated diets tend to include higher amounts of fats, which ultimately may be more impactful on health than the proteins themselves. Plant-based protein diets may inherently contain detrimental components. For example, soybean isoflavones are suggested to contribute to greater parasitic oocyst fecal output and reduce immune responsiveness in mice fed a soy-based diet compared to casein and whey protein fed groups (Ford et al., 2001). For this reason, crude protein diet studies make it difficult to identify the bioactive component responsible for the observed phenotype. A study in rats comparing protein from soy, casein, pork, beef, chicken, and fish indicates that protein source alters microbial composition (Zhu et al., 2015). Specifically, white meat (chicken and fish) increased beneficial *Lactobacillus* species. Blood levels of lipopolysaccharide-binding protein (LBP), a marker for lipopolysaccharide (LPS) endotoxemia, was found to be significantly higher in the soy protein diet group compared to fish, chicken, pork, beef, and casein protein fed groups. Further research is needed in controlled animal models to investigate isolated protein types and processing techniques on host digestion, microbiome and fermentation products to mechanistically link the impact of protein on infection resistance.

Dietary glutamine supplementation has proven to be an effective therapy to help restore intestinal integrity in patients with post-infectious associated irritable bowel syndrome (Zhou et al., 2018). Although glutamine significantly improved IBS scores compared to a placebo supplemented group, a larger cohort and mechanistic studies are warranted. The effect of glutamine supplementation may be associated with glutamines ability to enhance intestinal cell

proliferation (Wenkai Ren et al., 2014), decrease the Firmicute population, and activate innate immunity through NF-κB, MAPK, and PI3K-Akt signaling pathways (Chen et al., 2018). Similar effects have been observed with arginine supplementation (W. Ren et al., 2014). Over a 14-day study, daily supplementation of 30 g of L-glutamine to overweight individuals led to a significant decrease in Firmicute populations, including species from the genus *Dialister*, *Dorea*, *Pseudobutyrivibrio*, and *Veillonella* (Zambom de Souza et al., 2015). Since overweight individuals typically have a higher Firmicute/Bacteroidetes ratio than lean individuals (Koliada et al., 2017), a decrease in Firmicutes with glutamine supplementation suggests that dietary glutamine may play a beneficial role in restoring microbiota balance. In accordance, glutamine and arginine supplementation promoted the activation of innate immunity and lowered intestinal pathogen load in ETEC-infected mice (G. Liu et al., 2017). In humans, enteral glutamine administration in critically ill patients with severe trauma, burns, and sepsis significantly reduced the number of isolated enteric bacteria such as *Pseudomonas sp.*, *Klebsiella sp.*, *Escherichia coli*, and *Acinetobacter sp.*, all of which can contribute to pneumonia if transmitted to the lungs (Conejero et al., 2002; Sader et al., 2018). Enteral glutamine administration reduced bacterial overgrowth within the gastrointestinal tract, which may have reduced the chance of bacterial exposure to the lungs and explain the reduced incidence of pneumonia in patients. Moreover, a systematic review and meta-analysis concluded that glutamine-enriched enteral formulae can significantly reduce gut permeability in critically ill patients (Mottaghi et al., 2016). The requirement and importance of enteric glutamine has been extensively reviewed (Biolo, 2013), but requires further research in healthy subjects and animals models to understand the impact on the microbiome and enteric infection resistance.

Further emerging evidence suggests that numerous microbially-derived indoles from tryptophan catabolism can promote intestinal homeostasis by activating regulatory T cells (Tregs) through their interaction with the aryl hydrocarbon receptor (AhR) (Mezrich et al., 2010). Roager and Licht (2018) summarize known microbes responsible for producing tryptophan-derivatives that positively act on tight junctions, gastrointestinal motility, host metabolism, AhR to activate IL-22, along with their systemic anti-oxidative and anti-inflammatory properties (Roager & Licht, 2018). In this respect, dietary tryptophan likely contributes to infection resistance by priming host defense strategies. The importance of tryptophan is further supported by the ability of host dendritic cells to metabolize tryptophan into kynurenine using indoleamine 2,3-dioxygenase-1 (IDO1) in order to control host inflammation during a *C. difficile* infection (El-Zaatari et al., 2014). Kynurenine production during *C. difficile* infection is proposed to be beneficial as it reduces excessive IFNγ cytokine production by limiting neutrophil populations in the lamina propria (El-Zaatari et al., 2014). Clinically, these findings provide important insight into the use of IDO1 inhibitors for cancer treatment which would prevent kynurenine production, and increase the severity of *C. difficile* infection (El-Zaatari et al., 2014). Like tryptophan, threonine is another essential amino acid that must be obtained from diet with deficiencies leading to immune and barrier dysfunctions (Dong et al., 2018). Dietary threonine is essential for the production of mucin with deficient diets leading to altered mucosal integrity and persistent diarrhea in neonatal piglets (Law et al., 2007). The importance of dietary threonine for mucus production and structure may not only provide protection for host IECs but also could stimulate mucolytic bacteria with unknown functions (Figure 1.3).

Dietary protein source, amount and processing can alter their impact and effects within gastrointestinal environment. Clearly host protein digestion shares an intimate relationship with the gut microbiome and their fermentation products (Diether & Willing, 2019). A balanced macronutrient or low indigestible protein diet is recommended to discourage proteolytic bacteria from overproducing cytotoxic, genotoxic and carcinogenic by-products that disrupt intestinal integrity and increase the risk of infection (Ma et al., 2017).

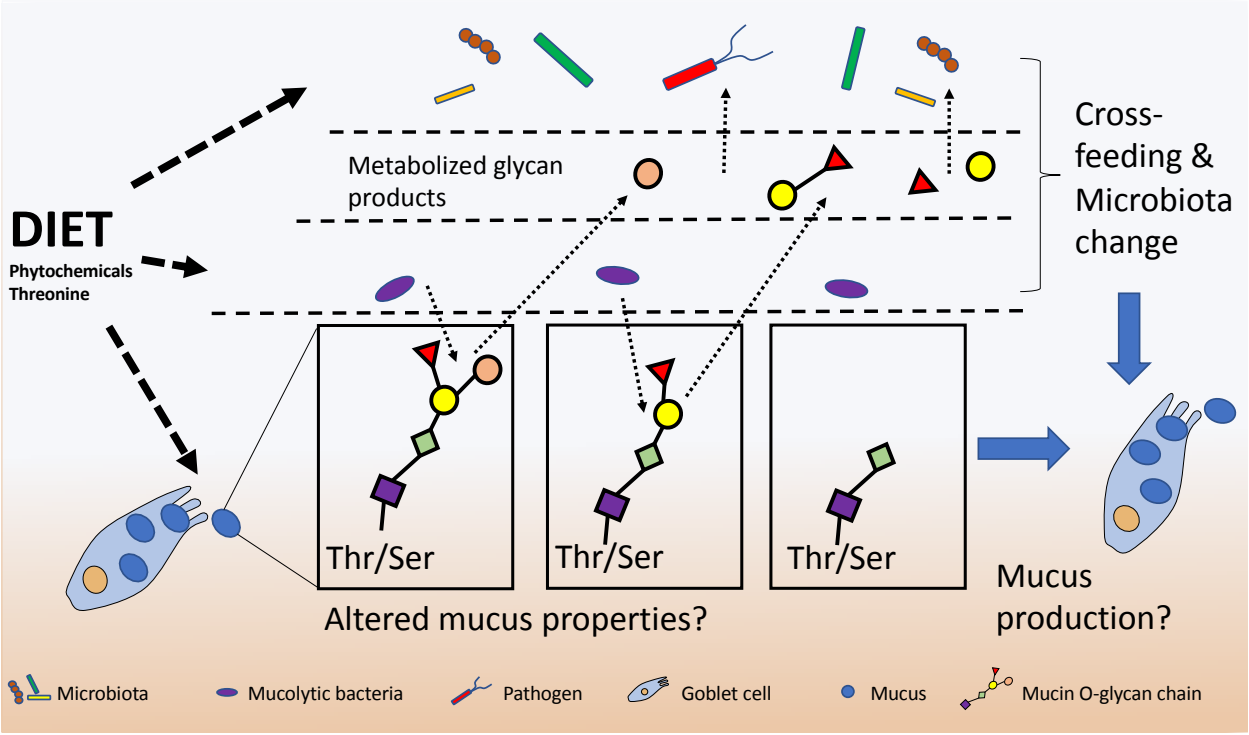


Figure 1.3: Diet alters host-microbiota-pathogen mechanisms of mucus production and consumption. Mucolytic specialists that digest the mucus O-glycans and subsequently cross-feed with other bacteria and pathogens can lead to further microbiota changes and alterations to mucosal integrity.

1.7 Phytochemicals

Plants synthesize a large pool of compounds known as phytochemicals to protect themselves from stress, predation, and infection. Complex mixtures of phytochemicals are found in the roots, seeds, leaves, bark, flowers, and fruit of plants and have been intensively studied for their antimicrobial, anti-inflammatory, and antioxidants activities (Ayseli & Ipek Ayseli, 2016). The physicochemical properties of phytochemicals give plants their unique colour, smell, and flavor profiles, and dictates their bioactivities and bioavailability within the gastrointestinal tract (Kemperman et al., 2010). Condensed tannins, mainly polymeric flavanols can act as antinutritional factors that reduce host digestion through enzyme inhibition and protein precipitation (Gilani et al., 2012). However, the consumption of phytochemicals is typically associated with beneficial health outcomes from their activities on the resident microbial population and host metabolism (Anhê, Varin, et al., 2015; Dueñas et al., 2015; Pandey & Rizvi, 2009). Phytochemicals are treated as xenobiotics by the host and because of this, the liver can reintroduce phytochemical derivatives to microbes through enterohepatic circulation, further complicating their effects on host health. Many studies fail to demonstrate and characterize absorbed phytochemical derivatives to investigate whether their impact on host are direct or indirect through the microbiota.

Research has focused on the use of phytochemicals as an alternative to antibiotics and as a dietary supplement to strengthen host pathogen resistance (Willing et al., 2018a). For instance, chickens fed a mixture of pepper (*Capsicum*) and turmeric oleoresin had less weight loss and reduced intestinal lesions scores in a necrotic enteritis disease model (S. H. Lee et al., 2013). The phytochemical mixture lowered intestinal but increased splenic proinflammatory cytokines/chemokines (IL-8, lipopolysaccharide-induced TNF-a factor, IL-17) levels altering

host immunity through immune cell differentiation, proliferation, apoptosis and NO production (S. H. Lee et al., 2013). Reactive nitrogen and oxygen species produced by peripheral leucocytes is an essential defence strategy against pathogens. In fish, dietary supplementation of a grass extract (*Cynodon dactylon*) to infected *Catla catla* carp stimulated reactive oxygen and nitrogen species production and decreased mortality in a dose depend manner (Kaleeswaran et al., 2011). Screening multiple phytonutrients revealed that the dietary flavonoid naringenin can act as an agonist on the AhR to induce regulatory T cells (Treg) that suppress allergy and autoimmune disease (H. K. Wang et al., 2012). Interestingly, phytochemicals such as indole-3-carbinol (I3C) present in cruciferous vegetables (e.g. broccoli, cabbage) act as ligands for AhR leading to the expansion of the anti-inflammatory IL-22 producing ILCs (Kiss et al., 2011). Functioning AhR has proven to be crucial for immunity because AhR-deficient mice failed to control *C. rodentium* infections (Qiu et al., 2012) . Moreover, mice fed a phytochemical-free diet had a reduced formation of lymphocyte aggregates and follicles, a similar phenotype as seen in AhR-deficient mice (Kiss et al., 2011). Dietary I3C supplementation protected against *C. difficile* infection through activation of AhR but also through unknown AhR-independent mechanisms likely caused by changes to microbial populations (Julliard et al., 2017).

Anti-adhesion properties are well sought after when studying the direct effects of phytochemicals on pathogen fitness. Cranberry extracts are documented to inhibit pathogenic *E. coli* adhesins (e.g. fimbriae) limiting their ability to attach to host cells (Luís et al., 2017; Nicolosi et al., 2014). The anti-adhesion activity of cranberry extract is attributed to the polyphenolic flavan-3-ol compounds known as A-type proanthocyanidins (PACs) (Howell, 2007). Cranberry A-type PACs reduced adherence of multiple strains uropathogenic *E. coli* and *Proteus mirabilis in vitro* (Nicolosi et al., 2014). However, *in vivo*, intestinal and microbial

PACs metabolites are found at higher concentrations in urine than the intact PACs and thus may be the bioactive metabolites responsible for the anti-adhesive properties (Peron et al., 2017). Interestingly, an analysis of urine phytochemical metabolites indicated that they change over-time due to multiple rounds of enterohepatic circulation modifications(Peron et al., 2017) with poorly understood activities (L. Tian et al., 2019). Moreover, cranberry PACs are thought to inhibit host and microbial enzymes (e.g. lipase, glycosidases) protecting against diet-induced obesity (Yokota et al., 2013a). PACs are associated with increased *Akkermansia sp*. abundance; however, it is unknown whether microbiota changes are a direct action of PACs or an indirect result of their effects on host metabolism (Anhê, Roy, et al., 2015). B-type PACs are known to be less inhibitory to both bacteria and host metabolism (Yokota et al., 2013a). Work from our group demonstrates that pea seed coats rich in B-type PACs led to a significant decrease in the Firmicutes population, increased fecal mucin content, and caused greater pathogen colonization in mice compared to a PAC-poor diet (Forgie, Gao, et al., 2019). B-type PACs may have led to improper mucus formation leading to a greater concentration of mucin excreted in feces. Phytonutrient supplementation is associated with increases in beneficial Clostridia species and can strengthen mucosal barrier function by increasing mucus production and thickness (Marta Wlodarska et al., 2015), protecting epithelial cells from invading pathogens and disease. Interestingly, a positive feedback loop may be established between mucolytic bacteria such as *Akkermansia sp.* that can degrade mucus O-linked glycans, thereby producing SCFAs that could stimulate goblet cells to secrete more mucus (Anhê, Varin, et al., 2015). Polyphenolic compounds may stimulate the microbiota directly or indirectly through modulation of mucus production; however, further research is needed to establish direct links between diet and infection resistance (Figure 1.3).

1.8 Vitamins and minerals

Micronutrients are essential for proper metabolic and immune function. Nutrient and mineral deficiencies, typical in those that are critically-ill and in developing countries, can lead to metabolic changes, oxidative damage, immunological defects, weakness, and death (Shenkin, 2006). The effects of essential minerals, including iron, zinc, copper, selenium, silver, sulfur, calcium, phosphorus, and magnesium have been shown to affect resident microbial populations and health outcomes in both animal and human studies (Skrypnik & Suliburska, 2018). Phagocytes have been shown to utilise the bactericidal actions of copper and zinc to enhance intracellular killing of pathogens (Djoko et al., 2015). For instance, mice fed a zinc-deficient diet and challenged with Enteroaggregative *Escherichia coli* (EAEC) had reduced leukocyte infiltration and increased virulence factors in luminal content, indicating an impaired immune response and increased infection severity (Bolick et al., 2015). Regular supplementation of vitamin C (1 to 2 g/day) and zinc (<100 mg/day) reduced the duration of the common cold by 8-14% and 33% respectively (Hemilä, 2017; Rondanelli et al., 2018). For vitamin C, prophylactic doses greater than 0.2 g/day alleviated respiratory associated problems, particularly in physically strained and stressed individuals; however, its use as a therapy to treat the common cold remains controversial (Douglas et al., 2008). In contrast, zinc supplementation studies support its use as a treatment option to reduce the duration and severity of the common cold (Hemilä, 2017). Vitamin D had the best overall protection against the common cold; however, baseline vitamin D levels and dose must be considered since lower doses and deficient individuals experienced the most benefit (Rondanelli et al., 2018). More mechanistic research is required to understand the impact of vitamins on immune responsiveness, especially with respect to the host-microbe gut axis in deficient and in excess conditions. Experiments in germ-free, conventionalized and

infectious *C. rodentium* mice models confirm that the microbiota influences vitamin D metabolism by lowering fibroblast growth factor (FGF) 23 through increased activation of TNF-α in the colon (Bora et al., 2018). The fact that the presence of the microbial community or mono-colonization with *C. rodentium* increases serum vitamin D levels highlights their role on host homeostasis, especially since vitamin D levels control calcium homeostasis and bone formation (Bora et al., 2018). Research suggests that proper regulation of vitamins and minerals is key for establishing a proper immune response and intestinal barrier function. Similar to vitamin and mineral deficiencies, excessive supplementation can impair a host ability to resist enteric infections by altering intestinal integrity or enhancing pathogen fitness.

Recently, oral iron and vitamin B12 supplementation are suggested to impair microbiota dependent infection resistance. A systematic review and meta-analysis comprising 6831 adult participants concluded that oral ferrous sulfate (iron) supplementation is associated with a significant increase in gastrointestinal side-effects compared to placebo and intravenous iron delivery (Tolkien et al., 2015). This reveals that the effects of iron supplementation are possibly initiated through the microbe-gut axis with unknown consequences and should be used cautiously. For instance, excessive luminal iron affects intestinal integrity through oxygen radical production, encourages pathogen virulence and alters microbial populations leading to pathogen overgrowth (Kortman et al., 2012; Natoli et al., 2009). In a dose dependent manner, iron increased epithelial invasion and translocation of *S.* Typhimurium in Caco-2 cells *in vitro* and reduced the survival of the nematode *Caenorhabditis elegans* infected with *S*. Typhimurium (Kortman et al., 2012, 2015). Regulation of luminal iron is extremely important for maintaining intestinal integrity and controlling pathogen expansion (Hurrell & Egli, 2010). Furthermore, lipocalin-2 is a protein produced by neutrophils and epithelial cells during inflammation that

directly limits bacterial iron uptake, reducing pathogen overgrowth and severity (Zhuoming Liu et al., 2013). Unlike iron, vitamin B12 is directly regulated in the gut by intrinsic factors for absorption and in excess, it can escape host absorption and affect microbial competition. The gut commensal bacteria *Bacteroides thetaiotaomicron* may compete against enterohemorrhagic *Escherichia coli* (EHEC) to sequester dietary vitamin B12 (Cordonnier et al., 2016a). *In vitro* competition assays show that *B. thetaiotaomicron* reduced EHEC shiga toxins but when co-cultured with a mutant *B. thetaiotaomicron* lacking a vitamin B12 transporter, EHEC had normal shiga toxin production (Cordonnier et al., 2016b). Microbial vitamin B12 transporters have different affinities towards vitamin B12 allowing them to compete with host cells and other microbes to take up exogenous vitamin B12 (Degnan, Barry, et al., 2014b; Wexler et al., 2018). More research is needed into micronutrient supplementation on host-microbe interactions towards pathogens, especially in the context of over-supplementation, which may be detrimental depending on the micronutrient balance and host intestinal homeostasis. Limiting the expansion of enteric pathogens can be accomplished by reducing their access to vitamin or minerals either through diet or stimulation of gut commensals to compete with pathogen for vital nutrients.

1.9 Conclusion

Pathogen resistance and tolerance requires tight host regulation of dietary components and subsequent microbial actions that together influence each other and host immunity. Undigested and unabsorbed dietary components are able to influence microbial populations and their fermentation by-products can indirectly contribute to infection resistance by modulating host intestinal integrity. Dietary intervention studies are difficult to control and compare due to seasonal variations in diets sources. We suggest that dietary intervention studies should include

diet backgrounds designed with macro- and micro- nutrients that stress and protect the gastrointestinal environment, as to give a proper assessment of that dietary component on host. In general, a balanced diet of SFA, MUFA, MACs, protein, phytochemicals, vitamins and minerals with limited sources of n-6 PUFAs, simple carbohydrates, BAPs, and iron may help restore intestinal homeostasis in compromised individuals. Dietary individuality makes it difficult to make general diet recommendations as each individual may have genetic, microbiota and unforeseen environmental factors that influence diet digestibility and utilization. Together, these factors ultimately provide the context to which dietary components may influence intestinal integrity and homeostasis.

1.10 Hypotheses and Objectives

This thesis aimed to investigate the direct and indirect effects of phytochemicals and vitamin B12 on intestinal integrity. These dietary components share common features, including the ability to promote health, undergo enterohepatic circulation and modulate microbial communities. Phytochemical and vitamin B12 supplements are available but are typically consumed in excessive amounts without considering the potential impact they may have on microbial communities in the gut. In particular, we focus on host-microbe interactions that alter intestinal integrity as determined through an enteric pathogen challenge with the following hypotheses and objectives.

1.10.1 Hypotheses

1. Pea seed coat proanthocyanidin fraction supplementation, in the context of a high fat diet, improves intestinal integrity and protects against early pathogen colonization.

2. Luminal mucin accumulation in the gut from phytochemical supplementation is dependent on the microbiota.

3. Excessive supplementation of vitamin B12 alters the functional activity of the gut microbiota creating a favorable environment for pathogen colonization and pathogenesis.

1.10.2 Objectives

1. To explore the impact of pea fractions, rich and poor in proanthocyanidins, on microbial community structure and ability to resist enteric infection (Chapter 3).

2. To determine the role of the microbiota in phytochemical-induced fecal mucin accumulation and how mucin contributes to overall microbial community structure (Chapter 4).

3. To characterize the function of the microbiota in response to high vitamin B12 concentrations in the colon as well as resistance to infection (Chapter 5).

2 CHAPTER 2: PEA POLYPHENOLICS AND HYDROLYSIS PROCESSING ALTER MICROBIAL COMMUNITY STRUCTURE AND EARLY PATHOGEN COLONIZATION IN MICE

2.1 Introduction

Intervention and epidemiological studies have demonstrated that consuming pea (*Pisum sativum*) seeds or their components can benefit metabolic, cardiovascular and gastrointestinal health in humans (Dahl et al., 2012). Benefits from pea consumption are typically associated with whole pea seed ingestion and attributed to the pea fiber concentrated within the pea seed coat (Whitlock et al., 2012). Whole pea seed or seed coat flours have been extensively shown to help with glucose control and weight in human and animal models (Hashemi et al., 2014, 2017; Marinangeli & Jones, 2011; Schäfer et al., 2003; Whitlock et al., 2012). Benefits are associated with the intake of dietary fiber with growing interest of health outcomes being mediated through their impact on the gut microbiota (Bibi et al., 2017; Desai et al., 2016; Eslinger et al., 2014; Monk et al., 2017).

Dietary fiber is a substrate for intestinal bacterial fermentation producing short chain fatty acids (SCFA(s)) (den Besten et al., 2013). SCFAs positively influence host metabolism and intestinal integrity (Tan et al., 2014). As a consequence, increasing fiber intake is an important dietary strategy to improve intestinal health and modify gut community structures. Compared with other pulses, including beans, chickpeas and lentils, pea specific fiber had a stronger bifidogenic effect in healthy Wistar rats (Queiroz-Monici et al., 2005). Pea supplementation has comparable health benefits to that of fructooligosaccharide supplementation, a well-established functional fiber (Eslinger et al., 2014; Slavin, 2013). SCFA products from fiber fermentation act

as fuel for epithelial cells, suppress inflammation and may strengthen the mucosal integrity through mucin-2 (Muc2) production, an important glycosylated protein of the mucus layer (Lucas López et al., 2016; Schroeder et al., 2017). Muc2 knockout mice models have confirmed the necessity of Muc2 to maintain gut homeostasis and reduce intestinal stress that would otherwise aggravate intestinal dysfunctions (Bergstrom et al., 2010). Accordingly, mice fed a fiber-depleted diet, typical of a western diet, resulted in a thinner mucus layer and enhanced pathogen susceptibility (Desai et al., 2016). Work from our research group has previously identified increased Muc2 expression in rats after consumption of a pea seed coat supplemented diet, suggesting enhanced mucosal protection (Hashemi et al., 2017). However, it remains to be determined whether the enhanced Muc2 expression associated with pea seed consumption enhances intestinal integrity and pathogen resistance.

In addition to fiber, specific pea cultivars are enriched with bioactive polyphenolic compounds. Polymers of flavan-3-ol subunits known as proanthocyanidins or condensed tannins concentrate within the pea seed coat along with the fiber (Ferraro et al., 2014; Jin et al., 2012). Condensed proanthocyanidins are considered as antinutritive factors that reduce the digestibility of dietary proteins and carbohydrates (Gilani et al., 2012); however, they have also been extensively studied for their health promoting properties in a variety of foods including peas, beans, nuts, fruits, spices, wines and teas, and praised for their antioxidant activity (B. Collins et al., 2016; A.-N. Li et al., 2014; Zhibin Liu et al., 2016; Lovisa et al., 2016; Quifer-Rada et al., 2016). Other bioactive properties of proanthocyanidins include anti-inflammatory, anti-microbial, protein precipitation, and enzyme inhibition (Balaji et al., 2016). Through these processes, proanthocyanidins reduce high fat diet (HFD)-induced intestinal inflammation in animal models and promote the growth of beneficial *Lactobacillus spp.* and *Bifidobacteria spp.*

(Cani et al., 2008; Cires et al., 2017). Processing polymeric proanthocyanidin-containing pea seed coats by acid hydrolysis releases their monomeric anthocyanidins, which are more readily absorbed and can have systemic effects (K. Yang et al., 2015). Acid hydrolyzed proanthocyanidin-rich pea seed coat consumption has been shown to increase proanthocyanidin-derived serum metabolites and improve β-cell function in glucose intolerant rats (K. Yang et al., 2015). Moreover, some phytochemicals enhance pathogen resistance by modifying antimicrobial peptide production, mucus layer thickness and growth of beneficial clostridia species (Marta Wlodarska et al., 2015). Despite the importance of proanthocyanidins, no studies to date have compared the benefits of pea cultivars with high and low seed coat proanthocyanidin content.

In the present study we hypothesize that pea fraction supplementation, in the context of a HFD, improves intestinal integrity and protects against early pathogen colonization. To test this, we fed mice various pea supplemented diets and challenged intestinal integrity with *Citrobacter rodentium,* a natural mouse specific pathogen. *C. rodentium* mimics the attaching and effacing pathology of human Enterohemorrhagic *Escherichia coli* (EHEC), inducing colonic inflammation in mice (Crepin et al., 2016). We show that not all pea cultivar seed coats equally protect gastrointestinal integrity and that their benefits are dependent on polyphenolic content and form.

2.2 Methods

2.2.1 Pea seed coat diets

Pea seed coat fractions supplemented in diets were sourced from two cultivars, proanthocyanidin-rich ‘Solido’ and proanthocyanidin-poor ‘Canstar’ varieties grown in Alberta, Canada. Both cultivars have been previously characterized for nutrient and polyphenolic content

(Ferraro et al., 2014). Crushed whole peas were passed through a 1.0-mm screen and larger embryo fragments were hand-picked from seed coats. Cleaned seed coats were then milled into flour with Retsch ZM200 ultracentrifugal mill (Retsch; Newton, Pa., USA) with a 0.25 mm ring sieve. Approximately 30 g of cleaned seed coats were ground at a time with a cooling period to prevent heat degradation. Seed coat flour fractions were added unprocessed or acid hydrolyzed to a high (20% w/w)-fat diet (HFD; Table 2.1) and low (6% w/w)-fat diet (LFD; Table 2.2). Acid hydrolysis was performed with an HCl acid solution as described in Yang *et al.* (K. Yang et al., 2015). Isocaloric diets were balanced for macronutrient and fiber content, with non-fermentable cellulose used for the control diets (Slavin, 2013). Proanthocyanidins were added at a final concentration of 0.4 % (w/w), half the concentration previously studied in rats (K. Yang et al., 2015). Mice consumed 0.37 g of pea seed coat flour per day from the 18 Kcal/day diet equating to an achievable 46 g of pea seed coat flour a day for a human consuming 2250 Kcal/day.

2.2.2 Animal feeding trial

All animal experiments were in accordance to the guidelines set by the Canadian Council on Animal Care. Animal use protocols were assessed and approved by the Health Sciences Animal Care and Use Committee at the University of Alberta. Six- to nine-week-old female C57Bl/6 mice (Jackson Laboratories) were randomly housed four per cage and handled in a biosafety cabinet under specific pathogen-free (SPF) conditions. Mice were allowed to acclimatize for one week with access to water and standard chow (PicoLab® Rodent diet #5053) ad libitum. Cages were randomly assigned to treatment diets supplemented with proanthocyanidin-rich (PA), hydrolyzed proanthocyanidin-rich (hPA), non-proanthocyanidin rich (NPA) and hydrolyzed non-proanthocyanidin rich (hNPA) fractions to a high fat diet.

Supplementation on a LFD background was evaluated to determine palatability of pea flours as a factor for weight gain. For three weeks, mice had ad libitum access to water and powdered diets in ceramic food cups (Figure 2.1). Body weights were taken each week and fecal samples were collected for microbiome analysis.

Table 2.1: HFD background composition of dietary treatments (g/kg).

Component	HFD		
	Control	PA or hPA	NPA or hNPA
Lard (Tenderflake)	190	190	190
Flaxseed oil	2	2	2
Corn oil (Mazola)	8	8	8
Casein	270	262	267
L-Methionine	2.5	2.5	2.5
Dextrose	214	214	214
Corn Starch	194	194	194
Cellulose	50	0	0
Mineral Mix	51	51	51
Vitamin Mix	10	10	10
Inositol	6.3	6.3	6.3
Choline Chloride	2.8	2.8	2.8
Solido (PA) seed coat		96.5	
Canstar (NPA) seed coat			71.5

Note: The nutrient content of both raw and acid processed cultivars were analyzed previously and adjusted accordingly. Treatment diets are equal in caloric density with a total content (w/w) of 20% fat, 26% protein, 40% carbohydrate, and 3-5% fiber. PACs content is 4.51% (w/w) of "Solido" PSC.

Table 2.2: LFD background composition of dietary treatments (g/kg).

Component	LFD		
	lfd_Control	lfd_PA or lfd_hPA	lfd_NPA or lfd_hNPA
Lard (Tenderflake)	57	57	57
Flaxseed oil	0.6	0.6	0.6
Corn oil (Mazola)	2.4	2.4	2.4
Casein	270	262	267
L-Methionine	2.5	2.5	2.5
Dextrose	279	279	279
Corn Starch	269	269	269
Cellulose	50	0	0
Mineral Mix	51	51	51
Vitamin Mix	10	10	10
Inositol	6.3	6.3	6.3
Choline Chloride	2.8	2.8	2.8
Solido (PA) seed coat		96.5	
Canstar (NPA) seed coat			71.5

Note: The nutrient content of both raw and acid processed cultivars were analyzed previously and adjusted accordingly. Treatment diets are equal in caloric density with a total content (w/w) of 6% fat, 26% protein, 55% carbohydrate, and 3-5% fiber. PAC content is 4.51% (w/w) of 'Solido' PSC.

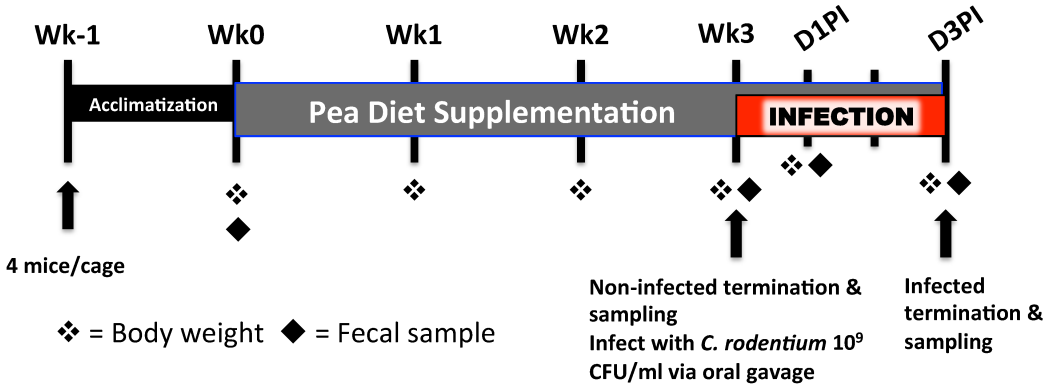


Figure 2.1: Experimental design diagram.

2.2.3 Early pathogen challenge model

All mice were checked by fecal plating prior to infection and confirmed to be free of commensal *Escherichia coli*, thus allowing accurate *C. rodentium* enumeration after infection. A single colony of *C. rodentium* (DBS100) was picked and incubated overnight at 37°C in Luria-Bertani (LB) broth (Sigma-Aldrich, Lennox) with shaking (200 RPM). The mice were infected by oral gavage with 0.1ml of overnight *C. rodentium* culture with approximately 1 x 10^9 colony forming units (CFU)/ml. Pathogen fecal load was determined at day three-post infection (D3PI) by collecting fresh fecal samples in 1ml of PBS and plating serial dilutions on MacConkey agar (BD Difco™). Bacterial colonies were counted after a 24 h incubation period at 37°C and normalized to fecal sample weight.

2.2.4 Sample collection

All mice were euthanized using carbon dioxide and sampling was done aseptically. Mouse ileum, cecum and colon tissues and content were snap frozen in liquid nitrogen and stored at -80°C until use.

2.2.5 Monocyte chemoattractant protein-1 (MCP1) Assay

Colon protein was extracted using a 2 cm piece of tissue excised between 2-4 cm from the rectal sphincter and homogenized in 300 µl of RIPA buffer which contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitor mini tablets (Pierce™; Thermo Scientific, Nepean, ON). The homogenates were centrifuged at 15,000 rpm for 10 min, and the supernatant protein concentration was determined using the Pierce™ BCA Assay Kit (Thermo Scientific). Samples were normalized to

100 μg of protein, as recommended by the manufacturer, and MCP1 was quantified with the MSD U-Plex Biomarker Group 1 (mouse) assay platform (Meso Scale Discovery, Gaithersburg, MD).

2.2.6 Reverse-transcription quantitative PCR (RT-qPCR)

RNA was extracted using the GeneJET RNA Purification Kit (Thermo Scientific) following a modified extraction protocol. Gut tissues excised between 1-2 cm from the distal end of the ileum and colon were homogenized in 600 μl of lysis buffer and bead beating was conducted in nuclease-free tubes with three metal beads at 4 m/s for 20 s (MP Biomedicals, Solon, OH, USA). Samples were treated with DNase for 15 minutes (Qiagen) prior to elution. The concentration of RNA was determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific) and samples were normalized to 1 μg of RNA for reverse transcription using the qScript Flex cDNA Synthesis Kit (Quanta Biosciences) following the random primer and oligo dt protocol. Quantitative PCR was performed using PercfeCTa SYBR Green Supermix (Quantabio) with primers listed in supplementary Table 2.3 (Curtis et al., 2014b; D. H. Lee et al., 1996) and conducted on an ABI StepOne^{TM} real-time System following the cycles: 95°C for 3 min and 40 cycles of 95°C for 10 sec, 60°C for 30 sec. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for calculating the fold change of gene expression relative to animals fed a control diet using the $2^{-\Delta\Delta Ct}$ method. Total bacteria primers (Table 2.3) were used to quantify cecal bacterial load and were normalized to the weight of cecal content used to isolate bacterial DNA.

Table 2.3: Real-time qPCR primer list.

Target Genes	Oligonucleotide sequences (5' - 3')	Product length	Ref.
Glyceraldehyde 3-phosphate	F: ATTGTCAGCAATGCATCCTG R: ATGGACTGTGGTCATGAGCC	109	Curtis et al., 2014b
Mucin 2	F: GCTGACGAGTGGTTGGTGAATG R: GATGAGGTGGCAGACAGGAGAC	135	Curtis et al., 2014b
Regenerating islet-derived protein 3 gamma	F: AAGCTTCCTTCCTGTCCTCC R: TCCACCTCTGTTGGGTTCAT	107	Curtis et al., 2014b
Regenerating islet-derived protein 3 beta	F: GGCTTCATTCTTGTCCTCCA R: TCCACCTCCATTGGGTTCT	106	Curtis et al., 2014b
Total Bacteria	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	179-210	D. H. Lee et al., 1996

Note: The annealing temperature used for all genes was 60°C.

2.2.7 Short chain fatty acids (SCFA) analysis

Snap frozen cecal content was weighed (~30mg/sample) and homogenized in 25% phosphoric acid. Samples were subsequently centrifuged at 15,000 rpm for 10 min and the supernatant was collected and passed through a 0.45μm filter. Isocaproic acid (23 μmol/ml) was added at a 1:4 ratio to samples as the internal standard and samples were analyzed on a Scion^{TM} 456-GC instrument.

2.2.8 Fecal Mucin Assay

Fecal pellets were pooled from four mice per cage with four cages per treatment, freeze-dried and ground to a powder. A fluorometric assay kit (Fecal Mucin Assay kit; Cosmo Bio co. LTD) that discriminates O-linked from N-linked glycoproteins was used to quantify mucin within the feces (Crowther & Wetmore, 1987).

2.2.9 DNA extraction and microbiome analyses

Total DNA was extracted from samples using the QIAamp® Fast DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) with an additional bead-beading step with ~200 mg of garnet rock at 6.0 m/s for 60 s (FastPrep-24™ 5G instrument, MP Biomedicals). A paired-end sequencing run was performed using the Illumina MiSeq Platform (2 x 300 cycles; Illumina Inc San Diego, CA) as described in a previous study (Ju et al., 2017). Amplicon libraries were constructed according to the protocol from Illumina (16S Metagenomic Sequencing Library Preparation) that amplified the V3-V4 region of the 16S rRNA gene (primers: Forward: 5' TCGTCGGCAGCGTCAGATGTGTATAAG AGACAGCCTACGGGNGGCWGCAG-3'; Reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The quality of the reads was assessed using FastQC. FASTTX-Toolkit was used to trim and filter poor quality reads. PANDAseq algorithm was used to merge pair-end sequence reads. Merged sequences were dereplicated and singletons removed with VSEARCH and filtered for chimeras using USEARCH (database "gold.fa") creating a reference database (Edgar et al., 2011; Rognes et al., 2016). Original merged reads were mapped to this reference database using usearch_global (VSEARCH) command with 97% identity and OTU table was created using the "uc2otutab.py" script. Taxonomy was assigned using QIIME 1.9.1 (Quantitative Insight into Microbial Ecology) toolkit default setup (Caporaso et al., 2010; McDonald et al., 2012; Q. Wang et al., 2007). Diversity analyses were done using 'core_diversity_analyses.py' command and normalized to a sampling depth set by the sample with the lowest number of reads (>1500). PD whole tree, Shannon and Chao1 diversity indices calculated with 'alpha_diverstiy.py' and plotted with R using ggplot2. Statistical significance for alpha diversity was determined in QIIME using

the default two-sample t-test with Monte Carlo permutations (999) to calculate the non-parametric *P*-value. Beta diversity was calculated using the Bray-Curtis dissimilarity metric and a principle coordinate analyses (PCoA) was plotted using the phyloseq package in R (McMurdie & Holmes, 2013). Clustering significance was determined using the 'betadisper' function for dispersion and 'pairwiseAdonis.dm' function for orientation as determined by Bray-Curtis distances (Anderson, 2006; Martinez Arbizu, 2017). Abundance heatmap was visualized in R with the 'pheatmap' function and significant genus differences were determined by permutational ANOVAs corrected with 'TukeyHSD' function for multiple comparisons.

2.2.10 Statistical analysis

Percent weight gain was calculated and analyzed by blocking for experiment using the block linear model with a Tukey-Kramer post-hoc test in SAS® University Edition. Unless otherwise stated, significance testing was conducted using GraphPad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA). Data were tested for normality of distribution and statistically significant differences were determined by one-way analysis of variance (ANOVA) for parametric and Kruskal-Wallis test for nonparametric data. Differences between dietary groups were corrected by conducting either Bonferroni's or Dunn's post-hoc comparison tests as appropriate. Spearman's correlation of microbial taxa abundances and SCFAs concentrations were analyzed and Bonferroni corrected using R software and visualized with 'pheatmap' function.

2.3 Results

2.3.1 Anthocyanidins reduced weight gain on a HFD

Hydrolyzed proanthocyanidins containing cultivar fraction supplementation on a HFD significantly reduced total weight gain ($P < 0.001$) over the three week long diet intervention period (Figure 2.2a). No effect was observed when dietary pea fraction flours were supplemented to a LFD compared to lfd_Control (Figure 2.2b). However, NPA and hNPA groups had higher weight gain compared to PA and hPA groups only on the LFD background ($P < 0.05$).

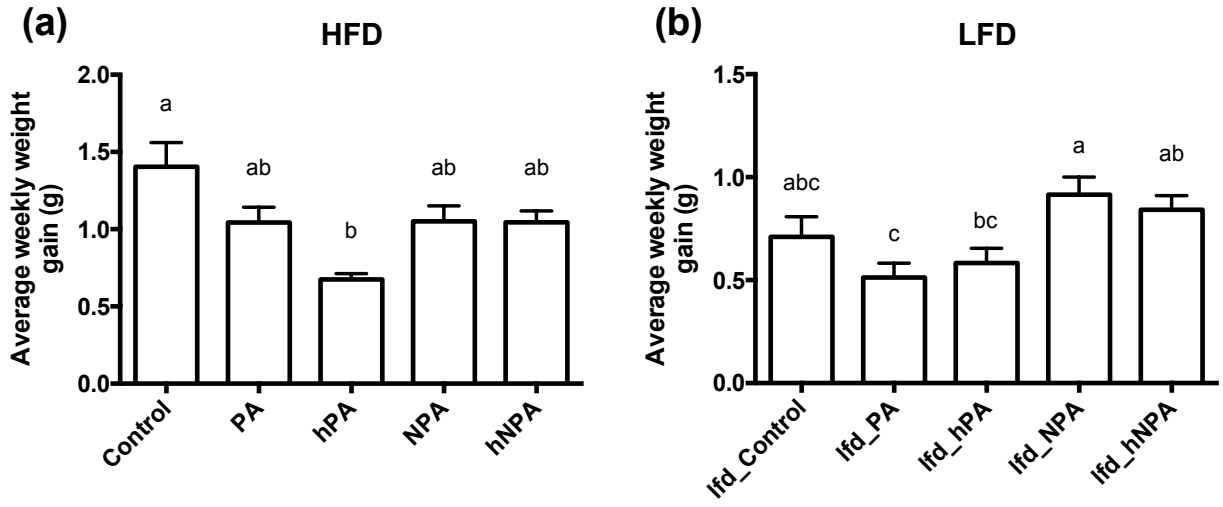


Figure 2.2: Anthocyanidins significantly affect weekly weight gain when supplemented on a HFD. The hPA diet significantly reduced weekly weight gain compared to control on a HFD but not on a LFD background; (a) Mice weights were gathered from two separate HFD non-infected animal trials (n=23-24/group); (b) mice weights gathered from LFD trial (n=16/group). Proanthocyanidin containing pea diets (lfd_PA and lfd_hPA) reduced weight gain compared to non-proanthocyanidin rich pea diets (lfd_NPA and lfd_hNPA). Analysis was done using a linear

mixed model design and blocked by experiment when appropriate. Significance indicated by different letters ($P < 0.05$; mean ± SEM).

2.3.2 Microbial community modifications were dependent on pea cultivar and processing

Pea polyphenolic content and acid processing altered microbial community structure within the ileum, cecum and colon (Figure 2.3). Principle coordinate analysis plots using Bray-Curtis dissimilarity metric indicate distinct microbial community clustering dependent on dietary treatment. Shannon and phylogenetic diversity indices (PD whole tree) indicate major effects on microbial diversity in the cecum of mice fed the PA diet. As in the cecum, the PA group maintained a significantly lower phylogenetic diversity in the colon compared to hPA and NPA groups. Beta-diversity statistical analyses are summarized in supplementary table S2 (Table 2.4). In the ileum, all treatment groups had similar distributions, but the PA group clustered away from control ($P = 0.05$). Cecal microbial communities of the PA group clustered separately from hPA ($P = 0.02$) and NPA ($P = 0.01$) groups. The hNPA was the only group with similar distribution ($P = 0.82$) and orientation ($P = 0.45$) to that of control. In the colon, microbial communities were drastically different in the pea supplemented groups, especially the PA group which clustered away from other treatment groups. This unique clustering of PA group corresponded with reductions in *Bifidobacteriaceae*, *Lactobacillaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Dehalobacteriaceae* and also increases in unclassified *Clostridiales*, *Turicibacteraceae*, and *S24-7* family members (Table 2.5). *Dehalobacterium sp*. and

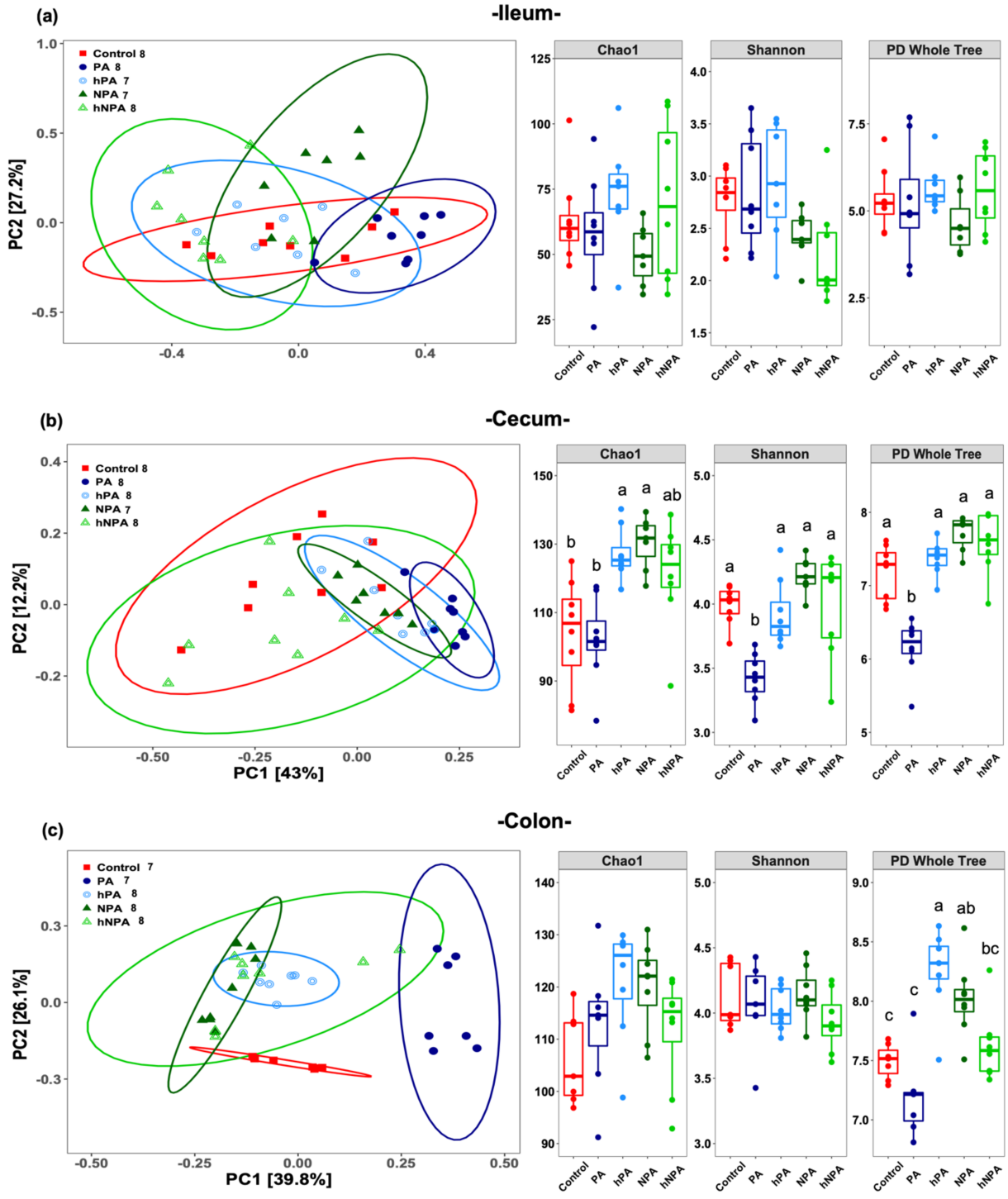


Figure 2.3: Microbial community structure was dependent on pea cultivar and acid hydrolysis. Ileal (a), cecal (b), and colon (c) principle coordinate analysis (PCoA) plots using Bray-Curtis

dissimilarity metric and alpha diversity indices of microbial communities from mice fed a HFD supplemented with pea seed coat fractions. Intact polymeric proanthocyanidins in the diet had distinct effects on microbial community structure in the ileum, cecum and colon (ADONIS: $P < 0.001$). Alpha diversity indices show a decrease in species richness and evenness, and taxonomic distances for the PA group in the cecum and colon. Ileal and cecal sample were from a separate trial than colon samples. Significance indicated by different letters ($P < 0.05$; mean ± SEM; n = 7-8/group).

Table 2.4: Microbial community analyses.

Region	Comparisons		Community distances	
			Dispersion	Location
	Pairs	R^2	(*P* - value)	(*P* - value)
Ileum	Control vs PA	0.29	0.73	0.05
	Control vs hPA	0.04	0.69	1
	Control vs NPA	0.21	0.29	0.16
	Control vs hNPA	0.21	0.89	0.1
	PA vs hPA	0.33	0.38	0.01
	PA vs NPA	0.32	0.09	0.02
	PA vs hNPA	0.54	0.63	0.01
	hPA vs NPA	0.2	0.51	0.23
	hPA vs hNPA	0.16	0.81	0.43
	NPA vs hNPA	0.27	0.42	0.07
Cecum	Control vs PA	0.49	0.01	0.01
	Control vs hPA	0.3	0.04	0.02
	Control vs NPA	0.24	0.08	0.01
	Control vs hNPA	0.15	0.82	0.45
	PA vs hPA	0.32	0.31	0.02
	PA vs NPA	0.4	0.08	0.01
	PA vs hNPA	0.5	0.01	0.01
	hPA vs NPA	0.18	0.55	0.09
	hPA vs hNPA	0.31	0.08	0.02
	NPA vs hNPA	0.27	0.13	0.01
Colon	Control vs PA	0.63	0.42	0.02
	Control vs hPA	0.49	0.2	0.01
	Control vs NPA	0.49	0.03	0.01
	Control vs hNPA	0.42	0.52	0.01
	PA vs hPA	0.64	0.01	0.01
	PA vs NPA	0.63	0.12	0.01
	PA vs hNPA	0.51	0.72	0.01
	hPA vs NPA	0.38	0.01	0.01
	hPA vs hNPA	0.17	0.21	0.07
	NPA vs hNPA	0.17	0.94	0.31

Note: Distances based off Bray-Curtis distance metric. Dispersion *P*-values were calculated with betadisper() using a permuted model that indicates a difference in dispersion between groups. Location *P*-values were calculated and adjusted using Bonferroni method with pairwise.adonis(), and indicates community structures cluster separately between groups.

Table 2.5: Fecal microbial community profiles after pea fraction supplementation on a HFD background. Represented as the average percent abundance of assigned taxonomy at the phyla and family level. Members that were <0.1% across mice were removed.

Taxonomy	Control		PA		hPA		NPA		hNPA		Adj. *P* - value
	(%)	sem	(%)	sem	(%)	sem	(%)	sem	(%)	sem	
p__Actinobacteria	**4.5**	0.74	**1.8**	0.5	**3.7**	0.76	**9.4** *	0.72	**5.4**	0.93	*0.0013*
f__Bifidobacteriaceae	**4.2**	0.71	**1.4** #	0.49	**3.1**	0.72	**8.7** *	0.63	**4.7**	0.93	*0.0051*
f__Coriobacteriaceae	**0.3**	0.05	**0.4**	0.04	**0.6**	0.09	**0.7** *	0.1	**0.7** #	0.08	*0.3758*
p__Bacteroidetes	**43.1**	2.26	**50.8** #	1.99	**38.7**	1.85	**23.4** *	1.52	**32.2** *	1.93	*0.0001*
f__Rikenellaceae	**1.7**	0.24	**4.2** *	0.59	**4.3** *	0.77	**1.5**	0.21	**1.9**	0.2	*0.0054*
f__S24-7	**41.4**	2.22	**46.6**	2.45	**34.3** #	1.27	**22.0** *	1.59	**30.4** *	1.78	*0.0003*
p__Firmicutes	**52**	1.83	**37.5** *	3.68	**38.5** *	1.24	**56.2**	3.52	**45.3**	4.55	*0.0073*
f__Bacillaceae	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	*1*
f__Planococcaceae	**0.4**	0.2	**0.1**	0.04	**0.5**	0.1	**0.3**	0.16	**0.5**	0.12	*0.3522*
f__Enterococcaceae	**0**	0.01	**0**	0	**0**	0	**0**	0.01	**0**	0.01	*0.3459*
f__Lactobacillaceae	**10.6**	2	**2.0** *	0.38	**10**	1.13	**15.4**	2.08	**12.7**	1.81	*0.0178*
f__Streptococcaceae	**0.6**	0.06	**0.5**	0.05	**0.9**	0.09	**1.4** *	0.19	**1.7** *	0.28	*0.0013*
f__Turicibacteraceae	**0**	0	**0.9** *	0.15	**0**	0	**0**	0	**0.2**	0.12	*0.0081*
o__Clostridiales;f__[1]	**15.7**	1.5	**19.5**	2.88	**6.8** *	0.75	**10.2** #	0.67	**7.0** *	0.83	*0.0014*
f__Clostridiaceae	**0.4**	0.11	**0.7**	0.23	**0.2**	0.05	**0.7**	0.3	**0.2**	0.05	*0.3627*
f__Dehalobacteriaceae	**0.2**	0.01	**0.0** *	0	**0.1** *	0.01	**0.1** *	0.02	**0.1** *	0.02	*0.0005*
f__Lachnospiraceae	**5.1**	0.43	**2.9**	0.37	**5.3**	0.35	**6.2**	0.79	**6**	1.08	*0.2894*
f__Peptostreptococcaceae	**1.9**	0.52	**1.1**	0.15	**1.8**	0.3	**2.1**	0.32	**2.9**	0.45	*1*
f__Ruminococcaceae	**13**	0.98	**7.7** *	0.84	**6.5** *	0.74	**6.3** *	0.39	**5.3** *	0.62	*0.0257*
f__Erysipelotrichaceae	**4**	0.7	**2.1**	0.36	**6.3**	1.2	**13.4** *	1.27	**8.7** #	1.91	*0.0042*
p__Proteobacteria	**0**	0.01	**0**	0	**0**	0	**0**	0	**0**	0	*0.0039*
f__Rhizobiaceae	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	*1*
f__Rhodobacteraceae	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	*1*
f__Enterobacteriaceae	**0**	0.01	**0**	0	**0**	0	**0**	0	**0**	0	*0.0008*
p__Tenericutes*	**0**	0	**0**	0	**0.1** *	0.02	**0.1** *	0.04	**0**	0	*0.0005*
f__Anaeroplasmataceae	**0**	0	**0**	0	**0.1** *	0.02	**0**	0	**0**	0	*0.0256*
o__RF39;f__[1]	**0**	0	**0**	0	**0**	0.01	**0.1** *	0.04	**0**	0	*0.0039*
p__Verrucomicrobia	**0**	0	**9.4**	4.62	**18.7** *	1.25	**10.5**	4.2	**16.8** *	3.97	*0.0611*
c__[Pedosphaerae];o__;f__[1]	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	*0.2214*
f__Verrucomicrobiaceae	**0**	0	**9.4**	4.62	**18.7** *	1.25	**10.5**	4.2	**16.8** *	3.97	*1*
p__Unassigned;Other	**0.3**	0.05	**0.5**	0.04	**0.3**	0.02	**0.4**	0.03	**0.4**	0.05	*0.4466*

[1]Unclassified order or family; Significant difference compared to Control [TukeyHSD (#) $P < 0.10$, (*) $P < 0.05$]; n = 7-8.

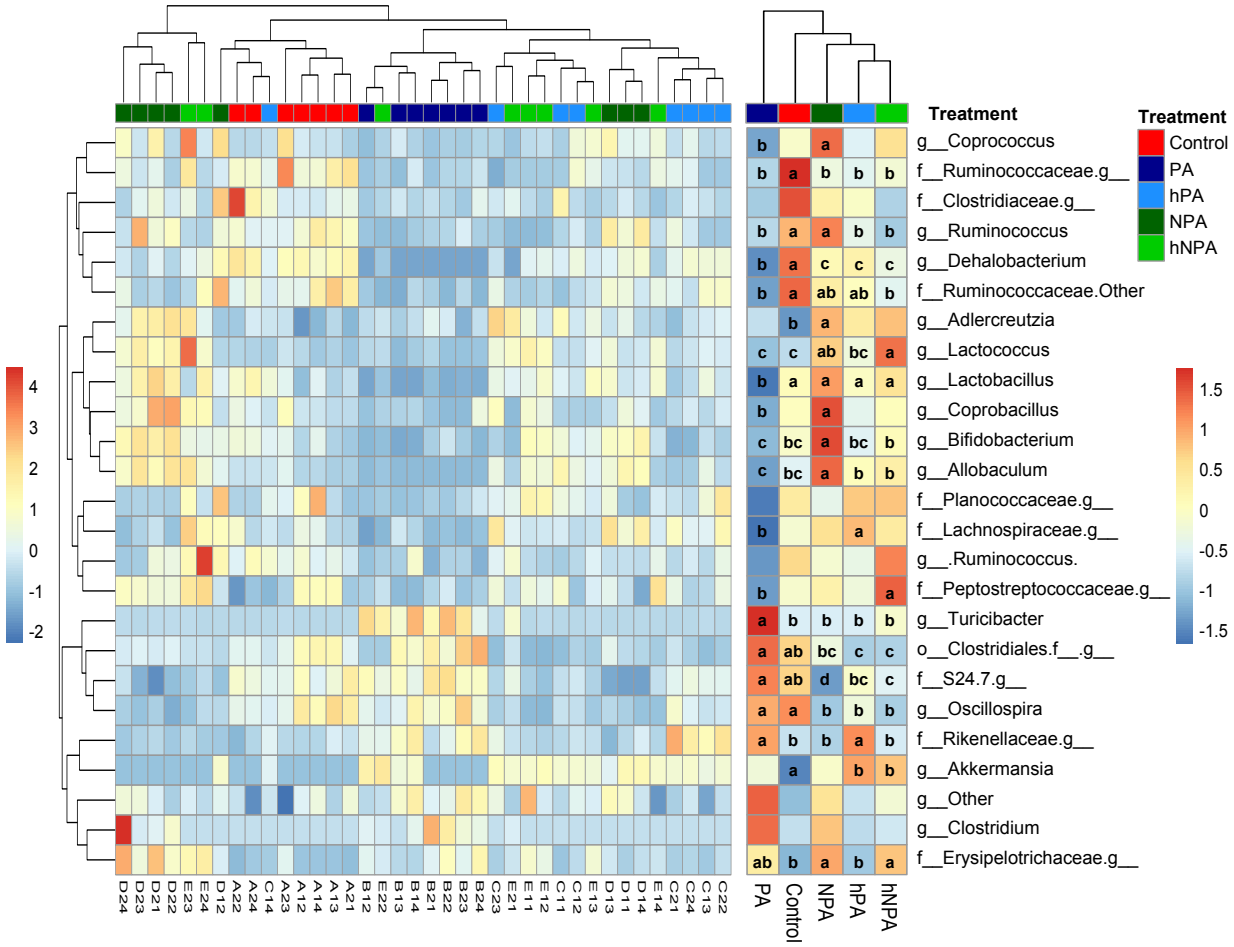


Figure 2.4: Colon microbial community heat map comparison with hierarchal clustering based on scaled relative abundance of OTUs assigned to genus level across individual mice and overall treatment average. Distinct impact on microbial community structure is evident in the PA diet compared to the other pea fraction diets supplemented to a HFD. This impact was reversed in the acid hydrolyzed hPA diet suggesting intact dietary proanthocyanidins have a direct effect on microbial communities. High and low abundant bacteria are highlighted from red to blue respectively. Statistical significance between treatments indicated by different letters ($P < 0.05$; n=7-8/group).

Allobaculum sp. were undetectable in the PA group but were detectable in all other treatment groups including the hPA group (Figure 2.4). In contrast, *Turicibacter sp.* was significantly higher in the PA group and lower or undetectable in all other groups. The NPA diet increased the population of *Bifidobacterium* relative to control; however, the PA group did not ($P < 0.05$). The Firmicutes to Bacteroidetes ratio was altered in favour of Bacteroidetes in the PA group to a final ratio of 0.74 ± 0.08 compared to Control (1.21 ± 0.10, $P = 0.19$), hPA (0.99 ± 0.07, $P = 0.54$), NPA (2.40 ± 0.24, $P < 0.001$), and hNPA (1.41 ± 0.19, $P < 0.05$) (Figure 2.5). Overall, the PA diet induced unique microbial modifications that disappeared in the hPA group and differed from the NPA and hNPA groups.

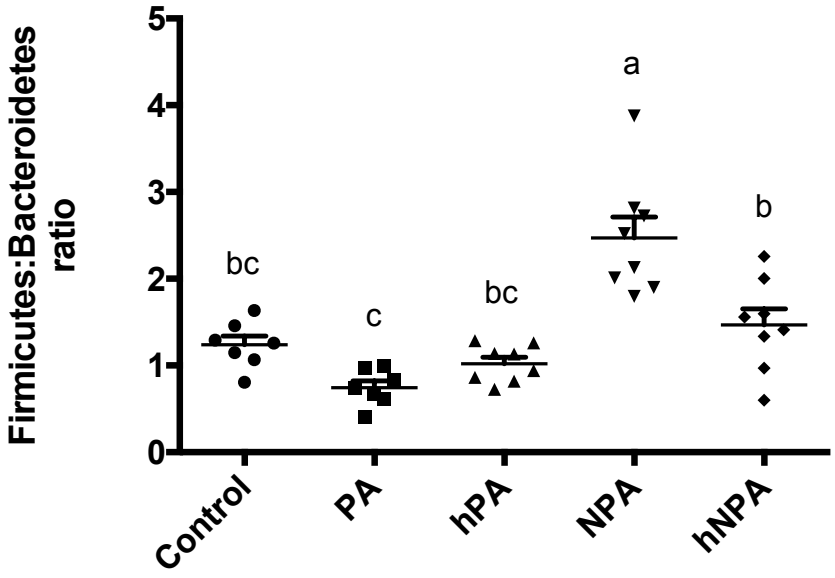


Figure 2.5: *Firmicutes* to *Bacteroidetes* ratio of mice colon microbiome after pea supplementation on a HFD.

2.3.3 SCFA production depended on pea cultivar and processing

Cecal fermentation of pea fractions on a HFD resulted in cultivar dependent variations in SCFA products. The PA group compared to the NPA group had significantly lower acetic ($P < 0.05$) and butyric ($P < 0.05$) acid concentrations. Similarly, the PA group had lower butyric acid ($P < 0.05$) compared to control. Raw and acid hydrolyzed cultivar groups had similar SCFA profiles with the exception of the caproic acid concentrations that were lower in PA compared to hPA ($P < 0.05$) (Figure 2.6a). Cecal bacterial load quantification using real-time qPCR revealed that the reduced SCFA in the PA group was not a result of reduced total bacteria (Figure 2.7). SCFA levels positively correlated with *Dorea* sp., *Dehalobacterium* sp., *Ruminococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., and an unclassified *Peptosteptococcaceae*, whereas *Bacteroides* sp. and *Sutterella* sp. negatively correlated with SCFA levels in the cecum (Figure 2.6b).

2.3.4 Innate defense activation depends on pea cultivar and processing

Addition of pea fractions to a HFD activated ileal Reg3 antimicrobial peptide gene expression and altered mucus homeostasis in the colon (Figure 2.8a). The hPA and hNPA groups had significantly greater Reg3γ gene expression ($P < 0.05$) than control, PA, and NPA. Reg3β was significantly higher in hPA ($P = 0.03$), NPA ($P = 0.05$) and hNPA ($P = 0.001$) and numerically higher but not significantly so in PA ($P = 0.10$) compared to control. No difference was observed in Muc2 gene expression in the colon (Figure 2.8b). The concentration of fecal mucin was greatest in PA with significantly less in the hPA, but both had much higher levels than HFD control, NPA, and hNPA (Figure 2.8c).

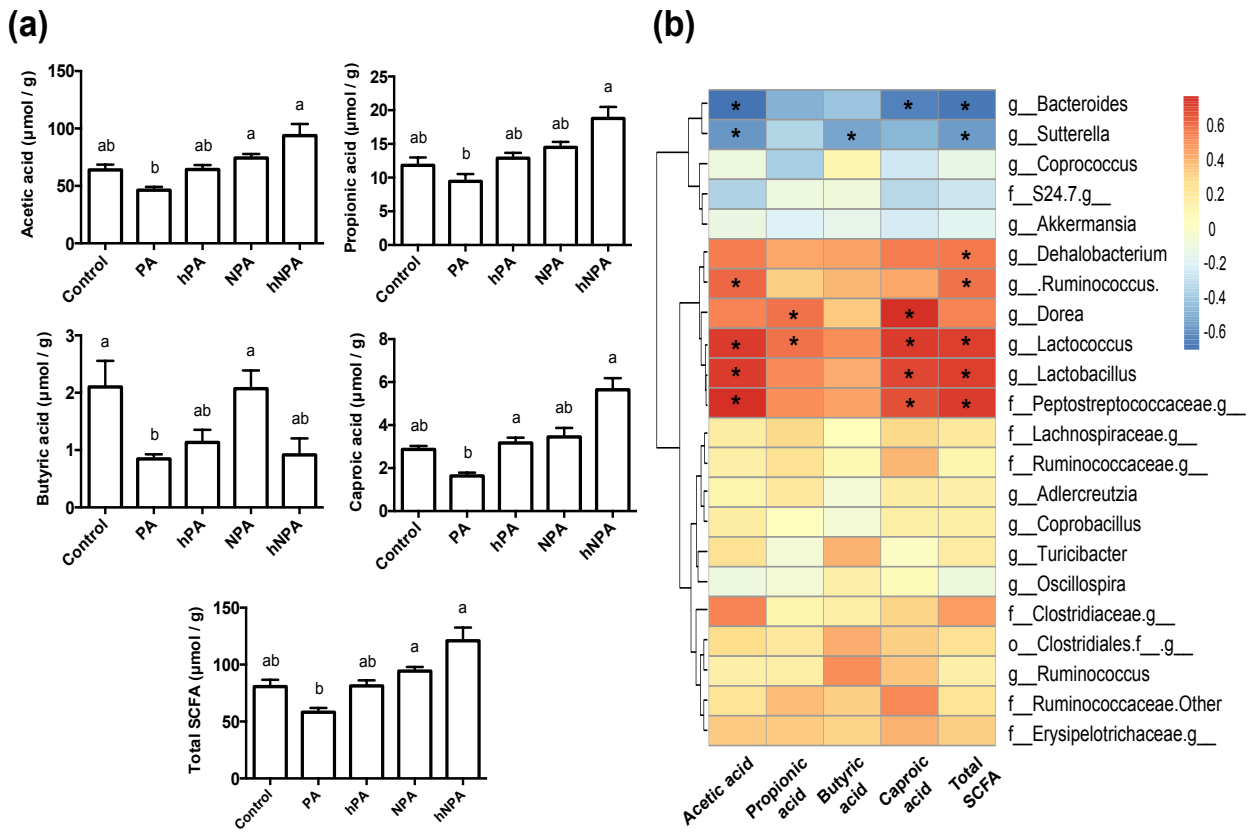


Figure 2.6: Pea fraction supplementation altered cecal short chain fatty acid (SCFA) production on a HFD. (a) Overall, the PA diet group shows reduced SCFA production compared to control and other pea fraction diets. Statistical significance between treatments indicated by different letters ($P < 0.05$; mean ± SEM; n=7-8/group). (b) Spearman correlation heatmap of cecal phylotype abundance and SCFA levels. Positive and negative correlations are shown from red to blue respectively. Those marked with * represent significant correlations with $P < 0.05$ after Bonferroni multiple testing corrections (n=36).

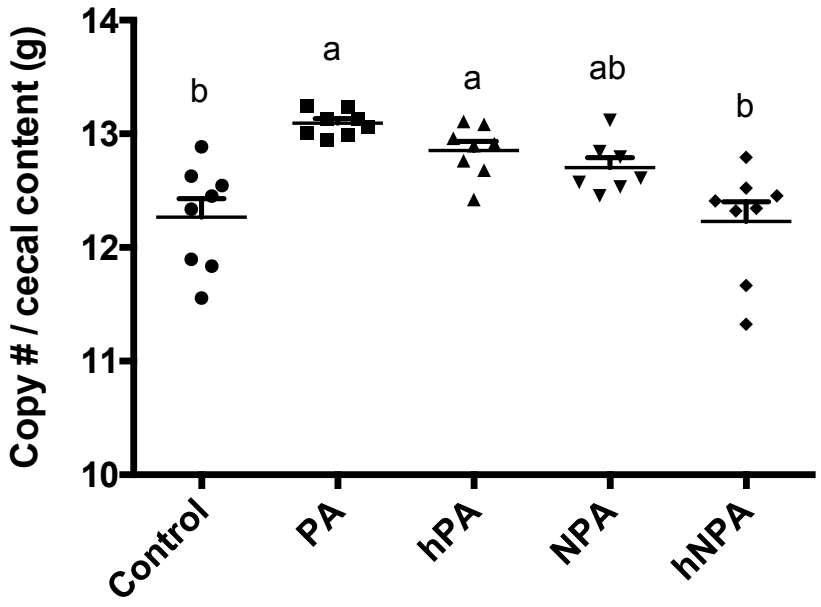


Figure 2.7: Total bacterial copy number in wet cecal content from mice fed a HFD supplementation with pea fractions. Proanthocyanidin-rich pea fractions had significantly higher total bacterial copy number compared to control. Statistical significance between treatments indicated by different letters ($P < 0.05$; n=7-8/group).

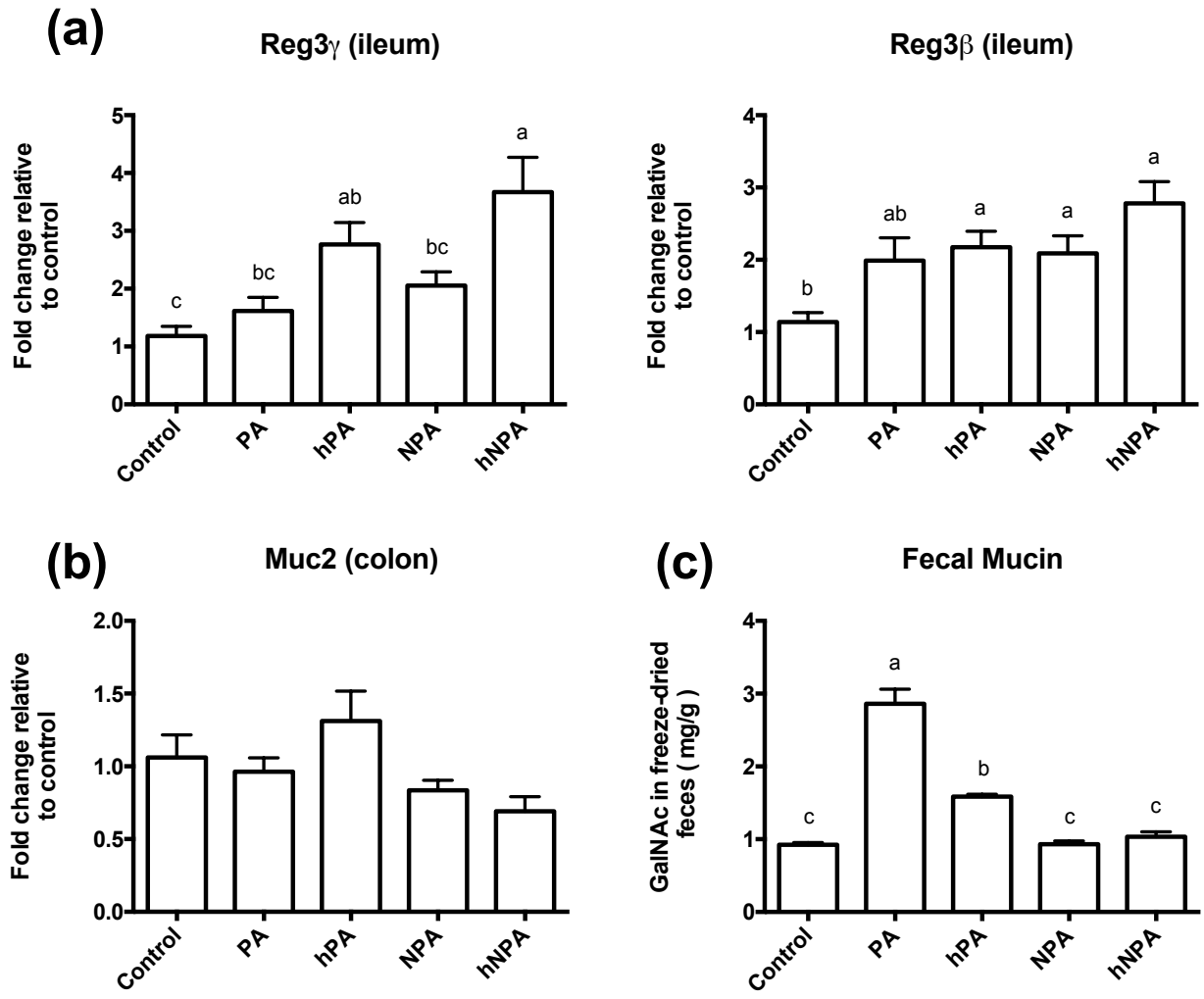


Figure 2.8: Reg3 antimicrobial peptide expression and mucin integrity altered after pea supplementation on a HFD. (a) Reg3β and Reg3γ show higher ileal expression levels regardless of pea cultivar combined from two separate trials (n=15-16/group). (b) Colon mucin-2 (Muc2) expression did not change with pea consumption (n=7-8/group); however, a significant proportion of mucin was quantified (c) within the fecal pellets of PA and hPA groups (n=4/group). Statistical significance between treatments indicated by different letters ($P < 0.05$; mean ± SEM).

2.3.5 Acid hydrolysis processing of pea seed coats restored pathogen resistance

Pathogen fecal load was determined to be highest in unprocessed cultivars PA ($P < 0.01$) and NPA ($P < 0.01$) compared to HFD control (Figure 2.9a). Both hydrolyzed diet variants (hPA and hNPA) did not show this increase and tended to reduce *C. rodentium* capacity to colonize, most notably between PA and hPA groups ($P < 0.08$). Colonization patterns of *C. rodentium* positively correlated with concentrations of monocyte chemotactic protein-1 (MCP1) in distal colon tissue collected three days post infection (Figure 2.9b & Figure 2.10). Dietary groups did not differ from control in MCP1 concentrations; however, the PA group was higher than hPA ($P = 0.06$) and hNPA ($P < 0.05$).

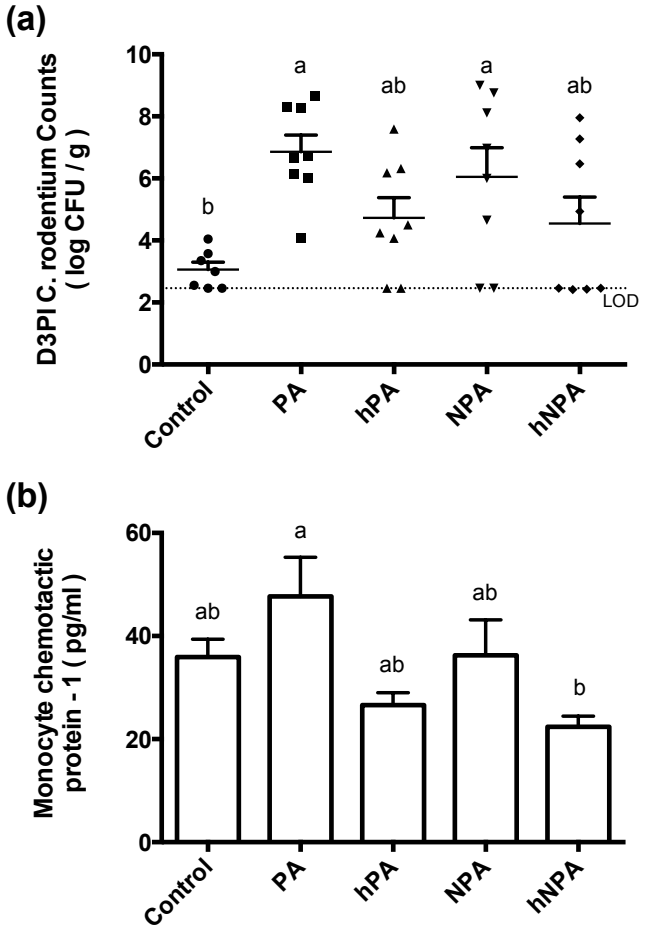


Figure 2.9: Unprocessed raw pea seed coat diets (PA & NPA) led to increased colon inflammation and pathogen load on a HFD. (a) *C. rodentium* load was significantly higher in raw pea seed coat supplemented group compared to control than their acid hydrolyzed fractions. Data was log transformed and normalized to fecal pellet weight. (b) Protein concentration of monocyte chemotactic protein 1 (MCP1) in infected mice colon tissue. Acid hydrolysis processing reduced pathogen colonization with similar reduction in MCP1 level in colon tissue, confirming pathogen induced tissue damage. Statistical significance between treatments indicated by different letters ($P < 0.05$; mean ± SEM; n=7-8/group).

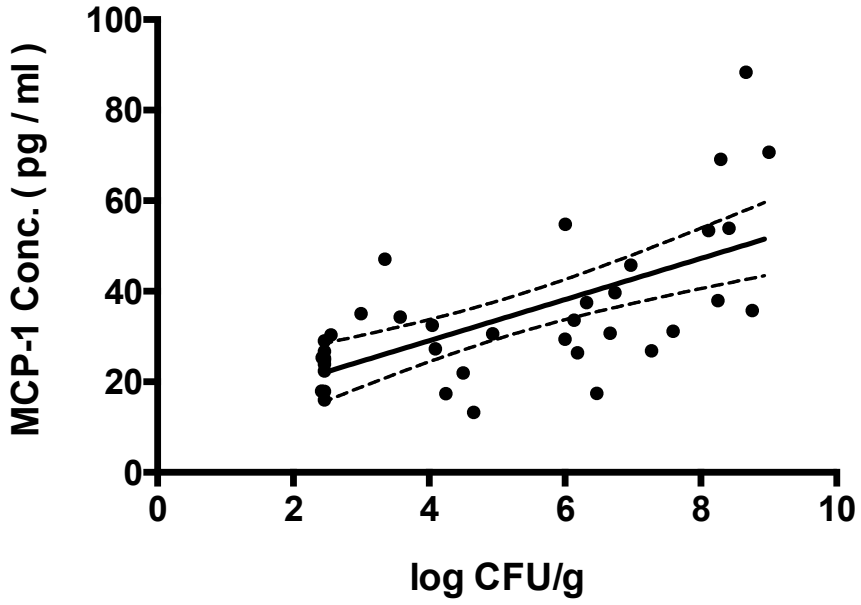


Figure 2.10: Spearman correlation of colon MCP-1 concentrations and *C. rodentium* fecal plating counts three days post infection (r = 0.662, $P < 0.001$; n=38).

2.4 Discussion

The present study demonstrates that the effects of supplementing pea seed coat fractions on host health and microbial community structure depend on pea cultivar and hydrolysis processing. In accordance with a previous study (K. Yang et al., 2015), supplementing hydrolyzed proanthocyanidin-rich pea seed coats reduced total percent weight gain on a HFD background. However, on a LFD background there was no impact of pea supplementation on weight gain compared to control, indicating that the benefits on a HFD are not a product of reduced palatability. Counter to the hypothesis, the HFD control had the lowest *C. rodentium* counts whereas pea supplementation yielded higher counts, especially when peas were consumed raw. Despite the fact that the HFD control group had the greatest weight gain, they also showed the lowest pathogen load. This is consistent with recent reports indicating that metabolic dysfunction, rather than weight gain, is linked to intestinal barrier dysfunctions and encourages enteric infections in mice (Thaiss et al., 2018a). Together, this suggests that the HFD control group may not have developed sufficient metabolic dysfunctions to increase pathogen susceptibility. In contrast, the addition of raw pea seed coat to the diet increased pathogen susceptibility while maintaining low body weights. Acid hydrolysis processing of both pea cultivars partly restored pathogen resistance. Surprisingly, the hydrolyzed proanthocyanidin-rich diet (hPA) reduced both weight gain and pathogen colonization compared to proanthocyanidin-rich diet (PA). Increased resistance after hydrolysis may be a result of an altered glucose metabolism, as benefits of hydrolyzed proanthocyanidin-rich pea seed coats have been previously proposed to improve pancreatic β-cell function, fat storage and insulin signaling mechanisms (K. Yang et al., 2015; K. Yang & Chan, 2017). Acid hydrolyzed proanthocyanidins monomers are shown to maintain antimicrobial and protein denaturing activity (Engels et al.,

2011) but have different kinetics of absorption and conversions during intestinal transit (Monagas et al., 2010). In addition, raw pea flours, regardless of cultivar, contain detrimental health components including phytate, lectins, and trypsin inhibitors, and acid hydrolysis may inactivate their negative effects in the digestive tract (Ayyagari et al., 1989). Accordingly, cooked pea seed coats have been shown to improve metabolic function and intestinal health compared to raw pea seed coat supplementation (Hashemi et al., 2017).

Dietary pea intervention had distinct effects on microbial community structure in the ileum, cecum and colon. The proanthocyanidin-rich diet (PA) had the greatest impact on microbial community structure, which became more prominent moving distally from the ileum to the colon. This could be explained by the polymeric structure of proanthocyanidins, which prevents their absorption in the upper gastrointestinal tract, allowing increased concentrations in the large intestine (Kemperman et al., 2010). Proanthocyanidins and flavon-3-ols both have antimicrobial and enzyme inhibition properties that can alter host physiology and commensal microbial populations (Cires et al., 2017; A.-N. Li et al., 2014). The antimicrobial activity directly impacts microbiota composition by selective inhibition of individual bacterial groups (Engels et al., 2011); enzyme inhibition indirectly impacts microbiota composition by inhibition of digestive enzymes, thus increasing the flow of carbohydrates and proteins to the large intestine, or by inhibition of microbial extracellular enzymes in the large intestine. This is supported by the severe reduction in alpha diversity in the cecal and colon contents of the PA group, which was much less apparent with in the hPA fed mice.

Pea seed coats from both the ‘Solido’ and ‘Canstar’ cultivars showed strong and distinct microbial modifying properties that were dependent on proanthocyanidin content and acid hydrolysis. Intact polymeric proanthocyanidin-rich pea supplementation lowered the *Firmicutes*

to *Bacteroidetes* ratio in the colon compared to the much higher ratio of the non-proanthocyanidin rich pea seed coat diets. Here we show that these drastic shifts were followed by higher colonization of *C. rodentium*. Resistance to infection has been associated with increases in *Bacteroidetes* and decreases in *Firmicutes* populations (Guan et al., 2016; Willing, Vacharaksa, et al., 2011). However, opposing evidence suggest shifts increasing *Firmicutes* abundance and localization towards intestinal epithelial cells is protective against pathogenic invaders (Baker et al., 2012). The inter-folding regions of the colon are enriched with the Firmicute families *Lachnospiraceae* and *Ruminococcaceae*, whereas *Bacteroidetes* families *Prevotellaceae*, *Bacteroidaceae* and *Rikenellaceae* are enriched in the luminal digesta (Gregory P. Donaldson et al., 2015). It is well established that competitive exclusion by commensals and the mucus barrier are the first defenses against pathogen colonization (Kamada et al., 2013). The reduction in *Firmicutes* and the associated reduction of SCFA concentrations may explain increased pathogen colonization in the raw proanthocyanidin-rich group. This is consistent with antibiotic modifications of microbial communities, which generally decrease richness, disrupt microbial community structure and exacerbate infection (Ju et al., 2017; Mullineaux-Sanders et al., 2017). Furthermore, the inherent increase of fecal mucin after proanthocyanidin-rich supplementation without increased Muc2 expression may highlight an ecological breakdown within the gut environment, decreasing pathogen resistance. Localization of *Firmicutes* to specific regions may be a basic phyla-wide function in pathogen recovery and clearance. However, these associations are most likely a product of key species with unique functions and cross-feeding rather than total phyla shifts.

Polymeric proanthocyanidins reduced the relative abundance of key commensals including *Lactobacillus* sp., *Bifidobacterium* sp., *Dehalobacterium* sp., *Allobaculum* sp., and

members of the *Ruminococcaceae* family while increasing *Turicibacter* sp., *Oscillospira* sp., and members of both the *S24-7* and *Rikenellaceae* families. Hydrolysis processing restored polymeric proanthocyanidin-dependent dysbiosis in all but the *Rikenellaceae* family, which stayed uniquely high in both raw and hydrolyzed fraction fed groups. In vitro assays performed with pea seed coat extracts from the 'Solido' cultivar confirms the direct inhibitory effect of proanthocyanidin fractions on *Allobaculum* sp. and *Ruminococcus* sp. mice gut isolates (Ares, 2017). Microbial shifts were associated with higher mucin content within the feces of the PA and hPA groups. We suspect the increase in mucin content resulted from a dysfunction in mucus layer formation because colon Muc2 gene expression did not differ between diet groups and may have also contributed to increased pathogen colonization. However, others have reported that fecal mucin content in a DSS-induced colitis model benefits intestinal health (Taira et al., 2015). The reduced in vivo effects after acid hydrolysis is most likely a product of increased absorption of monomeric units, limiting their interaction to microbial populations.

Improved intestinal integrity is linked to changes in antimicrobial peptide expression and SCFA production (Loonen et al., 2014; Tan et al., 2014). SCFA enhance intestinal function by providing colonocytes with a key source of energy (N. Singh et al., 2014). The switch from glucose to butyrate for cellular function reduces epithelial oxygenation that when present stimulates *C. rodentium* virulence (Lopez et al., 2016; Rivera-Chávez et al., 2016). Proanthocyanidins drastically reduced SCFA production compared to non-proanthocyanidin containing fractions and is likely a result of the antimicrobial effects of proanthocyanidin polymers reaching microbial populations. Decreased SCFA with proanthocyanidin supplementation could help explain the increased *C. rodentium* colonization and inflammation in these mice. Surprisingly, total bacterial load in the cecum was significantly higher in

proanthocyanidin supplemented mice indicating that the reduced SCFA production was not a product of reduced microbial populations. However, we acknowledge that this does not identify whether bacteria were alive or dead in the cecum. While the data indicates that early colonization and the pro-inflammatory response are worsened with pea supplementation, it is possible that pathogen clearance was impacted, which was not assessed in this study. Moreover, *Firmicutes* members positively correlated with high SCFA levels in the cecum, whereas key *Bacteroidetes* and *Proteobacteria* members were negatively correlated; again, indicating an important contribution from *Firmicutes* populations within the gut environment as it relates to intestinal integrity.

2.5 Conclusion

A major challenge in this study and other refined dietary interventions is identifying the direct and indirect health promoting mechanisms. The multifactorial dynamic relationship between gut commensal ecology and host defenses towards invading pathogens is essential in revealing the effects of diet on intestinal integrity. Peas certainly have a strong effect on microbial community structure and intestinal integrity that is highly dependent on proanthocyanidin content. This novel finding indicates that raw proanthocyanidin-rich pea fractions have negative effects on intestinal health, which are reduced following acid hydrolysis. Furthermore, hydrolyzed proanthocyanidins provide the best protection against high-fat diet induced weight gain, and ideally, only in the context of a high fat diet background.

3 CHAPTER 3: PHYTOCHEMICAL-INDUCED MUCIN ACCUMULATION IN THE GASTROINTESTINAL LUMEN IS INDEPENDENT OF THE MICROBIOTA

3.1 Introduction

The mucus layer of the mammalian gastrointestinal (GI) tract is a major part of the innate immune system. It is made of heavily glycosylated mucin glycoproteins produced by goblet cells and provides lubrication, hydration, and protection against pathogens and harmful substances that pass through the intestinal environment (Dhanisha et al., 2018). Two distinctive mucus layers exist: an inner layer devoid of bacteria and an outer loose layer filled with mucolytic and associated microbes that inhabit that particular niche (Jakobsson et al., 2015; Johansson et al., 2015; H. Li et al., 2015; Paone & Cani, 2020). Diet has been well recognized to alter host-microbe interactions in the GI tract that effect the integrity of the mucus layer and intestine (Forgie, Fouhse, et al., 2019). In particular, phytochemical consumption modulates mucus production, metabolism and has antimicrobial activities that directly affects the gut microbiota; however, the mechanism of their beneficial health outcomes is poorly understood.

Polyphenolic compounds make up the majority of the bioactive phytochemicals consumed in the human diet (Pandey & Rizvi, 2009). They are categorized as hydrolysable or condensed (non-hydrolysable) high molecular weight tannins (gallotannins & ellagitannins, and proanthocyanidins respectively) and low molecular weight polyphenols that include phenolic acids, flavonoids, lignans, stilbenes, and curcumins (Crozier et al., 2006). Because condensed tannins are resistant to host acid and enzyme hydrolysis, they are more likely to reach the gut microbiota compared to the quickly absorbed hydrolysable tannins and low molecular weight phytochemicals. Health benefits have been attributed to their free radical scavenging capacity to

neutralize inflammation-causing reactive oxygen species, as well as their direct antimicrobial effect on microbial communities, and the indirect production of bioactive polyphenolic catabolites by the microbiota (Kawabata et al., 2019; A.-N. Li et al., 2014).

Phytochemicals have an ability to alter mucus physiology, and along with their numerous forms and bioactivities have shown contradictory effects on resisting pathogen colonization and virulence in the GI tract (Forgie, Gao, et al., 2019; Marta Wlodarska et al., 2015). Phytochemical research has focused on improving intestinal barrier integrity with the mechanism of action hinting towards its ability to stimulate the mucus layer, with an increase in mucus production and thickness considered beneficial (Arike & Hansson, 2016; Bergstrom et al., 2010; Marta Wlodarska et al., 2015). However, thickness and expression of mucus-related genes does not necessarily mean that the mucus layer has formed properly for protection. In agreement, a previous study conducted by our group examining supplementation with peas (*Pisum sativuum*) rich or low in polyphenol content showed that the polyphenolic-rich pea diet increased the amount of fecal mucin in the lumen (Forgie, Gao, et al., 2019). Excess mucin in the GI lumen was associated with greater *Citrobacter rodentium* colonization and activation of a proinflammatory response (Forgie, Gao, et al., 2019). This diet-induced phenotype has been experimentally tested and confirmed *in vitro* to be caused by the ability of galloylated tannins and related compounds to directly cross-link with purified mucins, thereby altering the viscoelastic properties of mucus (Georgiades et al., 2014). Therefore, phytochemical consumption may increase the accessibility of mucus glycans to the gut microbiota, but to what extent this drives gut ecology has not been determined.

In this study, we investigated whether the presence of microbes is required for the previously observed increase in fecal mucin in response to polyphenol-rich pea seed coat

consumption. Whether changes in the gut microbiota drives the mucus phenotype or is a consequence of host-diet interactions remains unknown. We hypothesized that luminal mucin accumulation in the GI tract from phytochemical supplementation is dependent on the microbiota. We fed the proanthocyanidin (PA) and non-proanthocyanidin (NPA) high (20% w/w)-fat pea diets used in our previous study to germ-free (GF) mice and measured fecal mucin (Forgie, Gao, et al., 2019). In addition, we tested how the non-hydrolysable PA-diet compares to a hydrolysable red-osier dogwood (ROD; *Cornus sericea*) extract on fecal mucin and microbial communities when fed to conventional mice. ROD extracts have been shown in pig models to improve feed efficiency and promote gut resistance to invading pathogens (Koo et al., 2021); however, the underlying mechanism is poorly understood and possibly driven by changes to the mucus layer. The identification of mucin-degrading bacteria and their impact on the gut niche environment in response to dietary phytochemicals will help determine their contribution to gut ecology and health.

3.2 Methods

3.2.1 Animals and diet treatments

All animal experiments were conducted in accordance with guidelines set by the Canadian Council on Animal Care and approved by the Animal Care and Use Committee at the University of Alberta (Edmonton, AB, Canada). All mice used in this study were bred and maintained in the University of Alberta Axenic Mouse Research Unit. Mice were eight to ten-weeks-old and allowed to acclimatize on an autoclaved standard chow diet (5010 maintenance diet, LabDiet, St. Louis, MO, USA) for a week prior to dietary treatments. Table 3.1 provides formulations of the isocaloric treatment diets, which were balanced for macronutrients and

insoluble fiber with cellulose. Eight female GF Swiss-Webster mice were housed four per open-top cage in the same GF isolator (CEP Standard Safety, McHenry, Illinois, USA) and handling was done directly inside the isolator. GF mice were fed treatment diets containing pea seed coats flours rich ('Solido' cultivar; PA) and poor ('Canstar' cultivar; NPA) in proanthocyanidin content as describe previously (Forgie, Gao, et al., 2019). The diets provided to GF mice were prepared with 15 g instead of 10 g of vitamin mix per kg diet to account for the loss imposed by irradiating the diets to 10 kGY, which was done at the Cross-Cancer Institute at the University of Alberta. Germ free status following the diet intervention was confirmed by anaerobic and aerobic culture of fecal pellets at termination. To investigate the role of microbes and test a second polyphenolic source on the fecal mucin phenotype, we fed a control diet (Control), a PA diet (PA), and the ROD supplemented diet (DW) to conventional Swiss-Webster mice (Table 1). Mice were housed two to four per cage using the Tecniplast Isocage-P bioexclusion system (Buguggiate, VA, Italy) and all animal handling was done in a biosafety cabinet. Control and PA diet groups included two male and two female mice housed separately to determine if sex plays role in the fecal mucin phenotype, previously identified in female mice only (Forgie, Gao, et al., 2019). The spray-dried ROD extract (Red Dog Enterprises Ltd., Winnipeg, MB, Canada) was added to the dogwood (DW) diet at 4% as done in previous studies (Koo et al., 2021). ROD extracts can contain bioactive phenolic compounds at 4% to 22% depending on the season (Isaak et al., 2013). The addition of polyphenolic extracts was calculated based on the average total phenolic content of 10% in the ROD extract for the DW diet and 4.51% in the pea seed coat flour for the PA diet. The final amount of proanthocyanidin content in the diets was 0.4% per gram of diet. All diets were prepared aseptically as powdered diet at the University of Alberta and mice had *ad libitum* access to water and diet throughout the two-week long diet intervention. Body

weights were taken every second day and fecal samples were collected aseptically at beginning (day 0) and end (day 14) of the dietary treatment for conventional mice. All samples were stored at -80°C until use.

Table 3.1: HFD background composition of dietary treatments (g/kg).

	Germ-free		Conventional		
Component, g/kg	**NPA**	**PA**	**Control**	**PA**	**DW**
Lard (Tenderflake)	190	190	190	190	190
Flaxseed oil	2	2	2	2	2
Corn oil (Mazola)	8	8	8	8	8
Casein	267	262	270	262	267
L-Methionine	2.5	2.5	2.5	2.5	2.5
Dextrose	214	214	214	196	195
Corn Starch	194	194	193.4	180.7	180.4
Cellulose	0	0	50	0	45
Mineral Mix	51	51	51	51	51
Vitamin Mix	15	15	10	10	10
Inositol	6.3	6.3	6.3	6.3	6.3
Choline Chloride	2.8	2.8	2.8	2.8	2.8
Canstar (NPA) seed coat	71.5				
Solido (PA) seed coat		96.5		88.7	
Red osier dogwood (DW)					40
Total Weight (g)	**1024.1**	**1044.1**	**1000**	**1000**	**1000**
Fat / g	0.2	0.2	0.2	0.2	0.2
Protein / g	0.3	0.3	0.3	0.3	0.3
Carbohydrate / g	0.4	0.4	0.4	0.4	0.4
Insoluble fiber / g	0.04	0.05	0.05	0.05	0.05
Energy (At Water) kcal / g	4.4	4.3	4.5	4.4	4.4
% PACs / g	**0**	**0.4**	**0**	**0.4**	**0.4**

3.2.2 Fecal Mucin Assay

Fecal pellets from individual mice were pooled across daily fecal collections throughout the last four days of the two-week dietary treatment. Pooled fecal collections were subsequently freeze-dried and ground to a powder. A fluorometric assay kit (Fecal Mucin Assay kit; Cosmo Bio co. ltd, Carlsbad, CA, USA) that quantifies N-acetylgalactosamine (GalNAc), the reducing end sugar of the O-linked glycan chain, was used to determine the mucus content (Crowther & Wetmore, 1987).

3.2.3 Microbial community analyses

Total DNA was extracted from colon contents using the QIamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) with an additional bead-beating step using ~200 mg of garnet rock at 6.0 m/s for 60 s on a FastPrep-24 5G instrument (MP Biomedicals). Paired-end sequencing was accomplished using the Illumina MiSeq Platform (2x300 cycles; Illumina Inc., San Diego, CA, USA). Amplicon libraries were constructed according to the protocol from Illumina (16S Metagenomic Sequencing Library Preparation) that amplified the V3-V4 region of the bacterial 16S rRNA gene: 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Raw sequences were processed Quantitative Insight into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2019) pipeline using DADA2 to filter, trim and merge paired-end reads into amplicon sequence variants (ASVs). Phylogenetic trees were constructed using the qiime alignment (mafft; mask) and qiime phylogeny (fasttree; midpoint-root) function. Taxonomy was

assigned using the qiime feature-classifier classify-sklearn function using the SILVA v138 database trained for the specific amplicon region (Bokulich et al., 2018b). QIIME2 files (.qza) were imported into R using qiime2R (version 0.99.4) package and analyzed with phyloseq (version 1.34.0) package (McMurdie & Holmes, 2013). Sequences belonging to 'chloroplast' and 'mitochondria' were removed. In addition, '*Lactococcus*' sequences were dropped from the analysis because they were suspected to be a contaminant from casein (Bisanz et al., 2019; Kimoto et al., 2003). Numbers assigned to ASVs reflect their total counts from highest to lowest count across samples. Alpha diversity (Observed, Shannon, phylogenetic diversity (PD)) and beta diversity based on a Bray-Curtis dissimilarity index were done with rarefied reads at a count of 8444.

3.2.4 Statistical analysis

Significance testing and graphing for body weights and fecal mucin were done in GraphPad Prism 6 (Graphpad Software, LaJolla, CA. USA) using a t-test or a parametric anova corrected for multiple comparisons with Tukey's post-hoc test. Data was presented as mean ± standard error of the mean and letters were used to denote a significance when appropriate. Statistical significance for alpha diversity was determine with the anova TukeyHSD() correction function. Principal coordinate analyses (PCoA) was plotted using the phyloseq package and clustering significance was determined using the 'betadisper' function (Anderson, 2006) for dispersion and 'pairwiseAdonis.dm' function (Martinez Arbizu, 2017) for orientation. Differential abundance analysis was done with DESeq2 using non-rarefied reads and tree_glom() function to merge similar ASVs. The 'log2foldchange' of only the ASVs with a *P* value less than 0.05 were plotted with bolded ASVs signifying the significant adjusted *p* value < 0.10, < 0.05 (*), < 0.01 (**) and < 0.001 (***).

3.3 Results

3.3.1 Phenolic compounds increase mucin content in the gut independently of the microbiota

GF mice fed the PA diet had higher amounts of fecal mucin ($P < 0.05$) compared to the NPA control diet (Figure 3.1a). Phytochemicals directly increased fecal mucin in the GI tract independently of the microbiota. The presence of microbes did not alter this outcome as conventional mice displayed a similar increase in fecal mucin in the PA ($P < 0.05$), as well as the DW ($P < 0.05$) diet compared to control (Figure 3.1b). Polyphenolic diets did not affect the weights of conventional mice over the course of the two-week experiment (Figure 3.1c). Conventional female and male mice fed the Control and PA diets displayed no difference in weight gain or amount of fecal mucin. Although the limited sample size may not adequately represent the sex effect, the impact of phytochemicals on fecal mucin was not different between sexes.

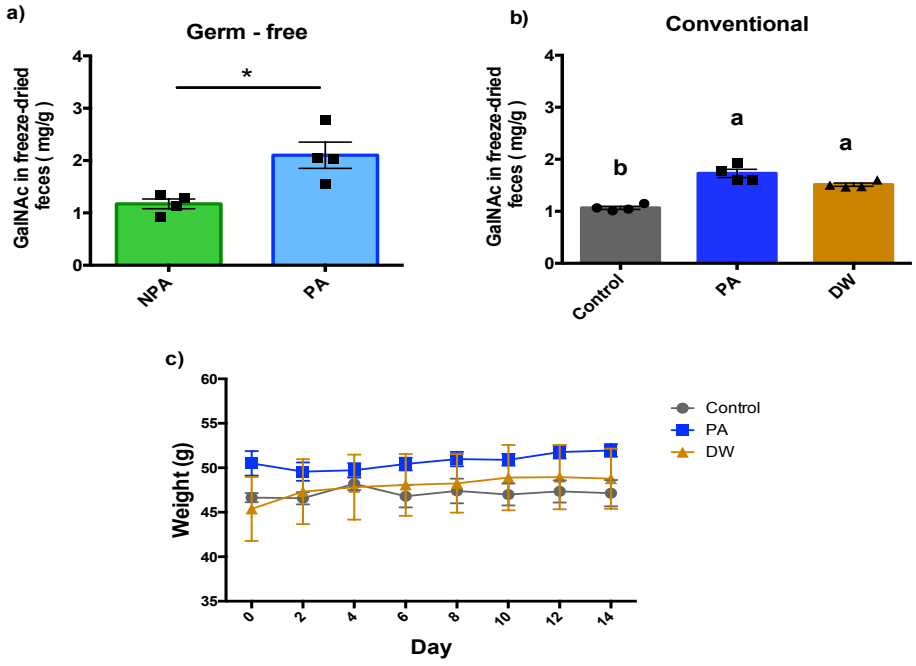


Figure 3.1: Phytochemical diets (PA and DW) increased the mucin content in the gastrointestinal tract independently of the microbiota. (a) Germ-free Swiss-Webster mice fed the PA diet also responded by increasing fecal mucin content, a novel finding that suggest phytochemicals act directly on host mucus chemistry independently of the microbiota (n = 4; * $P < 0.05$). (b) Dietary phytochemicals significantly increased fecal mucin content in PA ($P < 0.01$) and DW ($P < 0.05$) groups of conventional mice (n = 4). (c) Conventionalized Swiss-Webster mice weights were unaffected by dietary treatment (n = 4).

3.3.2 Changes in microbial composition in response to phenolic compound rich diets

Phytochemical diets associated with the fecal mucin phenotype revealed similar changes to microbial communities. PCoA was conducted using Bray-Curtis dissimilarity metric to visualize microbial communities before and after dietary treatment and identify overall differences between treatments. We analyzed microbial communities without separating female and male mice because sex did not appear to alter the amount of mucin recovered in the fecal samples. Microbial communities prior to diet intervention were not different between groups (Adonis unadjusted day 0 to control; PA: $R^2 = 0.10$, $P = 0.67$ & DW: $R^2 = 0.11$; $P = 0.56$) but clustered distinctly after 14 days (Adonis unadjusted day 14 to control; PA: $R^2 = 0.27$, $P = 0.11$ & DW: $R^2 = 0.79$, $P = 0.02$). All dietary treatments led to distinct changes in microbial communities (Adonis unadjusted day 0 to day 14; Control: $R^2 = 0.53$, $P = 0.05$; PA: $R^2 = 0.55$, $P = 0.04$ & DW: $R^2 = 0.76$, $P = 0.05$) (Figure 3.2a). Dispersion analysis between groups did not pass significance and shows that the within treatment variability was consistent between groups. PCoA of day 14 microbial communities revealed that both PA and DW diets had similar changes to microbial communities but were still distinct from one another. Principal component (PC) 1,

PC2, and PC3 explains 68.2%, 10.3%, and 8.8% respectively and when plotted as PC1vs PC2 (Figure 3.2b) and PC2 vs PC3 (Figure 3.2c) distinct clustering between treatments can be visualized. Alpha diversity metrics of day 0 and day 14 microbial communities show that all dietary treatments reduced the unique counts (Observed; $P < 0.05$); however, diversity as determined by Shannon index revealed that all but the DW group ($P < 0.05$) remained constant compared to both Control and PA groups (Figure 3.2d). The PD index revealed that Control and PA diets had lower microbial diversity ($P < 0.05$) at day 14 compared to day 0, whereas the DW diet maintained a similar diversity as at day 0 before diet treatment (Figure 3.2d).

The phytochemical diets drastically altered the colon microbiota as determined by differential expression of ASVs using DEseq2 compared to control (Figure 3.2e,f). This includes numerous ASVs assigned to taxa belonging to the Firmicutes phylum. Compared to the control group, the PA diet reduced *Romboutsia* and *Erysipelatoclostridiaceae* while increasing *Lachnospiraceae*, *[Clostridium] leptum, [Eubacterium] coprostanoligenes*, and a bacterium from the *Clostridia vadinBB60* group (Figure 3.2e). The DW diet reduced *Romboutsia,* many *Ruminococcaceae members, Oscillospiraceae, [Eubacterium] coprostanoligenes*, *Peptococcaceae*, and *Ethanoligenenaceae* members of Firmicute populations (Figure 3.2f). The DW diet increased *Akkermansia muciniphila*, *Parasutterella, Alistipes*, *Turicibacter*, and *Bacteroides thetaiotaomicron,* along with an unclassified member from the Muribaculaceae family.

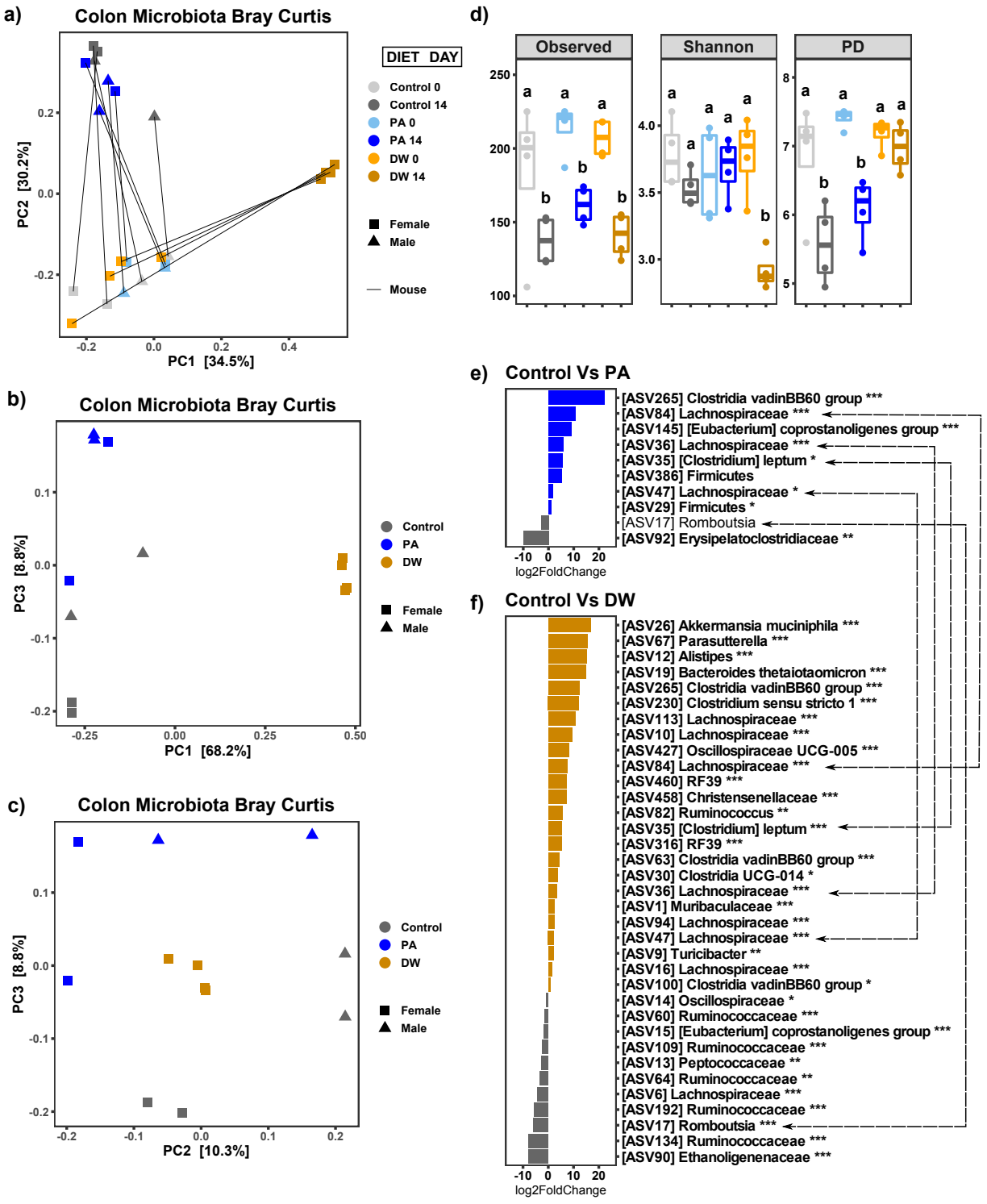


Figure 3.2: Phytochemical diets associated with the fecal mucin phenotype revealed similar changes to microbial communities of conventional mice. (a) Microbial communities prior to diet

intervention were not different between groups (Bray-Curtis PCoA; Adonis unadjusted day 0 to control; PA: $R^2 = 0.10$, $P = 0.67$ & DW: $R^2 = 0.11$, $P = 0.56$) but clustered distinctly after 14 days (Bray-Curtis PCoA; Adonis unadjusted day 14 to control; PA: $R^2 = 0.27$, $P = 0.11$ & DW: $R^2 = 0.79$, $P = 0.02$). All mice had similar microbial communities Control: $R^2 = 0.53$, $P = 0.04$; PA: $R^2 = 0.55$, $P = 0.05$ & DW: $R^2 = 0.76$, $P = 0.03$). At day 14, diet (b) Principal coordinate analysis plot of day 14 microbial communities using principal component 1 (PC1; 68.2%) and principal component 3 (PC3; 8.8%), along with (c) principal component 2 (PC2; 10.3%) plotted against PC3 shows a distinct but subtle similarity between dietary groups. (d) Alpha diversity metrics (Observed, Shannon, PD) showing day 0 and day 14 microbial communities shows that all treatment diets reduced diversity ($P < 0.05$), however, DW diet specifically reduced diversity (Observed and Shannon; $P < 0.05$) compared to both Control and PA groups. Differential expression of ASVs as determine by DEseq2 were plotted for (e) PA and (f) DW compared to Control group. Consistent ASVs that respond to PA and DW diets are noted with hashed arrow lines, this includes an increase in *Lachnospiraceae* and [*Clostridium*] *leptum* species along with a decrease in a *Romboutsia* species (n = 4; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.4 Discussion

Phytochemicals are secondary metabolites produced by plants to communicate with their environment. They are generally considered non-essential nutrients that contribute to physiology through numerous bioactive properties ranging from antioxidant, anti-inflammatory, and antimicrobial to protein chelation and enzyme inhibition (Balaji et al., 2016). Thousands of phytochemicals have been identified and are noted for their free radical scavenging capacity to

neutralize reactive oxygen species associated with inflammation and disease (A.-N. Li et al., 2014). Polymeric phytochemicals, such as proanthocyanidins, are the most abundant and bioactive polyphenols in our diet (Monagas et al., 2010). They are composed of polymeric flanva-3-ol monomers, known as condensed tannins and linked through double A-type and single B-type linkages (Monagas et al., 2010). The degree and type of polymerization, along with the galloylated moieties determine their physicochemical structure and thus their bioactivities on both the gut microbiota and host (Girard & Bee, 2020; Yokota et al., 2013b). The bioactive properties of polyphenolic compounds and their impact on physiology have made them good candidates for antibiotic alternatives and therapeutics against enteric infection (Willing et al., 2018b).

In this study, we determined that the increase in fecal mucin in response to polyphenol-rich diets observed previously (Forgie, Gao, et al., 2019; Taira et al., 2015) occurs independently of the gut microbiota. The inclusion of polyphenols in diet at 0.4% in this study appears safe, as no change in body weight was documented throughout the two-week dietary intervention. This is in accordance with previous studies that show that similar amounts of phytochemicals in diets do not negatively impact growth, and may even improve it (Forgie, Gao, et al., 2019; Koo et al., 2021). Despite being quite different with regards to phenolic compound composition, the PA and DW diets both led to increased fecal mucin, suggesting that flavan-3-ol condense tannins from peas, beans or fruit are not unique in their ability to increase fecal mucin (Forgie, Gao, et al., 2019; Taira et al., 2015; Tanaka et al., 2019). This occurs independently of the gut microbiota; however, more studies are required to determine the main phytochemicals or groups of chemicals responsible for the mucin phenotype. Our results provide *in vivo* support to the previous *in vitro* experiments showing that phytochemicals, particularly the galloylated polyphenols, directly

disrupt the viscoelastic properties of mucus by disrupting binding among mucin glycoproteins (Georgiades et al., 2014). In our previous study (Forgie, Gao, et al., 2019), the pea seed coat supplementation (PA diet) led to faster colonization of *C. rodentium*, a common enteric mouse pathogen, and we suspect that this is a consequence the direct impact of polyphenols on the mucus layer. Most studies have focused on the beneficial roles that phytochemicals have on health and has been extensively reviewed (Andersen-Civil et al., 2021); however, little is mentioned of the phytochemical-mucus interactions in the gut outlined herein. Therefore, in addition to confirming that phytochemical directly increase luminal mucin concentration, an analysis of the colonic microbial community was conducted, with a particular interest in mucolytic microbes that may benefit under these conditions.

Microbial community analysis revealed that pea seed coat and ROD-supplementation alters the gut microbiota. The PCoA plot analyses revealed that the DW diet substantially altered microbial composition compared to the PA diet. Shannon diversity and PD values of the colonic microbiome in the PA group is consistent with our previous experiment (Forgie, Gao, et al., 2019). The reduced Shannon diversity of the DW group could be explained by the antimicrobial properties of ROD phytochemicals; however, more research is required to confirm the direct antimicrobial actions of ROD supplementation. A study in pigs with a lower inclusion rate of 0.5% ROD extract showed no effect on ileal microbial alpha diversity (Shannon and Simpson) but a prebiotic effect on Lactobacillus species was noted along with no change to growth performance (Zheng et al., 2021). A study in weaned pigs challenged with *Escherichia coli* k88+ found that 2% and 4% ROD extract diets conferred beneficial effects on growth performance; however, microbial composition was not assessed (Koo et al., 2021). Phylogenetic diversity in the DW group was maintained at day 14, which could be explained by the increased abundance

of *Akkermansia muciniphila*, *Parasutterella* and *Turicibacter,* which only appeared in this DW group at day 14 and were not detected in any group at day 0. Although we did not detect these microbes in the sequencing data of Control and PA groups at day 14, they may have been present below our detection limit for 16s rRNA sequencing. The ROD extract effectively reduced the abundance of some species thereby opening up a niche for others. For this reason, we see higher PD values in the DW group compared to Control and PA groups.

Microbes that were enriched by both phenolic diets include *Lachnospiraceae* and [*Clostridium*] *leptum* species, which may reflect a response to increased mucin availability. Luminal mucin levels were confirmed greater in both PA and DW diet groups compared to Control. We characterized an increase in abundance of *A. muciniphila*, a well-known mucolytic microbe, in the DW group but not the PA group. Mucin supplementation has been confirmed in mice to encourage mucin degrading bacteria, such as *A. muciniphila*, and mitigates diet-induced microbiota perturbations (Pruss et al., 2021). Moreover, phytochemicals are well-known to increase the abundance of *A. muciniphila* and improved health outcomes (Anhê et al., 2016). The phytochemical-induced mucin phenotype may partly explain their increased abundance in the gut; however, the absence of *A. muciniphila* in the PA diet suggests other factors contributed to their increase in the DW group. A study in mice using jaboticaba fruit, which is high in flavan-3-ols, at 5%, 10%, and 15% of diet found an increase in *A. muciniphila* at only 10% and 15% (Soares et al., 2021) suggesting a dose-dependent threshold that supports their growth likely exists in the gut. The lack of *A. muciniphila* in the PA group of this present study is inconsistent with our previous study (Forgie, Gao, et al., 2019) and suggest mice did not receive the necessary dose of polyphenols to encourage *A. muciniphila* fitness in the gut. The moderate effect of the PA diet on microbial communities could be explained a loss in the antimicrobial

actions of pea phytochemicals after long-term storage. As a result, the PA diet had less severe impacts on microbial community structure as compared to the DW diet and suggested that the interactions among microbes are stable enough to prevent *A. muciniphila* expansion even with increased access to mucin glycans. In addition, both PA and DW diets led to a consistent decrease in abundance of *Romboutsia*, a species that is highly adapted to nutrient-rich environments (Gerritsen et al., 2017) and a potential marker of stability in the gut. The competitive advantage gained by mucolytic bacteria may have altered the nutrient-rich niches in the gut that genera like *Romboutsia* depend on for growth. The mucus layer supports microbial niches by directly providing glycans for energy and indirectly through cross-feeding from one microbe to another (H. Li et al., 2015). Further knowledge of these interactions will provide the foundational framework necessary to understand host-microbe stability and the role mucus plays in host health.

3.5 Conclusion

The production and maintenance of the mucus layer is a vital part of intestinal homeostasis. It has become clear that host mucus provides a foundation of host derived glycans that supports mutualism and commensalism among microbes in the gut. Understanding how phytochemicals influence the viscoelasticity of the mucus layer will help to determine the best therapeutic use of phytochemicals to promote health. This research provides insight into establishing the mechanisms involved in the ability of mucus to stabilize gut ecology and control microbial communities. Further studies are required to determine the specific phytochemical compounds and structure that induce mucus secretion and/or disrupt mucin binding and formation.

4 CHAPTER 4: EXCESSIVE ORAL INTAKE OF VITAMIN B12 ALTERS HOST-MICROBE INTERACTIONS THAT STIMULATE *CITROBACTER RODENTIUM* GROWTH AND VIRULENCE IN MICE

4.1 Introduction

Vitamin B12 (cobalamin) is a cobalt-containing corrinoid molecule required for fundamental biological processes in both humans and bacteria. It is made exclusively by microorganisms and belongs to a family of organometallic cofactors called cobamides (Kennedy & Taga, 2020). With few exceptions, such as ruminants which depend on the biosynthesis of cobalamin by resident microbes, most animals rely on its bioaccumulation in the food chain (Watanabe & Bito, 2018). Humans must obtain cobalamin through diet because the only significant population of microbes that could produce cobalamin resides in the colon, past the absorption site in the small intestine (Degnan, Taga, et al., 2014). Dietary sources of cobalamin in humans are mainly of animal origin, but supplements, fortified food products, fermented foods, and some plants and algae are available as alternatives (Watanabe, 2007).

Cobamides are essential for bacteria, playing a significant role in supporting enzyme activity in the cytosol and as coenzyme riboswitches that regulate gene expression in the nucleus (Tucker & Breaker, 2005). Genomic studies revealed that widespread cobalamin sharing between microbes can impact microbial growth and metabolism through numerous cobamide-/corrinoid- dependent enzymes and transporter proteins (Degnan, Barry, et al., 2014a; Nahvi et al., 2004; Sokolovskaya et al., 2020). Because of this, B12 and cobamide derivatives likely play a more critical role in microbe-microbe interactions that modulate microbial ecosystems than previously understood (Degnan, Taga, et al., 2014). For example, B12 uptake by the gut

commensal *Bacteroides thetaiotaomicron* was shown *in vitro* to limit the production of Shiga toxin-2 produced by Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) (Cordonnier et al., 2016a). This has been attributed to reducing the ability of EHEC to use ethanolamine (a breakdown product of lipid membranes and major metabolite found in the gut) by limiting the availability of cobalamin required to activate adenosylcobalamin-dependent ethanolamine ammonia-lyase. Regulation of ethanolamine metabolism influences the growth and/or virulence of several *Enterobacteriaceae* and Firmicute species (Rowley & Kendall, 2019). Therefore, it follows that competition among microbes for cobalamin can impact the activity of ethanolamine-utilizing bacteria. In addition, *B. thetaiotaomicron* creates competition by using a surface-exposed lipoprotein, which binds cobalamin with such affinity that it can remove it from intrinsic factor, a cobalamin transport protein necessary for absorption in humans and animals (Wexler et al., 2018). However, additional competitive and syntrophic interactions may exist between microbes for cobalamin in the gastrointestinal (GI) tract and to what extent this impacts bacterial pathogenesis in the gut remains poorly understood.

Daily oral cobalamin supplements can contain doses that far exceed (as high as 10,000 μg/tablet) the recommended daily amount of 2.4 μg/day in humans. High exposures are required when normal absorption is impeded and are generally considered safe. The practice of over-supplementing cobalamin to ensure adequate absorption is supported by the fact that no upper limit has been set for B12 supplementation. We hypothesized that excessive cobalamin supplementation alters the gut microbiota's functional activity, creating a favourable environment for pathogen colonization and pathogenesis. To test this, we supplemented B12 to mice in drinking water. We challenged the animals with *Citrobacter rodentium*, a natural mouse-specific pathogen that mirrors the attaching and effacing pathology seen in human EHEC

infections (Crepin et al., 2016). We evaluated the direct and indirect impact of excessive amounts of cobalamin on host-microbial interactions.

4.2 Methods

4.2.1 Mice and vitamin B12 supplementation

Animal experiments were conducted in accordance with guidelines set by the Canadian Council on Animal Care and approved by the Animal Care and Use Committee at the University of Alberta (Edmonton, AB, Canada). All mice were raised and maintained under specific pathogen-free or germ-free conditions. Six to seven-week-old female C3H/HeOuJ mice (Jackson Laboratories, Maine, USA) were randomly housed four or five per cage. Eight-week old female C57Bl/6J mice (University of Alberta, AB, Canada) were housed three per cage using the Tecniplast Isocage-P bioexclusion system (Buguggiate, VA, Italy). Eight-week old germ-free female C57Bl/6J mice were housed in an isolator (CEP Standard Safety, McHenry, Illinois, USA) with open cages in the University of Alberta Axenic Mouse Research Unit. All mice were allowed to acclimatize for one week with *ad libitum* access to water and standard chow containing approximately 0.08 mg of cyanocobalamin per kilogram of diet post-autoclaving (2020SX; Envigo-teklad, Indiana, USA). Calculated based on the average consumption of 5 g of diet per mouse per day, the standard chow diet alone contributed approximately 0.4 μg of cyanocobalamin. Mice received filter-sterilized drinking water supplemented with or without B12 in the form of cyanocobalamin (V2876, Sigma-Aldrich, St. Louis, MO, USA) at 40 μg/ml, approximately 100 times the amount in the diet. The recommended daily allowance of mice is approximately 0.05 μg/day based on a diet that contains 10 μg of B12 per kilogram and deemed adequate (Nation Research Council (US) Subcommittee on Laboratory Animal Nutrition, 1995).

In addition, different forms of B12 were investigated in conventional C57Bl/6J mice by supplementing cyanocobalamin and methylcobalamin (Thermo Fisher Scientific, Massachusetts, USA) at 10 µg/ml and 40 µg/ml in drinking water. In all experiments, cages were randomly assigned to treatment groups: control (CON) and B12 supplemented (cyanocobalamin at 10 µg/ml, CNCbl10 and 40 µg/ml, CNCbl40; methylcobalamin at 10 µg/ml, MeCbl10, and 40 µg/ml, MeCbl40). After two weeks of water treatment, survival (SURV) and early-stage pathogen colonization (EPC) experiments were performed in C3H/HeOuJ mice using *C. rodentium*. A twenty percent loss of initial body weight was selected as a humane endpoint for mice in the SURV experiment as previously described (Willing, Vacharaksa, et al., 2011). For the EPC experiment, after two weeks of B12/water treatment, two mice from each cage were euthanized (naïve_CON & naïve_CNCbl40). The remaining mice continued on water treatment for subsequent *C. rodentium* challenge (inf_CON & inf_CNCbl40). To confirm the pre-challenge B12-induced phenotypes observed in C3H/HeOuJ mice and to test the role of microbes, we additionally treated germ-free and conventional C57Bl/6J mice for two weeks (Figure 4.1).

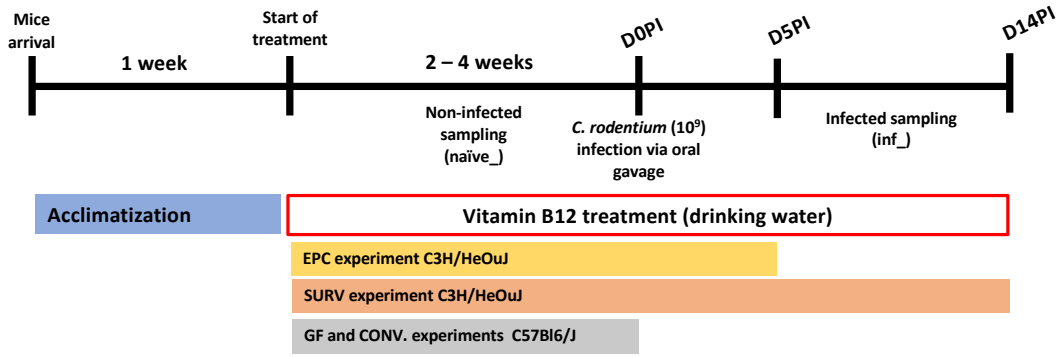


Figure 4.1: Experimental design diagram.

4.2.2 Water intake and B12 dose estimation

A pilot study (data not shown) was conducted to determine daily water consumption of mice supplemented with B12 (cyanocobalamin) in drinking water. Cyanocobalamin at 40 μg/ml or control drinking water was provided to mice (n = 15; five mice per cage) *ad libitum* and water consumption was monitored for a week. Drinking water was changed every two days and water consumption was measured. The water consumed per cage at each timepoint was considered a replicate and the average was used to compare water intake between treatments. B12 supplemented in drinking water did not impact water consumption. Daily water intake was approximately 3 ml per mouse per day. According to water intake, B12 supplemented in drinking water at 10 μg/ml and 40 μg/ml were estimated to reach a total dose of 30 μg and 120 μg per mouse a day, respectively.

4.2.3 *C. rodentium*-challenge model

From a glycerol stock, *C. rodentium* (DBS100) was plated on MacConkey agar (BD Difco, NJ, USA) and a single colony was picked and incubated overnight at 37°C in Luria-Bertani broth (Sigma-Aldrich) with shaking at 200 rpm. Mice were infected with 100 μl of the overnight culture (1 x 10^9 CFU/ml) by oral gavage. All mice were confirmed to be free of coliforms by plating a fecal sample on MacConkey agar prior to infection. Pathogen load was determined daily in fecal samples and in the GI tract at day 5 post-infection by plating serial dilutions of sample homogenates in 1 x PBS on MacConkey agar. Plates were incubated at 37°C overnight and colonies were counted and normalized to sample weight.

4.2.4 Sample collection

Fresh fecal samples were collected daily or every second day post-infection directly in 1 ml of sterile 1 x PBS for plating. All mice were euthanized using carbon dioxide and sampling was done aseptically. Prior to infection, fecal samples were collected from mice for baseline microbiome analysis. Mouse tissues and intestinal content (ileum, cecum and colon) were snap-frozen in liquid nitrogen and stored at -80°C until use.

4.2.5 Intestinal vitamin B12 level measurement

Snap-frozen cecal and colonic digesta samples were weighed and homogenized with two rounds of beating (30 s at 4 m/s with a cooling step on ice) in a proprietary buffer provided by Calgary Laboratory Services: Diagnostic and Scientific Research Centre (Calgary, AB, Canada). Samples were subsequently centrifuged at 10,000 rpm and the supernatant was collected and stored at -20 °C. Vitamin B12 was quantified via electrochemiluminescence using the Roche Diagnostics Vitamin B12 II assay performed on the Roche Diagnostics e602 (Calgary Laboratory Services). This technique measures total vitamin B12 using a ruthenium-label recombinant porcine intrinsic factor as the reporter probe.

4.2.6 Short chain fatty acid (SCFA) analysis

Snap-frozen cecal content was thawed on ice, weighed (30 mg/sample) and homogenized in 600 µl of 25% phosphoric acid. Samples were centrifuged at 15,000 rpm at 4°C for 10 min and the supernatant was passed through a 0.45 µm syringe filter (Fisher). A 200 µl aliquot of filtered sample was combined with 50 µl of internal standard (23 µmol/ml, isocaproic acid) and analyzed on a Scion 456-GC instrument.

4.2.7 Cecal microbial metatranscriptome analysis

Total RNA was extracted from frozen cecal samples as previously described (Just et al., 2018). Approximately 50 mg of frozen cecal content was added to 0.1 mm glass bead-containing tubes (PowerBead Tubes, Qiagen) prefilled with 300 µl RLT buffer (RNeasy mini kit, Qiagen) supplemented with β-mercaptoethanol (10 µl/ml, Sigma-Aldrich) and 1 ml Trizol (Invitrogen). Cell disruption was accomplished using a FastPrep®-24 bead-beating machine (MP biomedicals) with two rounds of beating (30 s at 6.5 m/s). After incubating for 5 min at room temperature, samples were centrifuged (1 min, 12,000 x g, 4°C) and supernatants were transferred into tubes containing 300 µl of chloroform, vortexed and incubated for 3 min. After centrifugation (15 min, 12,000 x g, 4°C), the upper aqueous phase was carefully collected and transferred into a new tube containing 1 ml of freshly prepared 70% ethanol solution, mixed by pipetting, and loaded onto a RNeasy spin column (RNeasy mini kit, Qiagen). RNA extraction and on-column DNA digestion (Qiagen) were completed as described by the manufacturer's protocol. The quality and quantity of RNA were measured using an Agilent Bioanalyzer. Samples with an RNA integrity number (RIN) ≥ 7.0 were used to generate metatranscriptome libraries at Génome Québec Innovation Centre (Montréal, QC). Samples were diluted to 100 ng/µl and host rRNA-depletion (NEBNext® Human/Mouse/Rat) was conducted. The libraries were sequenced as 100 bp paired-end reads on a NovaSeq 6000 system (Illumina).

Analysis of unfiltered raw data (~35M read average per sample) was completed using the Simple Annotation of Metatranscriptomes by Sequence Analysis 2.0 (SAMSA2) pipeline (Westreich et al., 2018) as follows: PEAR (version 0.9.10) to merge reads, Trimmomatic (version 0.36) to trim low quality reads, ShortMeRNA (version 2.1) to remove rRNA, and

DIAMOND (version 2.0.2) to annotate mRNA data to the RefSeq database (O'Leary et al., 2016). The merging step resulted in ~25M merged reads per sample. Bacterial rRNA made up ~9M reads per sample, and ribodepleted reads (mRNA transcripts) led to ~2M annotated reads with ~13M unknown reads per sample. In addition, the SEED Subsystems hierarchical database (Bokulich et al., 2018a) was used to categorize and compare functional activities of the microbiota. Analysis of annotated reads was completed using the DESeq2 package and visualized in R with the ggplot2 package.

4.2.8 Cytokine and chemokine assays

Protein was extracted using a 2-cm piece of distal colon and homogenized in 300 µl of Meso Scale Discovery lysis buffer with protease and phosphatase inhibitors as described in the assay protocol. The homogenates were centrifuged at 15,000 rpm for 10 min, and the protein concentration in the supernatant was determined using the Pierce Bicinchoninic Acid assay Kit (Thermo Scientific). Sample homogenates from naïve mice and infected mice were loaded into wells at 150 µg and 100 µg of total protein, respectively. The U-Plex Biomarker Group 1 (mouse) assay platform (Meso Scale Discovery, Gaithersburg, MD, USA) was used to measure interferon gamma (INFγ), interleukins (IL1β, IL4, IL6, IL10, IL12/IL23p40, IL17A, and IL22), keratinocytes-derived chemokine (KC), tumour necrosis factor alpha (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinase-9 (MMP9), chemokine protein known as regulated on activation normal T cell expressed and secreted (RANTES), interferon gamma-induced protein-10 (IP10), monocyte chemoattractant protein-1 (MCP1), and macrophage inflammatory proteins (MIP1α, MIP2, and MIP3α). Final concentrations were presented as pg/ml in 100 µg of total colon protein.

4.2.9 Microbial community analyses

Total DNA was extracted from ileum, cecum, and colon contents using the QIamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) with an additional bead-beating step using ~200 mg of garnet rock at 6.0 m/s for 60 s on a FastPrep-24 5G instrument (MP Biomedicals). Amplicon libraries were constructed according to the protocol from Illumina (16S Metagenomic Sequencing Library Preparation) that amplified the V3-V4 region of the bacterial 16S rRNA gene: 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG - 3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Paired-end sequencing was accomplished using an Illumina MiSeq Platform (2 x 300 cycles; Illumina Inc., San Diego, CA). Raw sequences were processed with Quantitative Insight into Microbial Ecology 2 (QIIME) (Bolyen et al., 2019) pipeline using the divisive amplicon denoising algorithm 2 (DADA2) to filter, trim and merge paired-end reads into amplicon sequence variants (ASVs). Ribosomal RNA data from cecal metatranscriptomic sequencing was processed as pre-merged single-end reads in QIIME2 using deblur denoise-16S function and trimmed at 160 bp. Phylogenetic trees were constructed using the qiime alignment (mafft; mask) and qiime phylogeny (fasttree; midpoint-root) function. Taxonomy was assigned using the qiime feature-classifier classify-sklearn function using the SILVA v138 database trained for the specific amplicon region (Bokulich et al., 2018b). QIIME2 files (.qza) were imported into R using qiime2R (version 0.99.4) package and analyzed with phyloseq (version 1.34.0) package (McMurdie & Holmes, 2013).

Alpha diversity (Observed, Shannon, Phylogenetic diversity (PD)) and beta diversity (weighted and unweighted UniFrac) indices were analyzed with rarefied samples (ileum at 21,497, cecum at 34,012, and colon at 7,435 reads) in C3H/HeOuJ mice. In addition, we analyzed the cecal microbial community by analyzing the rRNA (rarefied at 1,247,061 reads) from the metatranscriptome sequencing data. Statistical significance for alpha diversity indices was determine with ANOVA and Tukey correction. Principal coordinate analyses (PCoA) was plotted using the phyloseq package and clustering significance was determined using the 'betadisper' function (Anderson, 2006) for dispersion and 'pairwiseAdonis.dm' function (Martinez Arbizu, 2017) for orientation. Differential abundance analysis was done with DESeq2 using non-rarefied reads and tree_glom (or tax_glom for cecum rRNA) function. Plotted ASVs were assigned according to their lowest classifiable taxonomic rank and are distinguishable by their corresponding ASV number from most to least abundant. The 'log2foldchange' of only the ASVs with a *P* value less than 0.05 were plotted with bolded ASVs signifying the significant adjusted *P* value < 0.10, < 0.05 (*), < 0.01 (**) and < 0.001 (***).

4.2.10 *In vitro* culture experiments

All *in vitro* culture experiments were done in an anaerobic chamber (5% CO_2, 5% H_2, and 90% N_2) and cultures were incubated at 37 °C without shaking. *B. thetaiotaomicron* was isolated from C3H/HeOuJ mice fecal samples by serially diluting in 1 x PBS with 0.1% L-cysteine and plating on pre-reduced brain heart infusion (BHI; Difco) agar plus 10% calf blood (Cedarlane, ON, Canada) supplemented with 200 µg/ml of gentamicin (Curtis et al., 2014a). Isolates were identified by amplifying and Sanger sequencing the 16S rRNA gene, and sequences were matched using BLAST web-based tool (Boratyn et al., 2013). *C. rodentium* (10 µl of overnight culture) was inoculated alone or in competition with *B. thetaiotaomicron* (100 µl

overnight culture) in 10 ml of pre-reduced low-glucose Dulbecco's modified Eagle's medium (Gibco life technologies, Grand Island, NY, USA) supplemented with cyanocobalamin at 0 ppm, 0.01 ppm and 15 ppm, which was subsequently incubated for 6 hours. Overnight cultures grown from a single colony of *C. rodentium* grown in Luria-Bertani broth at $1.4x10^7$ CFU/ml and *B. thetaiotaomicron* grown in BHI broth (Difco) at $5.5x10^7$ CFU/ml were used as inoculums. Counts were determined by plating on MacConkey agar for *C. rodentium* and the BHI with calf blood agar for *B. thetaiotaomicron*. Total RNA was immediately extracted from 1 ml of pelleted cells with 1 ml of Trizol reagent and purified using the spin columns as described above.

4.2.11 Reverse-transcription quantitative PCR

Colon tissues were homogenized in 600 µl of lysis buffer via bead beating and RNA was extracted using the GeneJET RNA Purification Kit (Thermo Scientific). Sample were treated with DNase as manufacturer's protocols. RNA samples extracted from both colon tissue and *in vitro* culture experiment were reverse transcribed using the qFlex cDNA Synthesis Kit (Quanta Bioscience). Primers used for quantitative PCR (Table 4.1) were previously validated (Curtis et al., 2014a; Kumar & Sperandio, 2019; Oshikiri et al., 2019). The qPCR was performed using PercfeCTa SYBR Green Super-mix (Quantabio) conducted on an ABI StepOne real-time System following the cycles: 95°C for 3 min and 40 cycles of 95°C for 10 s, 60°C for 30 s. Gene expression was calculated using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method that showed the fold change relative to a housekeeping gene.

4.2.12 Statistical analysis

Significance testing was conducted using GraphPad Prism 6 (Graphpad Software, La Jolla, CA, USA). Student's t-test or ANOVA was used for parametric and Kruskal-Wallis test

was used for nonparametric data. Data were presented as mean ± standard deviation. Survival curve analysis was done using Mantel-Cox test with data up to day 10 post-infection. Differences between multiple treatments were corrected by conducting either the Bonferroni's, Tukey's, or Dunn's post-hoc comparison test.

Table 4.1: Real-time qRT-PCR primer list.

Target Genes		Oligonucleotide sequences (5' - 3')	Product length	Ref.
ActB	F:	TGACAGGATGCAGAAGGAGA	131	Oshikiri et al., 2019
	R:	GCTGGAAGGTGGACAGTGAG		
IL12A	F:	CCACCCTTGCCCTCCTAAAC	132	Oshikiri et al., 2019
	R:	GTTTTTCTCTGGCCGTCTTCA		
IL12B	F:	GGGACATCATCAAACCAGACCC	239	Oshikiri et al., 2019
	R:	GCCTTTGCATTGGACTTCGG		
Rpoa	F:	ACGTCAGCCGGAAGTGAAAGAAGA	86	(Curtis et al., 2014a
	R:	AGCGGACAGTCAATTCCAGATCGT		
Ler	F:	ACAGTTTGAATCTCCTGCTCACGC	98	(Curtis et al., 2014a
	R:	AATTCGCCCACAACAAGCCCATAC		
Tir	F:	ATCAGATATCTCGCAAGCTCG	134	Kumar & Sperandio, 2019
	R:	CAACTCCATCTCCCATTCCTG		
EspA	F:	ACGAGGTAACAACCATGCGAGTGT	87	Kumar & Sperandio, 2019
	R:	CTGCCTGGCATTGCTTTCCAGAAT		

Note: The annealing temperature used for all genes was 60°C.

4.3 Results

4.3.1 Cyanocabalamin supplementation enhances early-stage colonization of *C. rodentium* and pathogenesis in C3H/HeOuJ mice

The amount of total cobalamin in cecum and colon contents of mice were determined to be 1000 times greater in cyanocobalamin-supplemented mice than control ($P < 0.01$) (Figure

4.2a). Higher cobalamin levels resulted in a more rapid and consistent colonization of *C. rodentium* as determined by daily fecal enumeration (Figure 4.2b), and in both ileal and cecal contents at day 5 post-infection (Figure 4.2c). Differences in pathogen load were greatest at day 3 post-infection ($P < 0.05$). The more rapid and consistent colonization of *C. rodentium* seen in the EPC experiment was confirmed in the SURV experiment. Mice receiving cyanocobalamin in

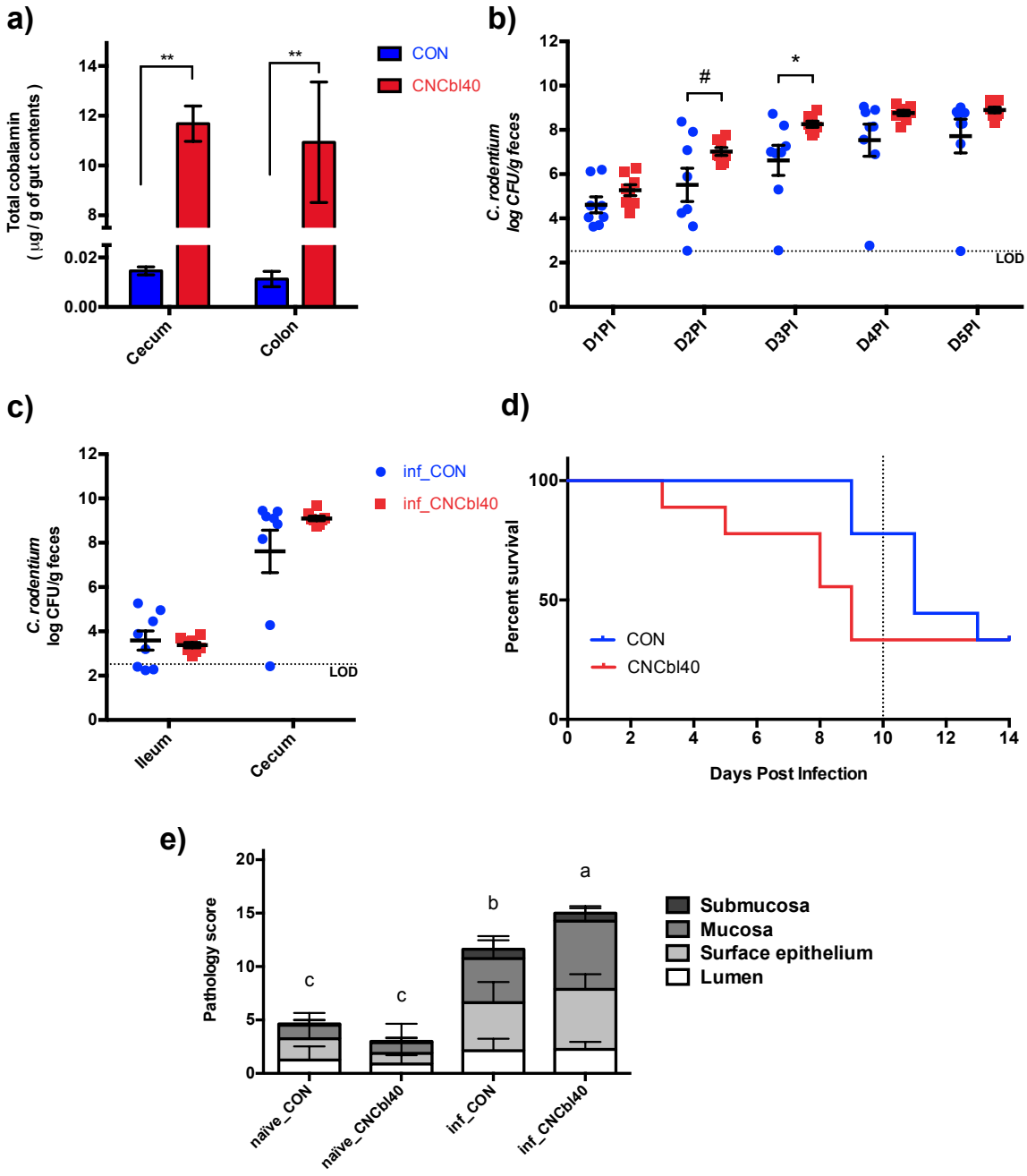


Figure 4.2: Cyanocobalamin supplementation enhanced *C. rodentium* colonization and pathogenesis in C3H/HeOuJ mice. (a) Supplementing cyanocobalamin in drinking water increased cecal and colon levels of cobalamin by ~1000 times (n = 15; * $P < 0.05$, ** $P < 0.01$). (b) Daily fecal enumeration of *C. rodentium* in the EPC experiment indicated increased colonization burden at day 2 post-infection (D2PI) and D3PI from cyanocobalamin supplementation (n = 8; # $P < 0.10$, * $P < 0.05$). (c) Enumeration of *C. rodentium* at D5PI in the ileum and cecum was more consistent with cyanocobalamin supplementation but colonization levels were similar (n = 8). (d) Consistent with the more rapid colonization, the SURV experiment revealed an earlier onset of mortality, reducing mice survival over the first 10 days post-infection (n = 9; $P < 0.05$; Mantel-Cox test). (e) Colon pathology scores at D5PI in naïve and infected mice was significantly higher in mice supplemented with cyanocobalamin as a result of increased mucosal damage (n = 8; $P < 0.05$; limit of detection (LOD)).

excess had an earlier onset of mortality that started at day 3 compared to day 9 in control (Figure 4.2d). Survival curves between CON and CNCbl40 were significantly different at day 10 ($P = 0.03$), although significance was lost by day 14 post-infection ($P = 0.14$). Consistent with the increased pathogen load, mice in the inf_CNCbl40 group terminated 5 days post infection had higher colon pathology scores compared to inf_CON ($P < 0.05$) (Figure 4.2e). Cyanocobalamin water supplementation alone induced no visible tissue damage prior to infection (Figure 4.2e). Overall, colonization and pathogenesis of *C. rodentium* in C3H/HeOuJ mice was enhanced following cyanocobalamin supplementation in drinking water.

4.3.2 Cobalamin supplementation alters the Firmicutes and Proteobacteria populations within the GI tract of C3H/HeOuJ mice

Cyanocobalamin supplementation at 40 µg/ml in drinking water caused a shift in microbial composition in the cecum and colon, but not ileum, favoring Proteobacteria species and altering the dynamics of the low-abundance Firmicutes (Figures 4.3 & 4.4). Species richness, indicated by observed counts, and phylogenetic distance analyses showed that B12 treatment led to lower colonic diversity prior to pathogen challenge. Principal Coordinate Analysis (PCoA) plots using weighted and unweighted UniFrac distance metrics showed distinct clustering of microbiomes in naïve and infected mice (pairwise Adonis; Table 4.2). Differences in unweighted UniFrac, but not weighted UniFrac, revealed that low-abundance community members were impacted in the cecum ($P < 0.05$) and colon ($P < 0.01$) in naïve_CNCbl40 mice compared with naïve_CON mice. The severity of microbial disruption induced by infection was more pronounced in the inf_CNCbl40 as compared to the inf_CON group. Both weighted ($P < 0.01$) and unweighted ($P < 0.05$) UniFrac metrics revealed a difference between naïve_CNCbl40 and inf_CNCb140 in the colon, whereas no difference was observed between infected and uninfected control groups (weighted P=1.0, unweighted P=0.81). In the cecum, the gut microbial communities between naïve_CON and inf_CON were different ($P < 0.01$) based on unweighted UniFrac metric, whereas naïve_CNCbl40 and inf_CNCbl40 groups were different ($P < 0.01$) by weighted UniFrac metric.

Differences in beta diversity observed in the colon and cecum were largely explained by changes in Firmicute and Proteobacteria populations in both naïve and infected mice as determined by DEseq2's differential expression analysis (Figure 4.4). In advance of infection, an uncultured bacterium belonging to the *Clostridia vadinBB60* group and a *Lachnospiraceae* were

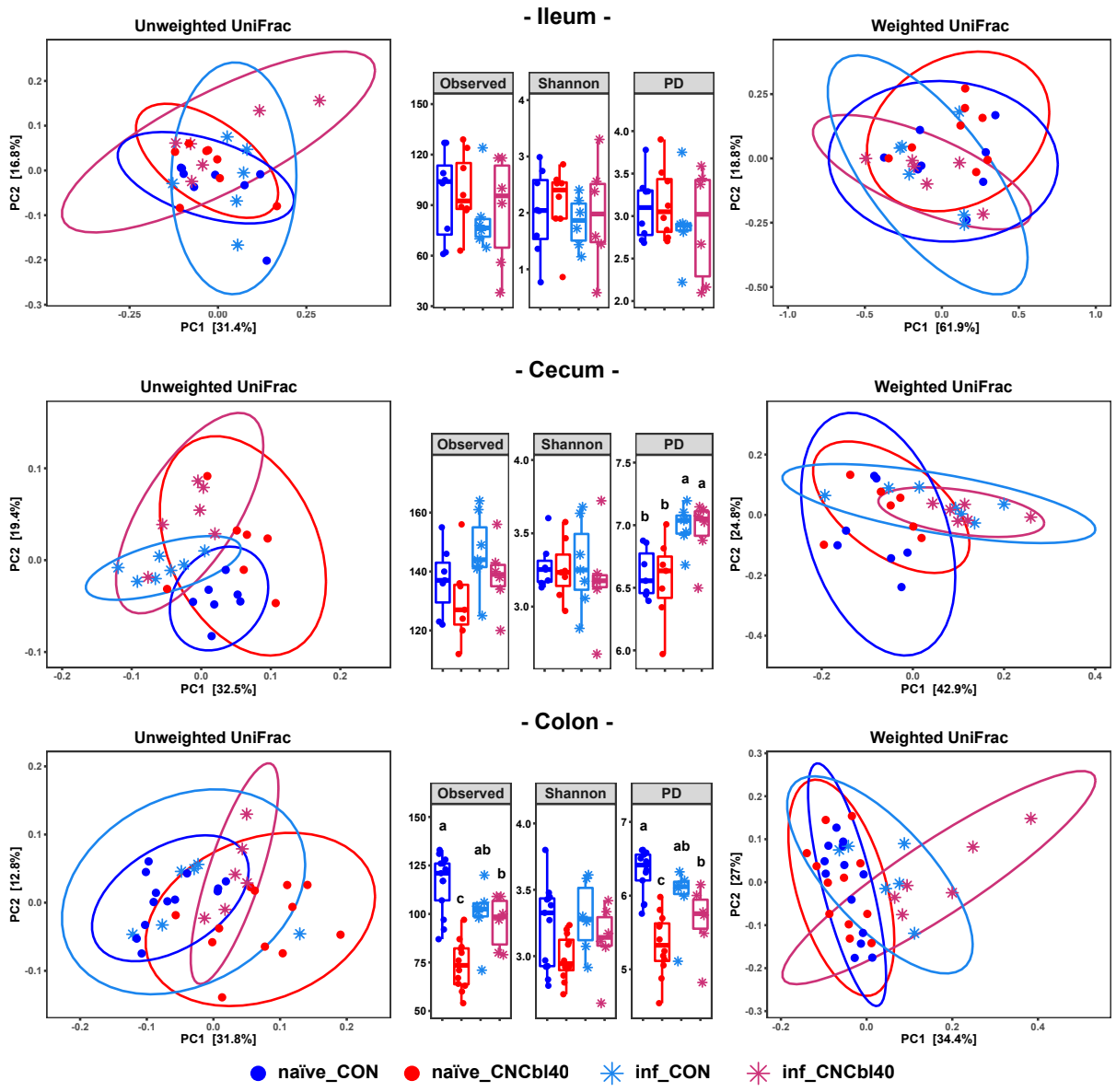


Figure 4.3: Principal coordinate plots based on weighted and unweighted UniFrac and alpha diversity indices (Observed, Shannon, PD) in the ileum, cecum and colon in C3H/H3OuJ mice. Distinct clustering was noted in the unweighted UniFrac analyses (see Table 4.2) and the colon microbiota was characterized by reduced diversity (Observed and PD) from cyanocobalamin supplementation (n = 7-12; $P < 0.05$).

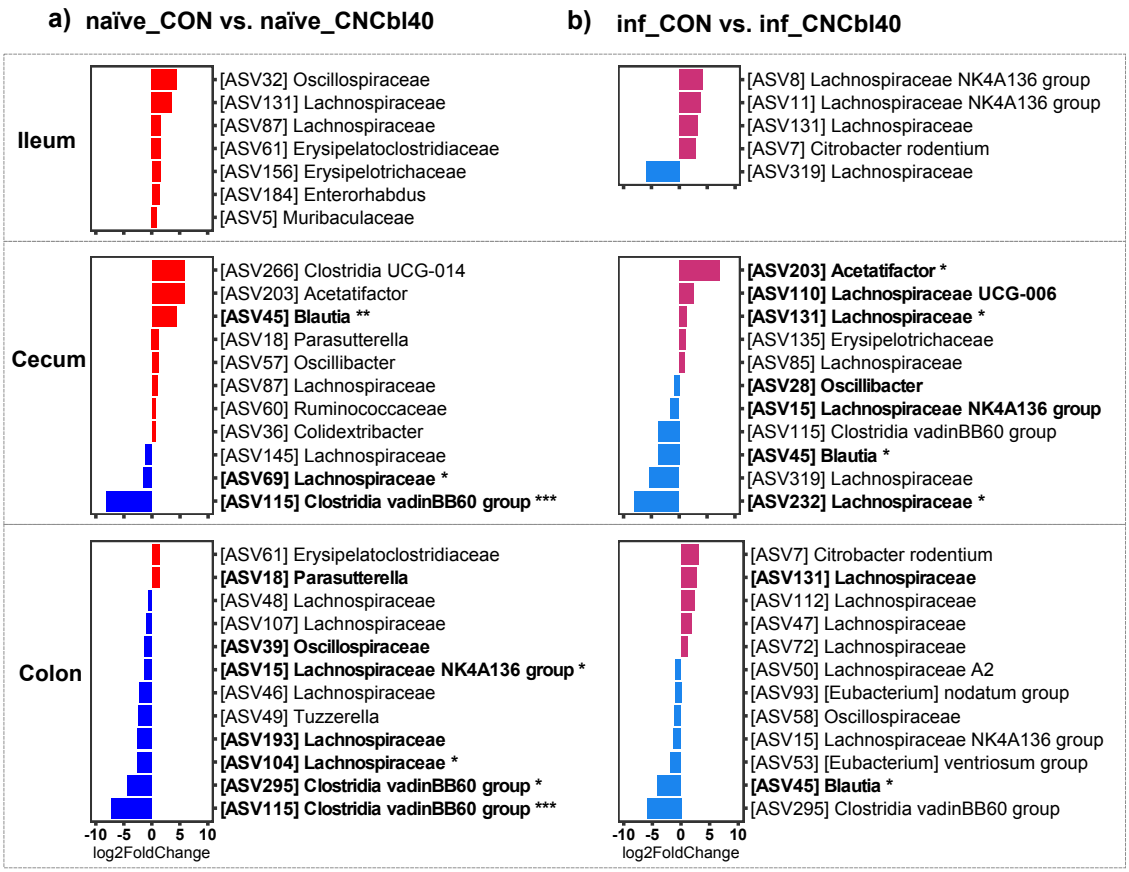


Figure 4.4: Cyanocobalamin supplementation altered the Firmicutes population throughout the GI tract in C3H/HeOuJ mice as determined by DESeq2 differential expression. The control group had a greater abundance of Firmicutes, including *Lachnospiraceae* species and *Clostridia vadinBB60* group bacterium in the cecum and colon (a) pre-infection and (b) post-infection (n = 6-8; only ASVs with a *P*-value less than 0.05 were plotted; adjusted *P*-value were used for significance; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 4.2: Summary of beta-diversity analyses of microbial communities.

Region	Comparisons	Weighted UniFrac			Unweighted UniFrac		
		Disp.	Ori.	R^2	Disp.	Ori.	R^2
Ileum	naïve_CON vs naïve_CNCbl40	0.98	1	0.05	1	1	0.06
	inf_CON vs inf_CNCbl40	1	1	0.03	1	1	0.11
	naïve_CON vs inf_CON	1	1	0.03	1	1	0.07
	naïve_CNCbl40 vs inf_CNCbl40	1	0.49	0.19	0.88	1	0.09
	naïve_CON vs inf_CNCbl40	1	1	0.09	0.97	0.4	0.13
	naïve_CNCbl40 vs inf_CON	1	1	0.12	0.89	0.53	0.12
Cecum	naïve_CON vs naïve_CNCbl40	1	1	0.06	0.13	**0.02**	0.2
	inf_CON vs inf_CNCbl40	0.84	1	0.1	1	0.25	0.17
	naïve_CON vs inf_CON	1	0.15	0.23	0.48	**0.01**	0.3
	naïve_CNCbl40 vs inf_CNCbl40	0.9	**0.01**	0.37	0.83	0.1	0.2
	naïve_CON vs inf_CNCbl40	0.79	**0.01**	0.38	0.54	**0.01**	0.37
	naïve_CNCbl40 vs inf_CON	1	0.35	0.16	0.84	**0.01**	0.32
Colon	naïve_CON vs naïve_CNCbl40	0.92	1	0.05	0.25	**0.01**	0.28
	inf_CON vs inf_CNCbl40	1	0.17	0.21	0.96	1	0.12
	naïve_CON vs inf_CON	0.95	1	0.08	0.33	0.81	0.09
	naïve_CNCbl40 vs inf_CNCbl40	0.74	**0.01**	0.36	0.91	**0.04**	0.13
	naïve_CON vs inf_CNCbl40	0.96	**0.01**	0.34	0.1	**0.01**	0.26
	naïve_CNCbl40 vs inf_CON	0.73	0.34	0.13	1	**0.05**	0.16
Colon [SURV vs ECP]	CON vs CNCbl40	0.21	0.7	0.08	**0.01**	**0.01**	0.16
	naïve_CON vs naïve_CNCbl40	0.88	1	0.05	**0.02**	**0.01**	0.27
	naïve_CON vs CON	0.31	**0.01**	0.67	0.76	**0.01**	0.8
	naïve_CNCbl40 vs CNCbl40	0.73	**0.01**	0.6	0.44	**0.01**	0.56
Cecum [RNAseq]	naïve_CON vs naïve_CNCbl40	0.38	1	0.06	0.29	0.6	0.09
	inf_CON vs inf_CNCbl40	0.71	0.85	0.1	0.53	0.32	0.1
	naïve_CON vs inf_CON	0.06	0.77	0.11	0.16	**0.01**	0.19
	naïve_CNCbl40 vs inf_CNCbl40	1	0.85	0.1	0.73	**0.05**	0.16
	naïve_CON vs inf_CNCbl40	0.4	0.08	0.21	0.87	**0.01**	0.25
	naïve_CNCbl40 vs inf_CON	0.74	1	0.07	1	0.08	0.14

Note: Dispersion (Disp.) *p*-values were calculated with betadisper() using a permuted model that indicates a difference in dispersion between groups. Orientation (Ori.) *p*-values and R^2 values were calculated with pairwise.adonis() and adjusted using bonferroni (perm=999) method, and indicates significantly different clustering between groups.

significantly lower in the cecum ($P < 0.001$ and $P < 0.05$, respectively) and colon ($P < 0.001$ and $P < 0.05$, respectively), whereas *Parasutterella* was higher in the colon ($P < 0.10$) of the naïve_CNCbl40 group compared with the naïve_CON group (Figure 4.4a). A *Blautia* bacterium was the only significant microbe that increased ($P < 0.01$) in the cecum but not in the colon of naïve_CNCbl40 group. Consistent with the higher fecal *C. rodentium* counts; mice in the inf_CNCb140 group had numerically higher numbers of *C. rodentium* compared to the inf_CON group in the ileum and colon (Figure 4.4b). In the cecum, *Acetatifactor* and *Lachnospiraceae* species increased ($P < 0.05$) while *Blautia* and other *Lachnospiraceae* species decreased ($P < 0.05$) in infected mice supplemented with cyanocobalamin. The colon of inf_CNCbl40 mice had lower levels of *Blautia* ($P < 0.05$) as well as numerically lower levels of species belonging to *Clostridia vadinBB60* group, *Oscillospiraceae*, *Lachnospiraceae* A2, and [Eubacterium] groups compared to inf_CON. These results indicate that cyanocobalamin supplementation encourages the growth of Proteobacteria (*Parasutterella* and *Citrobacter*) species and impacts the dynamics of the low-abundance Firmicute species in the gut.

Consistent shifts in beta diversity in response to B12 supplementation were observed in the SURV and EPC experiments prior to pathogen challenge (Figure 4.5). However, there were notable differences in community composition between experiments including the absence of *Akkermansia muciniphila, Bacteroides thetaiotaomicron*, and *Parasutterella* species in the SURV experiments (Figure 4.6), which are relevant to the immune phenotypes discussed below. SCFA analysis on cecal content from the SURV experiment revealed that B12 supplementation had no impact on the SCFA concentrations (Figure 4.7). Despite differences in baseline microbiomes, the species richness (observed counts) and phylogenetic diversity index were lower ($P < 0.05$) in mice supplemented with B12 in both experiments (Figure 4.5c). In addition

to numerically lower levels of *Tuzzerella* species, the *Clostridia vadinBB60* group were consistently reduced ($P < 0.01$) by B12 supplementation (Figure 4.5d,e). Due to the absence of *Parasutterella* in the SURV experiment, the higher relative abundance of the genus ($P < 0.10$) was only detected in the EPC experiment.

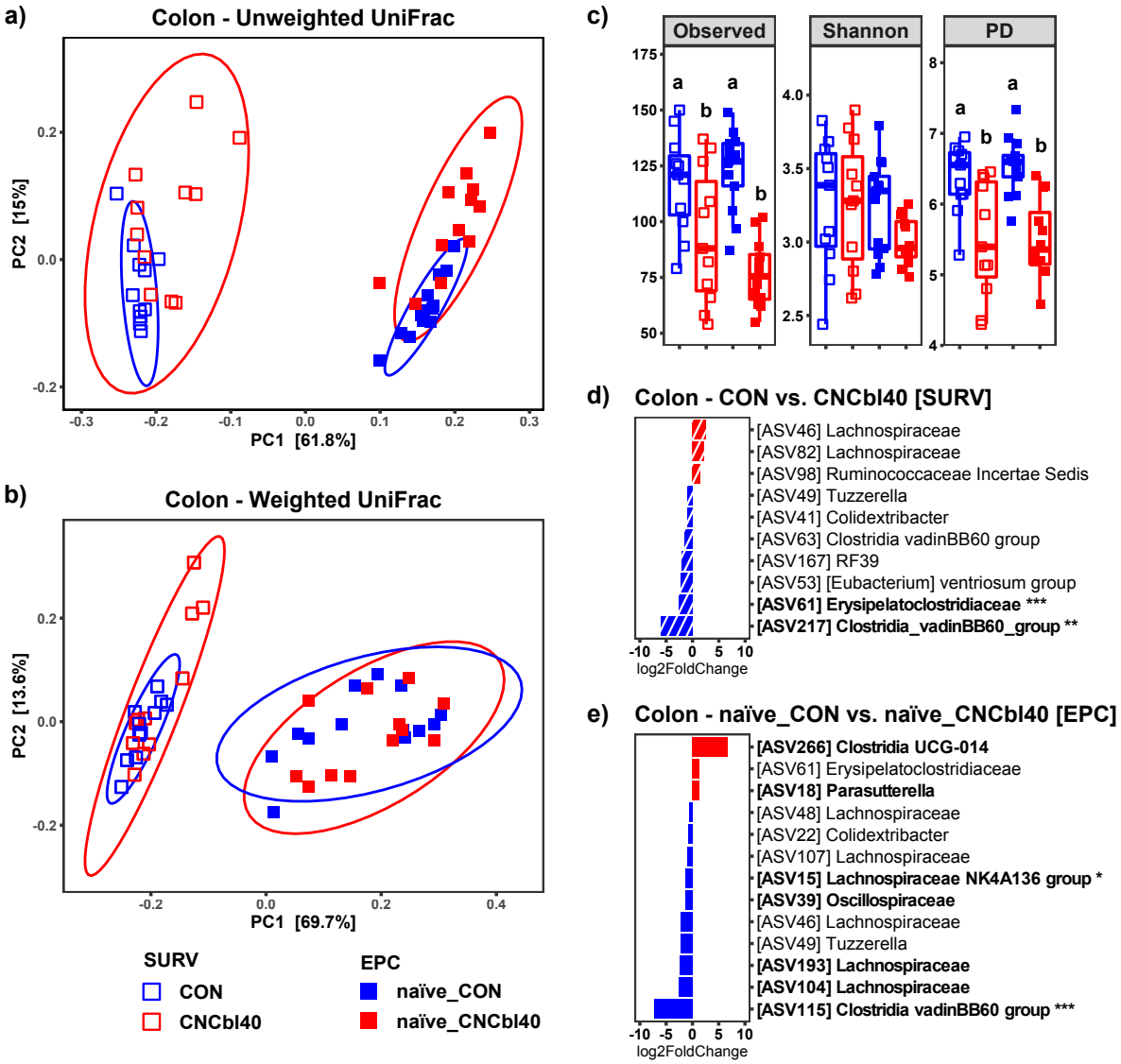


Figure 4.5: Comparison of microbial communities in naïve C3H/HeOuJ mice from SURV and ECP experiments. (a) Unweighted UniFrac PCoA plot and (b) weighed UniFrac comparison shows a similar pattern in microbial community clustering (see Table 4.2). (c) The changes were

associated with a consistent reduction in alpha diversity (Observed and PD) regardless of experiment (n = 10-12; $P < 0.05$). Differential expression determine by DEseq2 analysis also showed that the Firmicute populations (*Clostridia vadinBB60* group) were impacted by cyanocobalamin in both mice harboring (d) SURV and (e) EPC gut microbiomes (n = 10-12; bolded taxa represent a trend ($P < 0.10$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

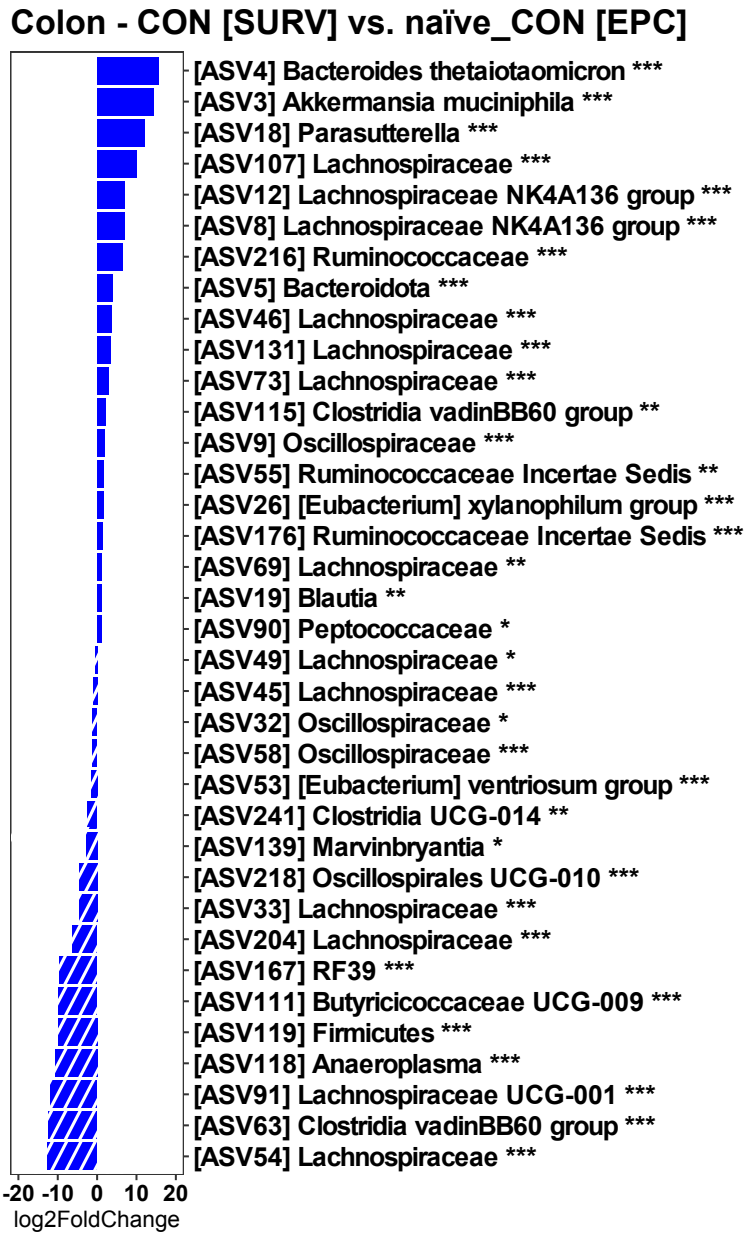


Figure 4.6: Differential expression analysis of naïve C3H/HeOuJ mice from the SURV and EPC experiments. A complete absence of major taxa differences included *Bacteroides thetaiotaomicron, Akkermansia muciniphila* and *Parasutterella* (n = 13-14; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

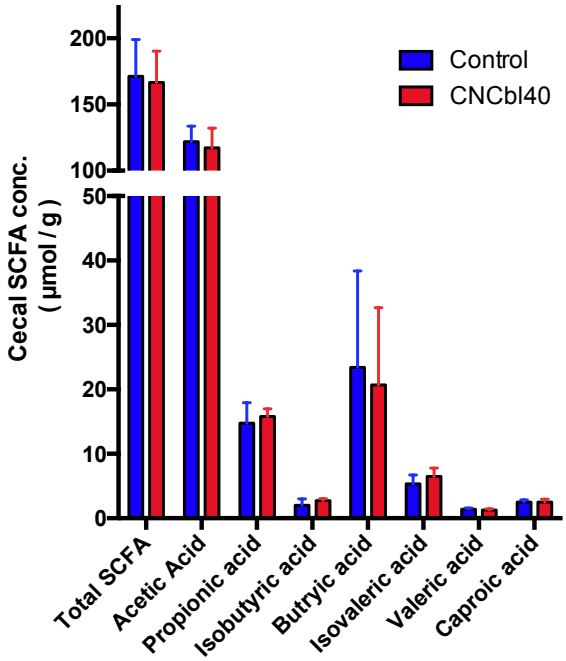


Figure 4.7: Cecal SCFA profiles of naïve C3H/HeOuJ mice from the SURV experiment (n=6).

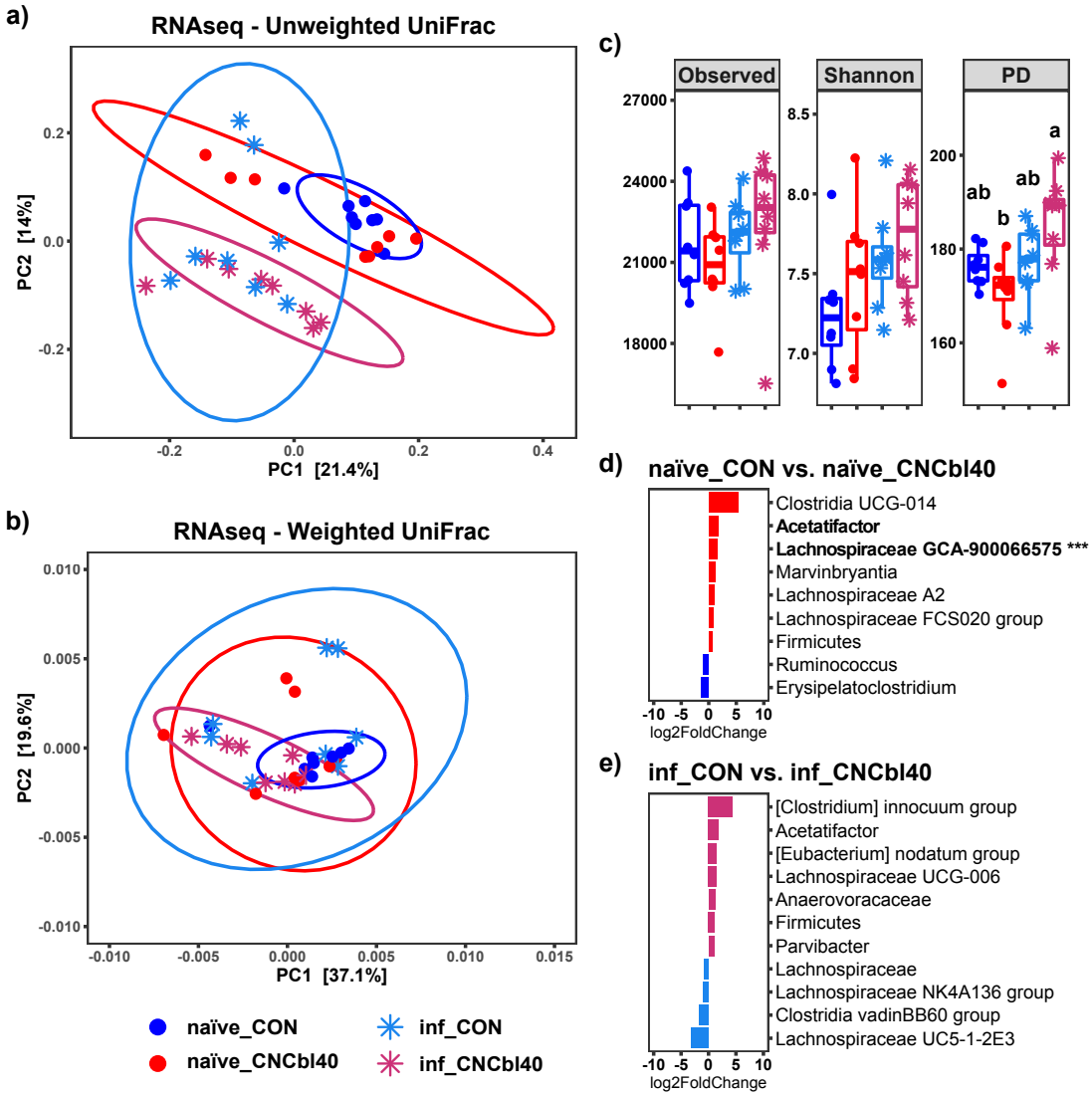


Figure 4.8: Cecal microbiota analysis of ribosomal rRNA data pulled from SAMSA2 metatranscriptome analysis. Distinct clustering of microbial communities in (a) unweighted but not (b) weighted UniFrac PCoA plots (see Table 4.2). (c) Alpha diversity as determine by Observed, Shannon and PD metrics showed that diversity (PD only) increased post-infection for cyanocobalamin-supplemented mice compared to control ($P < 0.05$). Differential expression analysis using DEseq2 of microbial taxa confirmed that the Firmicutes populations were altered

from cyanocobalamin supplementation in (d) naïve and (e) infected mice (n = 6-8; bolded taxa represent a trend ($P < 0.10$); *** $P < 0.001$).

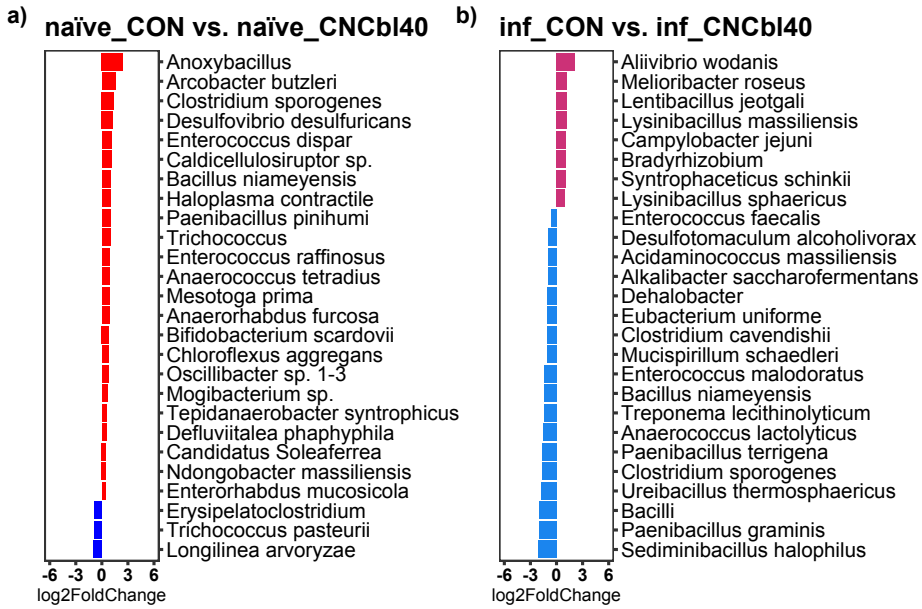


Figure 4.9: Differential expression analysis of the active microbial community as determine by cecal metatranscriptomics of C3H/HeOuJ mice. Cyanocobalamin supplementation increased the abundance of numerous active members before infection (a); however, mice at D5PI receiving regular drinking water (b) displayed greater numbers of different active members. All plotted taxa have a non-adjusted *P* value less than 0.05 (n = 8).

We analyzed changes in the active cecal microbiota using ribosomal RNA sequences identified in the metatranscriptome data (Figure 4.8). Unweighted UniFrac showed a clear separation between the microbiotas of naïve treatment groups and inf_CNCbl40 group, whereas some mice from the inf_CON group remained similar to naïve treatment groups (Figure 4.8a;

Table 4.2). No difference was observed with the weighted UniFrac analysis (Figure 4.8b). Alpha diversity metrics revealed no change in observed counts or Shannon diversity indices. However, the PD metric was numerically lower in the naïve_CNCbl40 group, which was consistent with the 16S rRNA gene amplicon datasets moving from the cecum to colon. Interestingly, the inf_CNCbl40 group became more diverse ($P < 0.05$) than the naïve_CNCbl40 group as determined by the PD metric (Figure 4.8c). Overall, changes in community composition with B12 supplementation and infection, including *Clostridia vadinBB60* and *Acetatifactor*, are consistent with 16s rRNA gene amplicon data (Figure 4.8d,e). Differential expression analysis of the active microbial community in the cecum did not reveal significant changes; however, does suggest restructuring of population dynamics in relation to B12 supplementation (Figure 4.9).

4.3.3 Cyanocobalamin supplementation altered the Firmicute population dynamics in the cecum

Transcriptome analysis revealed that cyanocobalamin treatment led to changes in overall microbial activity pre- and post-pathogen challenge (Figure 4.10). The naïve_CNCbl40 group had significantly lower expression of citrate:sodium symporter ($P < 0.01$) and a noteworthy decrease in methyltetrathydrofolate-corrinoid methyltransferase (unadjusted $P < 0.001$, adjusted $P = 0.64$), a cobalamin-specific enzyme (Figure 4.10a). Post-pathogen challenge revealed that the inf_CNCbl40 group had lower expression of flagellin domain protein ($P < 0.01$), 3N domain protein-glycosyl hydrolase family ($P < 0.05$), flagellar biosynthesis protein A ($P < 0.10$), and reverse transcriptase ($P < 0.10$), whereas enzymes glucose-1-phosphate thymidylyltransferase ($P < 0.01$) and D-alanine--poly(phosphoribitol) ligase ($P < 0.10$) were enriched (Figure 4.10b). The SEED subsystems pathway analysis (level 3) showed a trend for increased expression of genes related to the carotenoid's pathway ($P < 0.10$) in the naïve_CON group. At the same time, mice

supplemented with B12 had greater expression of genes related to the lipopolysaccharide assembly ($P < 0.05$) and catechol branch of beta-ketoadipate ($P < 0.10$) pathways. Genes related to Gram-positive competence and putrescine utilization pathways were numerically higher in the naïve_CON group. In addition, coenzyme B12 biosynthesis pathways were favored in the naïve_CNCbl40 group (Figure 4.11a). The main pathways enriched in the inf_CON group were related to triacylglycerol metabolism, acetyl-CoA fermentation to butyrate, and autoinducer 2 (A1-2) transport and processing (lsrACDBFGE) pathways. The inf_CNCbl40 group displayed microbial activity that favored the UDP-N-acetylmuramate from Fructose-6-phosphate biosynthesis pathway (Figure 4.11b).

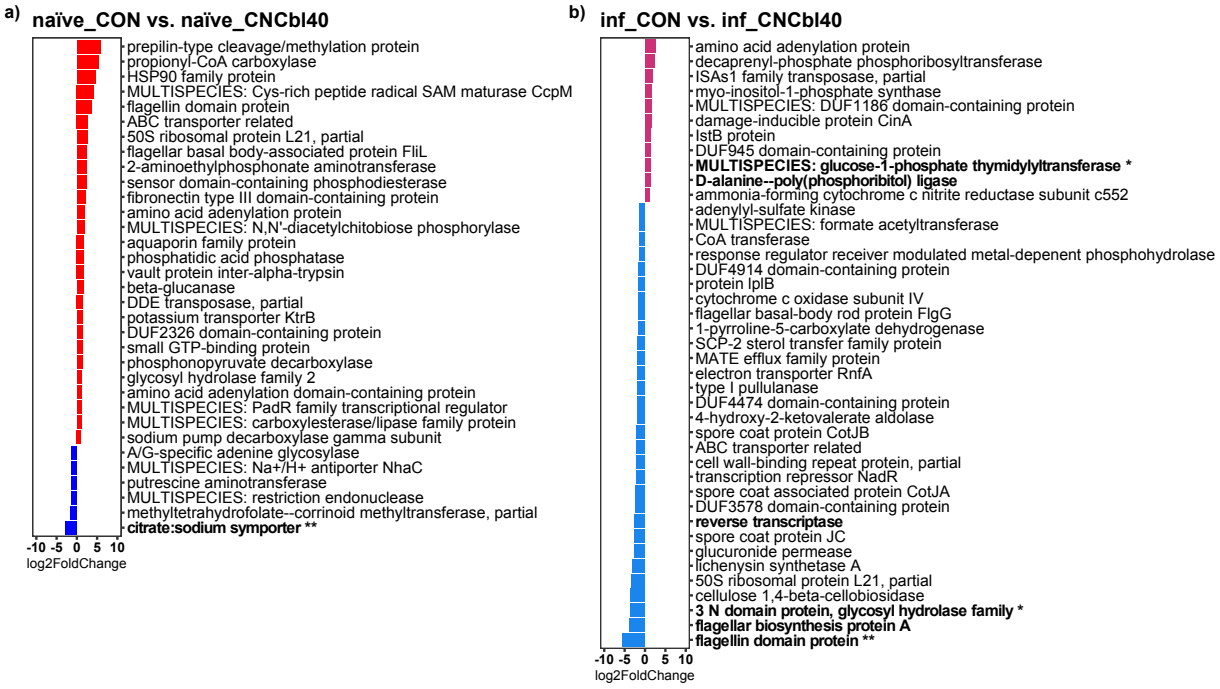


Figure 4.10: Cecal metatranscriptome analysis revealed key changes in the overall activity of the microbiota. (a) Naïve and (b) *C. rodentium*-challenged mice supplemented with cyanocobalamin

displayed altered functional activities related to metabolism (citrate:sodium symporter) and motility (flagellin domain protein), which was related to the Firmicute populations (n = 8; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$, ** $P < 0.01$).

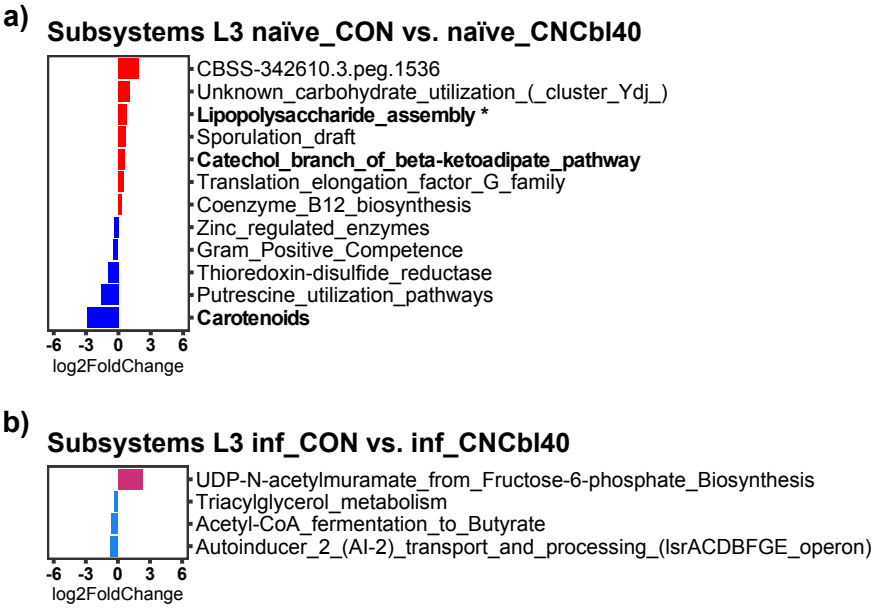
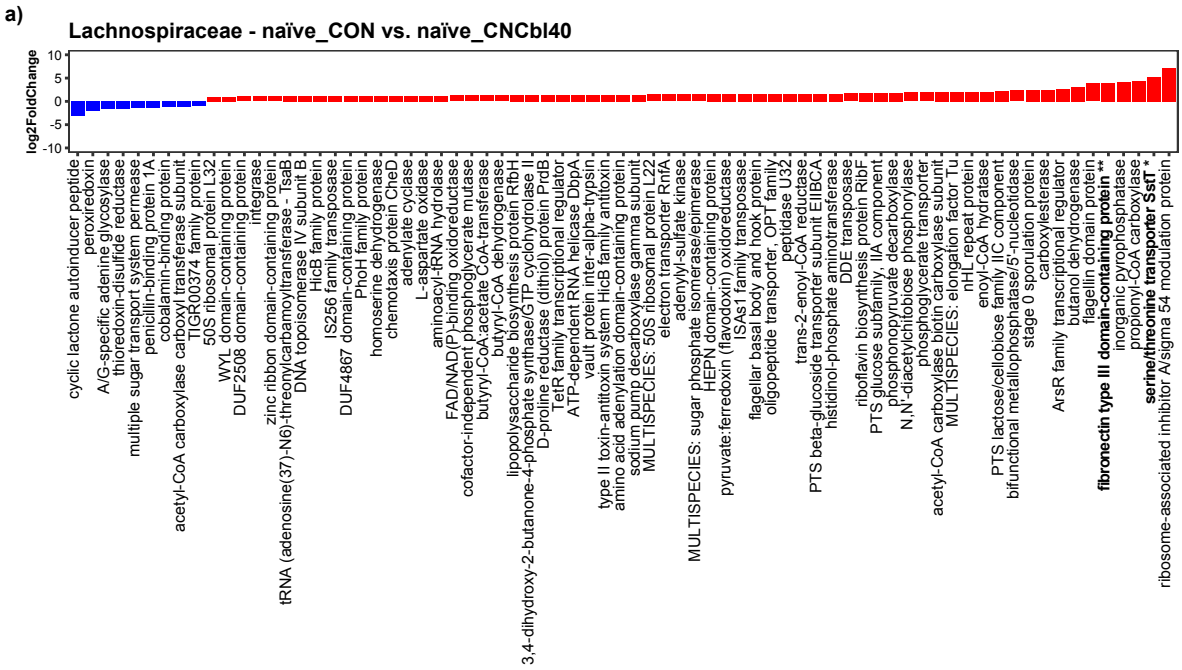
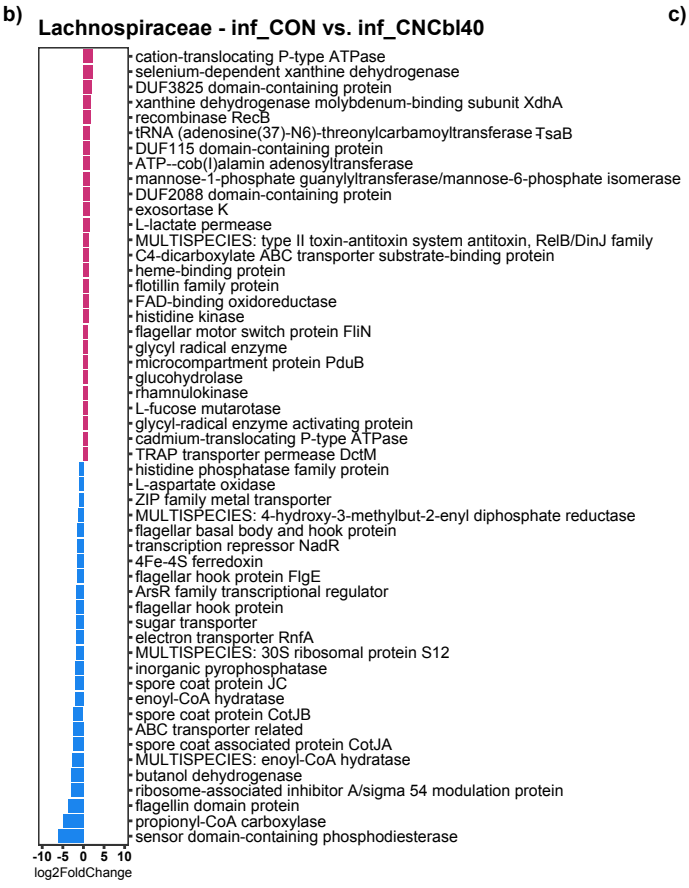


Figure 4.11: Pathway analysis of the C3H/HeOuJ cecal microbiota's functional activity using the SEED subsystems at level 3 from SAMSA2 pipeline. Cyanocobalamin supplementation altered various increased pathways related to Gram-negative while decreasing genes related to Gram-positive bacteria in (a) naïve mice but not in (b) *C. rodentium*-challenged mice (n = 8; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$).

The majority of these changes were attributed to *Lachnospiraceae* species pre-infection and *C. rodentium* post-infection. Therefore, we extracted and compared the metatranscriptome data annotated to "*Lachnospiraceae*" and "*Citrobacter*" (Figure 4.12). *Lachnospiraceae* species

drastically altered their activity in response to a gut environment saturated with cyanocobalamin. In naïve mice, cyanocobalamin treatment enhanced the expression of fibronectin type III domain-containing protein ($P < 0.01$) and serine/threonine transporter SstT ($P < 0.05$), along with favoring numerous notable genes: type II toxin-antitoxin system HicB family antitoxin, flagellin domain protein, inorganic pyrophosphatase, propionyl-CoA carboxylase, and ribosome-associated inhibitor A/sigma 54 modulation protein (Figure 4.12a). In addition, cobalamin-binding protein and cyclic lactone autoinducer peptide genes of *Lachnospiraceae* were favored in the naïve_CON group (Figure 4.12a). *Lachnospiraceae* species were more active in the inf_CON group than in the inf_CNCbl40 group, with a notable increase in ribosome-associated inhibitor A/sigma 54 modulation protein, flagellin domain protein, propinyl-CoA carboxylase, and sensor domain-containing phosphodiesterase. Of particular interest, ATP- cob(I)alamin adenosyltransferase and MULTISPECIES: type II toxin-antitoxin system antitoxin, RelB/DinJ family genes were enriched in the inf_CNCbl40 group (Figure 4.12b). Cyanocobalamin supplementation changed the activity of *C. rodentium* and notably enhanced expression of numerous virulence genes: *E. coli* secretion protein A (EspA) and D (EspD), translocated intimin receptor (Tir), intimin, and *E. coli* attaching and effacing gene B (EaeB) (Figure 4.12c). Interestingly, the gene related to glucose utilization, family 31 glucosidase, was favored in the inf_CON group along with transcriptional regulator HdfR and DUF4150 domain-containing protein (Figure 4.12c).


a)
Lachnospiraceae - naïve_CON vs. naïve_CNCbl40
log2FoldChange
10
5
0
-5
-10
cyclic lactone autoinducer peptide
peroxiredoxin
A/G-specific adenine glycosylase
thioredoxin-disulfide reductase
multiple sugar transport system permease
penicillin-binding protein 1A
cobalamin-binding protein
acetyl-CoA carboxylase carboxyltransferase subunit
TIGR00374 family protein
50S ribosomal protein L32
WYL domain-containing protein
DUF2508 domain-containing protein
integrase
zinc ribbon domain-containing protein
tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase - TsaB
DNA topoisomerase IV subunit B
HicB family protein
IS256 family transposase
DUF4867 domain-containing protein
PhoH family protein
homoserine dehydrogenase
chemotaxis protein CheD
adenylate cyclase
L-aspartate oxidase
aminoacyl-tRNA hydrolase
FAD/NAD(P)-binding oxidoreductase
cofactor-independent phosphoglycerate mutase
butyryl-CoA:acetate CoA-transferase
butyryl-CoA dehydrogenase
lipopolysaccharide biosynthesis protein RtbH
3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II
D-proline reductase (dithiol) protein PrdB
TetR family transcriptional regulator
ATP-dependent RNA helicase DbpA
vault protein inter-alpha-trypsin
type II toxin-antitoxin system HicB family antitoxin
amino acid adenylation domain-containing protein
sodium pump decarboxylase gamma subunit
MULTISPECIES: 50S ribosomal protein L22
electron transporter RnfA
adenylyl-sulfate kinase
MULTISPECIES: sugar phosphate isomerase/epimerase
HEPN domain-containing protein
pyruvate:ferredoxin (flavodoxin) oxidoreductase
ISAs1 family transposase
flagellar basal body and hook protein
oligopeptide transporter, OPT family
peptidase U32
trans-2-enoyl-CoA reductase
PTS beta-glucoside transporter subunit EIIBCA
histidinol-phosphate aminotransferase
DDE transposase
riboflavin biosynthesis protein RibF
PTS glucose subfamily, IIA component
phosphonopyruvate decarboxylase
N,N'-diacetylchitobiose phosphorylase
phosphoglycerate transporter
acetyl-CoA carboxylase biotin carboxylase subunit
MULTISPECIES: elongation factor Tu
nHL repeat protein
enoyl-CoA hydratase
PTS lactose/cellobiose family IIC component
bifunctional metallophosphatase/5'-nucleotidase
stage 0 sporulation protein
carboxylesterase
ArsR family transcriptional regulator
butanol dehydrogenase
flagellin domain protein
fibronectin type III domain-containing protein **
inorganic pyrophosphatase
propionyl-CoA carboxylase
serine/threonine transporter SstT *
ribosome-associated inhibitor A/sigma 54 modulation protein


b)
Lachnospiraceae - inf_CON vs. inf_CNCbl40
cation-translocating P-type ATPase
selenium-dependent xanthine dehydrogenase
DUF3825 domain-containing protein
xanthine dehydrogenase molybdenum-binding subunit XdhA
recombinase RecB
tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase TsaB
DUF115 domain-containing protein
ATP--cob(I)alamin adenosyltransferase
mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase
DUF2088 domain-containing protein
exosortase K
L-lactate permease
MULTISPECIES: type II toxin-antitoxin system antitoxin, RelB/DinJ family
C4-dicarboxylate ABC transporter substrate-binding protein
heme-binding protein
flotillin family protein
FAD-binding oxidoreductase
histidine kinase
flagellar motor switch protein FliN
glycyl radical enzyme
microcompartment protein PduB
glucohydrolase
rhamnulokinase
L-fucose mutarotase
glycyl-radical enzyme activating protein
cadmium-translocating P-type ATPase
TRAP transporter permease DctM
histidine phosphatase family protein
L-aspartate oxidase
ZIP family metal transporter
MULTISPECIES: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase
flagellar basal body and hook protein
transcription repressor NadR
4Fe-4S ferredoxin
flagellar hook protein FlgE
ArsR family transcriptional regulator
flagellar hook protein
sugar transporter
electron transporter RnfA
MULTISPECIES: 30S ribosomal protein S12
inorganic pyrophosphatase
spore coat protein JC
enoyl-CoA hydratase
spore coat protein CotJB
ABC transporter related
spore coat associated protein CotJA
MULTISPECIES: enoyl-CoA hydratase
butanol dehydrogenase
ribosome-associated inhibitor A/sigma 54 modulation protein
flagellin domain protein
propionyl-CoA carboxylase
sensor domain-containing phosphodiesterase
-10 -5 0 5 10
log2FoldChange

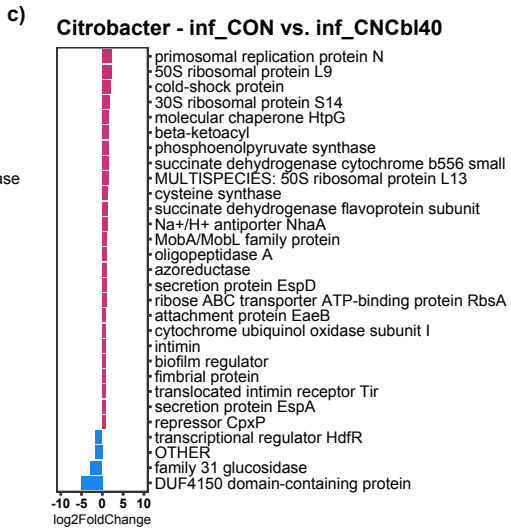

c)
Citrobacter - inf_CON vs. inf_CNCbl40
primosomal replication protein N
50S ribosomal protein L9
cold-shock protein
30S ribosomal protein S14
molecular chaperone HtpG
beta-ketoacyl
phosphoenolpyruvate synthase
succinate dehydrogenase cytochrome b556 small
MULTISPECIES: 50S ribosomal protein L13
cysteine synthase
succinate dehydrogenase flavoprotein subunit
Na+/H+ antiporter NhaA
MobA/MobL family protein
oligopeptidase A
azoreductase
secretion protein EspD
ribose ABC transporter ATP-binding protein RbsA
attachment protein EaeB
cytochrome ubiquinol oxidase subunit I
intimin
biofilm regulator
fimbrial protein
translocated intimin receptor Tir
secretion protein EspA
repressor CpxP
transcriptional regulator HdfR
OTHER
family 31 glucosidase
DUF4150 domain-containing protein
-10 -5 0 5 10
log2FoldChange

Figure 4.12: Filtered analysis of cecal metatranscriptomic data revealed that cyanocobalamin supplementation impacted the functional activity of *Lachnospiraceae* family members and *Citrobacter rodentium.* Prior to infection, the functional activity of the *Lachnospiraceae* family (a) shows that cyanocobalamin treatment increased the expression of numerous genes, including fibronectin type III domain-containing protein and serine/threonine transport SstT. (b) After exposure to *C. rodentium*, the *Lachnospiraceae* family members of the inf_CON and inf_CNCbl40 groups displayed distinct activities. *Citrobacter* specific gene expression (c) was more pronounced in the inf_CNCbl40 group than inf_CON with notable signals of increased virulence gene expression, while control mice had more family 31 glucosidase activity (n = 8; bolded taxa represent a trend ($P < 0.10$); * $P < 0.05$, ** $P < 0.01$).

4.3.4 Excess cyanocobalamin does not directly impact *C. rodentium* virulence expression *in vitro*

Previous studies have shown altered virulence expression of Shiga toxin-producing EHEC *in vitro* in response to *B. thetaiotaomicron* competition for cobalamin (Cordonnier et al., 2016a; Curtis et al., 2014a), therefore, we tested *C. rodentium* virulence expression when grown alone or in competition at physiologically relevant concentrations of cobalamin (Figure 4.13). Because luminal cobalamin levels increased from 0.01 ppm to approximately 15 ppm with supplementation, we evaluated how cyanocobalamin at 0 ppm, 0.01 ppm and 15 ppm impacted *C. rodentium* growth and virulence. The competition assay revealed that *C. rodentium* maintains steady levels regardless of cobalamin exposure. In contrast, *B. thetaiotaomicron* numbers increased ($P < 0.05$) with additional cyanocobalamin at 0.01 and 15 ppm (Figure 4.13a),

indicating that there was competition for B12. When *C. rodentium* was grown alone, cyanocobalamin treatment lowered *C. rodentium* abundance from 8.0 to 7.5 log CFU/ml ($P < 0.05$) in a dose-dependent manner (Figure 4.13b). Neither the competition assay nor when *C. rodentium* was grown alone revealed a change virulence gene in the expression of virulence factors of the locus of enterocyte effacement (LEE) operon, which included the LEE-encoded regulator (Ler), Tir, and EspA, and were not found to be different in the competition assay (Figure 4.13c,d) or when *C. rodentium* was grown alone (Figure 4.13d).

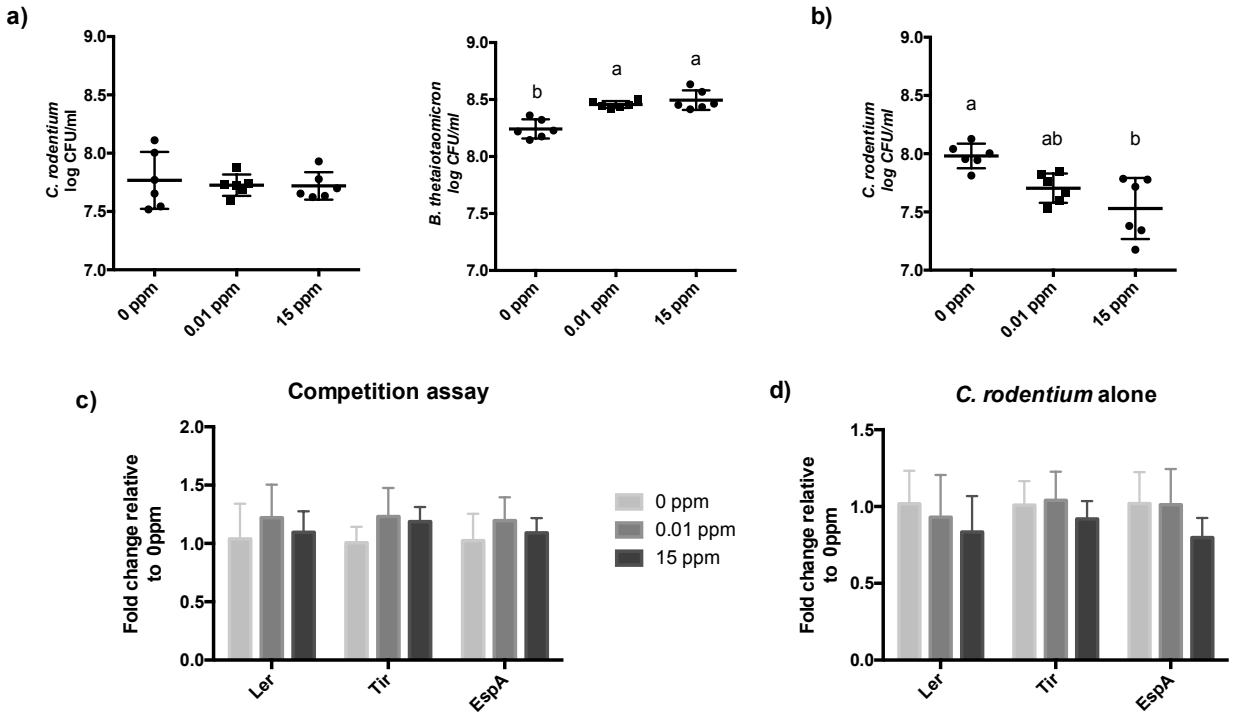


Figure 4.13: Cyanocobalamin alone was unable to directly alter *C. rodentium* growth or virulence *in vitro* at physiological relevant concentrations. (a) Enumeration of *C. rodentium* and *B. thetaiotaomicron* grown in competition for 6 h anaerobically, and (b) of *C. rodentium* grown alone. Expression of gene related to virulence (Ler, Tir, and EspA) did not differ between

treatments when *C. rodentium* was grown in (c) competition or (d) alone at different concentration of cyanocobalamin (n = 6; $P < 0.05$).

4.3.5 Cobalamin supplementation alters colonic cytokine profiles of naïve and infected C3H/HeOuJ mice

To determine host response to cyanocobalamin treatment, cytokine and chemokine biomarkers were measured in the colon tissue of naïve and infected mice of the EPC experiment (Figure 4.14). Naïve mice supplemented cobalamin in excess had greater concentrations of cytokines IL-12/23p40 ($P < 0.001$), IL-4 ($P = 0.06$), and IL-17A ($P < 0.05$) in colon tissue (Figure 4.14a; Figure 4.15). The inf_CNCbl40 group had increased levels of IFNγ ($P < 0.05$), IL-10 ($P < 0.05$), IL-17A ($P < 0.01$), and GM-CSF ($P < 0.05$) compared to inf_CON group (Figure 4.14b; Figure 4.16). These results suggest that the impact of cyanocobalamin impacted immune activation in advance of infection.

4.3.6 Colonic p40 subunit increased with cobalamin treatment depending on microbiota status

The IL-12/23p40 measurement could represent either the IL-12 or IL-23 cytokines. Therefore, we measured gene expression of IL12A (p35) and IL12B (p40) in the colon. IL12B was significantly enriched ($P < 0.01$) in the naïve_CNCbl40 group compared the naïve_CON group, but no difference was observed with IL12A (Figure 4.17a). In agreement, IL-12p70 cytokine levels, a heterodimer of p35 and p40 subunits were below the assay's detection limit in the colon (data not shown). The increase in IL-12/23p40 levels was not observed in the SURV experiment mice (Figure 4.17b), indicating the response is likely dependent on a specific

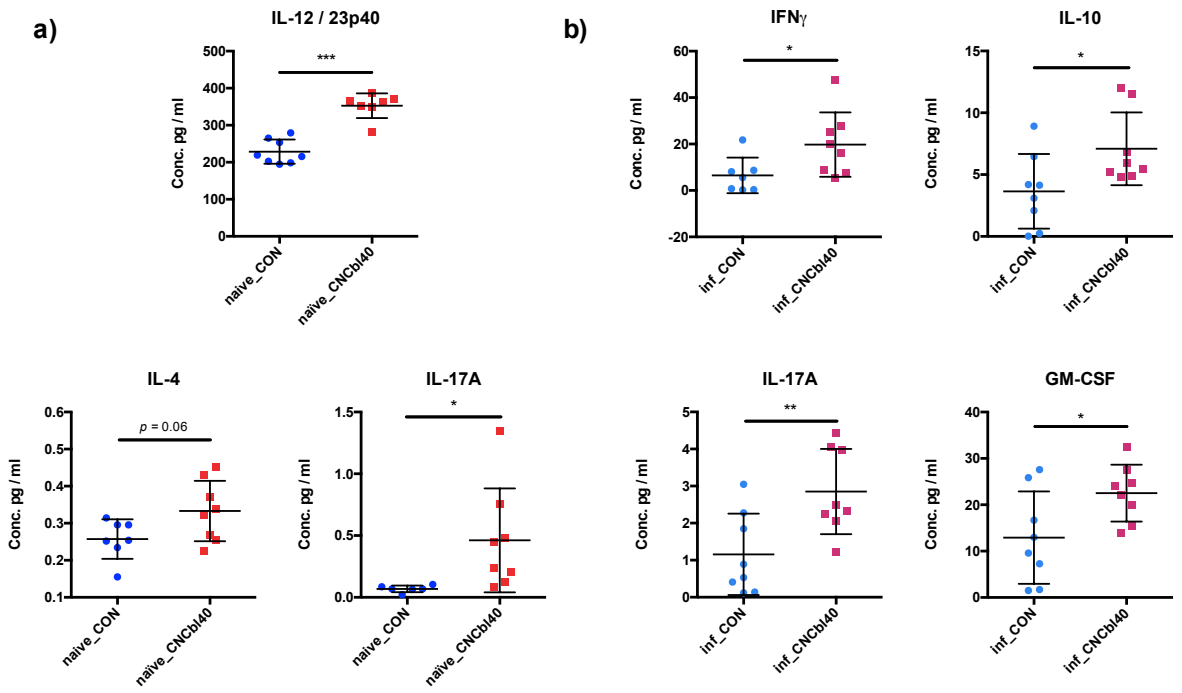


Figure 4.14: Significant host biomarker concentrations determined in colon tissue homogenates of C3H/HeOuJ mice. (a) Cyanocobalamin supplementation enhanced IL-12/23p40, and IL-17A cytokines of naïve mice, with a higher trend of IL-4 levels ($P = 0.06$) compared to control. (b) *C. rodentium* challenge led to a significant increase in IFNγ, IL-10, IL-17A, and GM-CSF in the cyanocobalamin-supplemented mice (n = 8; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

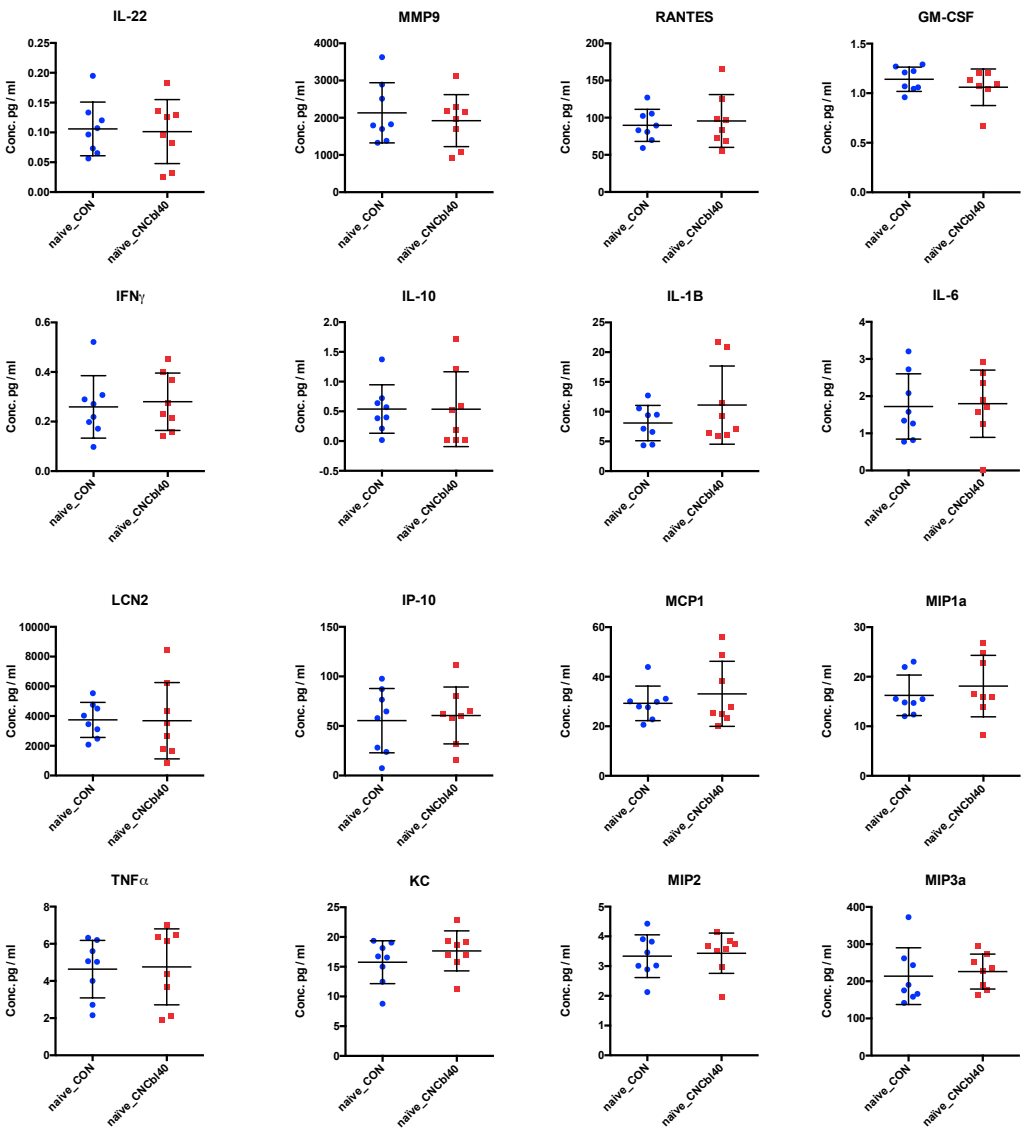


Figure 4.15: Colon biomarkers in naïve C3H/HeOuJ mice supplemented with cyanocobalamin (n = 8).

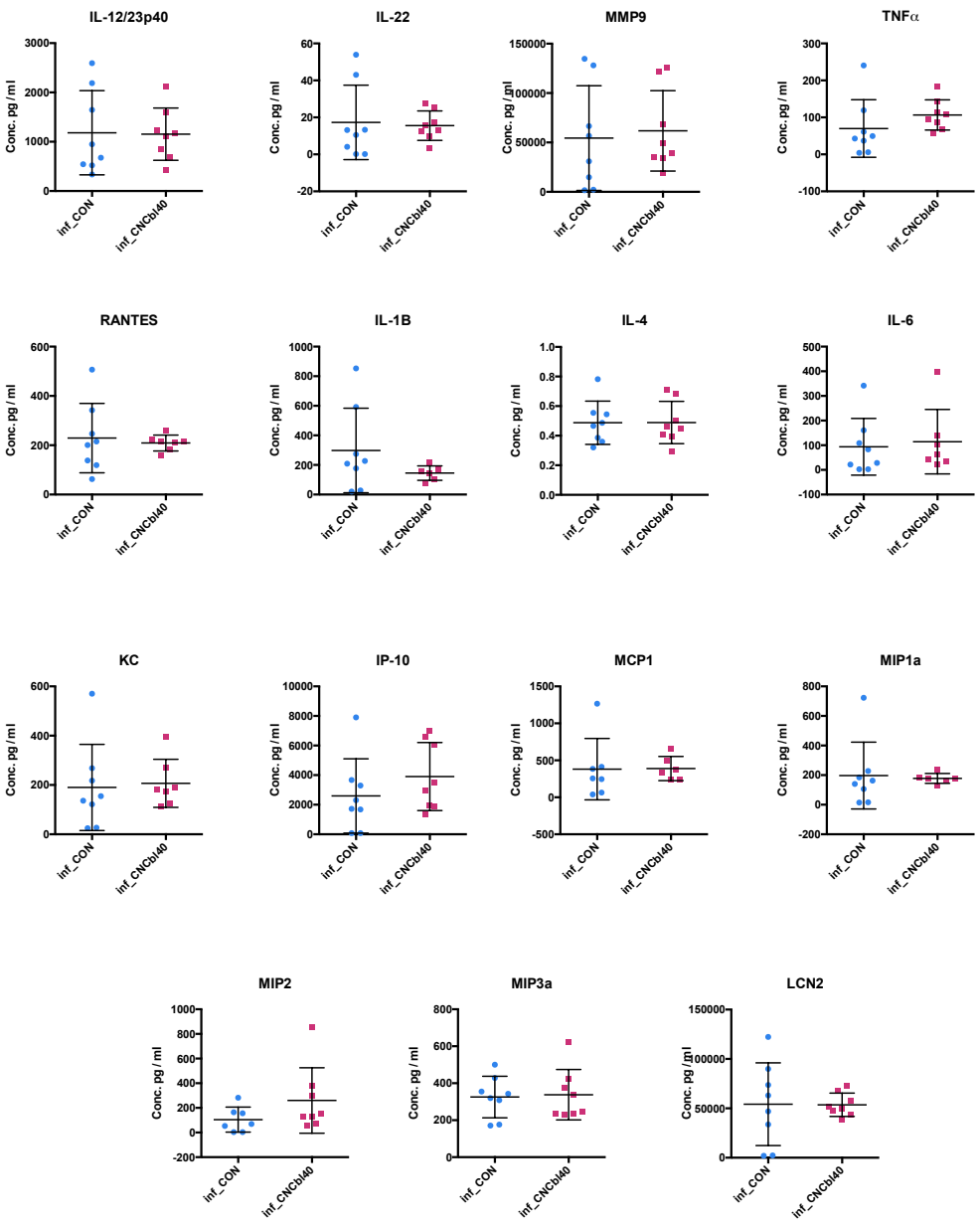


Figure 4.16: Colon biomarkers in *C. rodentium*-challenged C3H/HeOuJ mice supplemented with cyanocobalamin (n = 8).

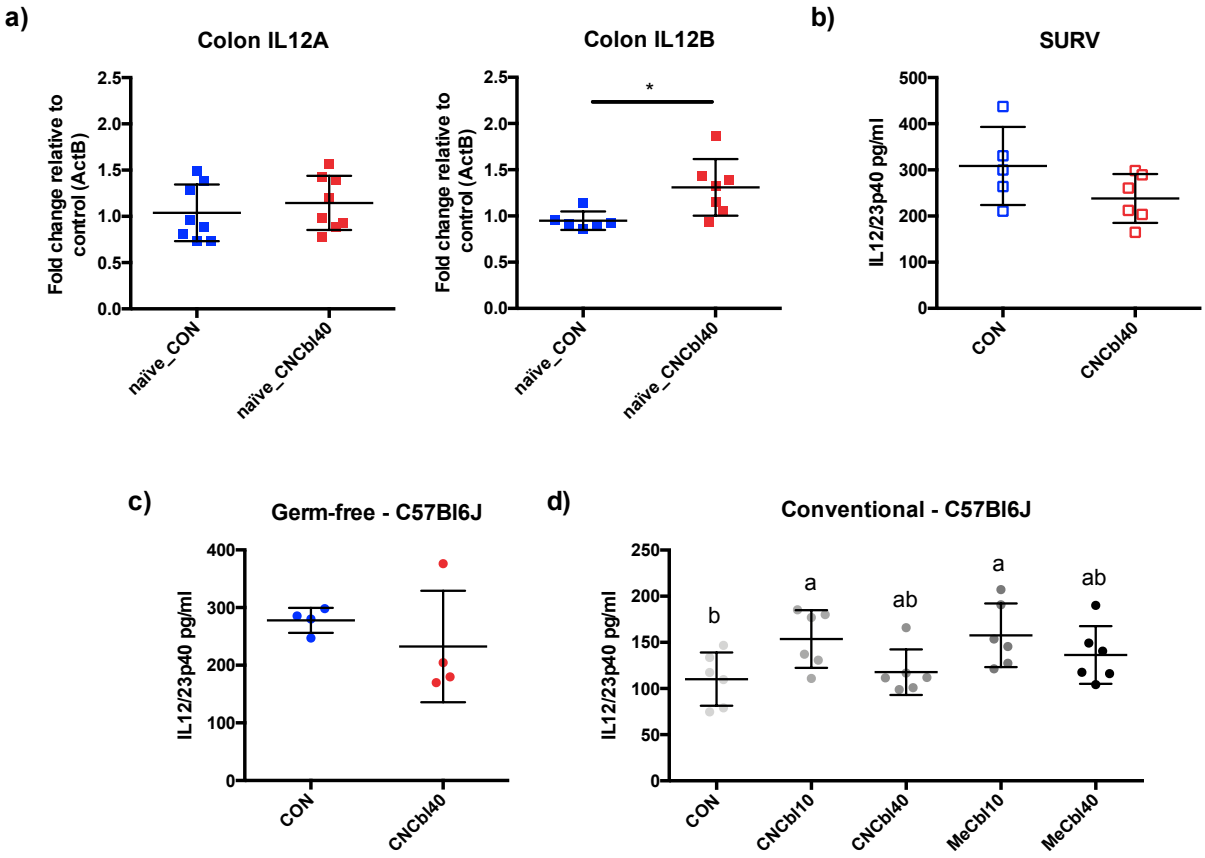


Figure 4.17: Pre-infection levels of IL-12/23p40 in colon tissues were directly related to microbiota structure and independent of related mice genotypes. (a) Gene expression was significantly higher for IL12B, the p40 subunit coding gene, but not for the IL12A (p35) gene (n = 8) in the EPC experiment. (b) The SURV experiment did not display the increase in IL12/23p40 (n = 5 - 6), nor did (c) germ-free C57Bl/6J mice (n = 4; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (d) Cyanocobalamin and methylcobalamin supplementation at 10 µg/ml and 40 µg/ml increased IL/23p40 protein levels in the colon of conventional C57Bl/6J mice (n = 6; $P <$ 0.05) compared to control.

component of the microbiome. As such, we tested whether a microbial community was required for the induction of IL-12/23p40. Indeed, cyanocobalamin treatment did not increase IL-12/23p40 protein levels in germ-free C57Bl/6J mice (Figure 4.17c), although it did increase in conventional C57Bl/6J mice (Figure 4.17d). We also wished to rule out the potential of cyanide as compared to other forms of B12. Conventionalized C57Bl/6J mice displayed similar increases in IL-12/23p40 protein levels when they received either cyanocobalamin or methylcobalamin. Colon IL12/23p40 protein levels were greatest in the CNCbl10 ($P < 0.05$) and MeCbl10 ($P < 0.05$) groups, while CNCbl40 and MeCbl40 were numerically higher (Figure 4.17c).

4.4 Discussion

We demonstrate that excessive cobalamin levels in the gut can alter the functional dynamics of the microbiota and host immune signaling, providing an environment supportive of pathogen colonization. It is well established that host metabolism, including host diet, environmental factors, and immune status, drives the ecological environment of the GI tract (Anhê et al., 2020; Cabral et al., 2019; Forgie, Fouhse, et al., 2019). Perturbations in activities of the gut microbiota, otherwise known as a gut dysbiosis, are typically described along with several diseases (Geuking, 2016; Kho & Lal, 2018). A study using antibiotic treatment to deplete butyrate-producing microbes revealed that this type of dysbiosis supports *Enterobacteriaceae* expansion and inflammation through suppression of the peroxisome proliferator-activated receptor gamma (PPAR-γ) homeostatic signaling pathway (Byndloss et al., 2017). The activation of PPAR-γ has been shown to prevent gut inflammation by regulating macrophage and T cell populations (Guri et al., 2010; Ul Hasan et al., 2019). Many studies using antibiotics and diet to induce a dysbiosis have associated it to increased mucosal inflammation with a shift in pathogen

and/or pathobiont activity (Buret et al., 2019; Devkota et al., 2012; Seregin et al., 2017; Willing, Russell, et al., 2011). Environmental factors and cooperative-metabolic strategies in the host can promote host-microbiota mutualism and promote the shift of a virulent pathogen to an avirulent passenger of the gut (Sanchez et al., 2018). In the present study, the effect of high cobalamin levels on gut host-microbe interactions may help to explain the enhanced *C. rodentium* colonization, reduced survival and increased mucosal damage in cyanocobalamin-treated mice.

Cyanocobalamin treatment had a subtle impact on overall microbiota structure but a distinct impact on Firmicute populations in cecum and colon. A study using C57Bl/6J mice concluded that B12 supplementation minimally impacts microbial community structure under healthy conditions, but does after DSS-induced colitis treatment (Lurz et al., 2020). Under these conditions, the researchers saw a decrease in numerous Firmicutes species, including the *Lachnospiracaea* family members. Still, more interestingly, they saw an increase in *Enterobacteriaceae* species in mice supplemented with B12. Data supports our findings that naïve mice supplemented with cyanocobalamin consistently showed decreased alpha diversity due to the reduced abundance of Firmicutes species and an increase in *Parasutterella* species. Upon *C. rodentium* challenge, we found that mice supplemented with cyanocobalamin had a greater quantity of *C. rodentium* with a notable change to numerous *Lachnospiraceae* species. In particular, cyanocobalamin treatment consistently reduced *Clostridia vadinBB60* group, *Lachnospiraceae* NK4A136 group, and other *Lachnospiraceae* species in both the SURV and EPC experiments. Similar changes to the microbiota were found to increase the ability of *Salmonella* to colonize the gut (Perez-Lopez et al., 2019). Researchers found that gnotobiotic mice harboring greater abundances of *Clostridia vadinBB60* and *Lachnospiraceae* NK4A136 groups, as well as *Ruminococcaceae* members had enhanced colonization resistance to a

Salmonella challenge (Perez-Lopez et al., 2019). In humans, high levels of *Clostridia vadinBB60* has also been associated with low *Escherichia-Shigella* (Lappan et al., 2019). The interactions among these Firmicutes and other commensal microbes have been shown to contribute to a host's ability to resist pathogen colonization and pathogenesis in *Salmonella*, *Clostridium difficile*, EHEC, and *C.* infections (Cameron et al., 2018; Jacobson et al., 2018; Mullineaux-Sanders et al., 2017; Ross et al., 2016; Thiemann et al., 2017). Therefore, we looked at the activity of the cecal microbiota in naïve and infected mice to better understand the impact of cyanocobalamin on the integration of *C. rodentium* into the resident gut community.

The activity of the gut microbiota, as determined through cecal metatranscriptome, was altered by cyanocobalamin treatment, indicating that cobalamin transport and utilization may be altered in some microbes. The citrate:sodium symporter, which was more expressed in naïve_CON than naïve_CNCbl40 group, is present only in a few pathogenic and commensal microbes in the GI tract, and is required for citrate fermentation (Martino et al., 2018). Interestingly, the increased citrate utilization by the gut commensal and opportunistic pathogen *Enterococcus faecalis* improved their pathogenic behavior in *Galleria mellonella* larvae (Martino et al., 2018). Following cyanocobalamin supplementation, the loss in citrate:sodium symporter expression suggested an overall reduction in citrate metabolism by commensal microbes. This would leave more citrate for *C. rodentium* and/or open a niche that is typically filled by citrate-metabolizing microbes in naïve mice. The persistence of commensals in various niches of the gut likely contributes to a host's ability to resist pathogen colonization through competitive exclusion (Ghoul & Mitri, 2016), and the enhanced citrate metabolism could be a good indication of microbe-microbe mutualism. In addition, the increased expression of lipopolysaccharide assembly genes implies that Gram-negative microbes were more active with

cyanocobalamin supplementation. This was matched by an increase in genes related to the catechol branch of beta-ketoadipate pathway, which may be related to *Pseudomonas* species, as they have been shown to degrade B12 (Scott et al., 1964). The increase in 'coenzyme B12 biosynthesis' was related to genes involved in cobalamin transport, a result of microbes recycling cobalamin from excessive supplementation. In contrast, genes related to 'Gram-positive competence' were elevated in the control group, along with putrescine utilization and carotenoids pathways. These pathways have been associated with colonic immunity and microbial mutualism that help to maintain GI homoeostasis (Nakamura et al., 2019, 2021; Pointon et al., 2010b; Tofalo et al., 2019). Cyanocobalamin supplementation led to distinct changes to overall cecal microbiota activity, including changes to various enzymes, transcription regulators, and transporters, and may indirectly explain the enhanced colonization of *C. rodentium*. In agreement, culture experiments showed no direct impact on *C. rodentium* growth or virulence at physiological relevant cobalamin levels. Interestingly, *C. rodentium* had enhanced expression of 'family 31 glucosidase' in control mice, a feature that may explain the nature of this pathogen's metabolism and increased virulence gene expression with cyanocobalamin supplementation. In fact, bacterial glucose metabolism and host niche adaptations that increase glucose levels in the intestine have been shown to control pathogen virulence (Anhê et al., 2020), and attenuate virulence in *C. rodentium* (Sanchez et al., 2018). Moreover, B12 may directly contribute to *C. rodentium* metabolism through a unique mechanism involving microbially derived 1,2-propanediol, which has been shown to control virulence and ability to colonize the host effectively (Connolly et al., 2018). A notable change in flagellin domain protein, related to *Lachnospiraceae* species, may represent a greater necessity for these organisms to be motile. This may be connected to fibronectin type III domain-containing protein and serine/threonine

transporter, both of which have been shown to be important for cell binding (Alahuhta et al., 2010; Pointon et al., 2010a) and anabolic reactions (Klewing et al., 2020), respectively. We suspect that this is a sign of niche displacement among *Lachnospiraceae* species and likely other Firmicutes in response to cyanocobalamin supplementation and pathogen challenge.

The cobalamin-induced changes in microbial composition and activity described above directly contributed to increased IL12/IL23p40 subunit levels. The cytokines IL-12 and IL-23 play a central role in T cell-mediated regulation, and their use as therapeutic targets has highlighted their importance to host defense and inflammatory disease (Hamza et al., 2010; Shi et al., 2021; Teng et al., 2015). The activation of IL12p40 has previously been attributed to hypoxia-inducible factor-1, a key regulator of mucosal inflammation by controlling Th1/Th17 response (Marks et al., 2017). Contrary to our results, researchers provide evidence of IL12p40 being protective against *C. rodentium.* Still, they noted a decrease in IL17, which in our mice was elevated in both a naïve and infected cyanocobalamin-supplemented groups. Previous studies have shown that the microbiota influences Th17 response in the gut (Ivanov et al., 2009) and that this intestinal inflammation can enhance pathogen colonization (Raffatellu et al., 2008). In fact, a study with similar microbial changes to our control mice caused by knocking out the Class I-restricted T cell-associated molecule gene was associated with lower Th17 response and reduced *Salmonella* colonization (Perez-Lopez et al., 2019). Although IL17 plays a key role in protecting against *C. rodentium* infection (Ishigame et al., 2009), we found that the increased level of IL17 and IL12/23p40 in colon tissue prior to infection was a sign of immune dysregulations and this led to greater colonization. Post-infection we found greater levels of IFNγ and IL17A when supplemented with cyanocobalamin. An immune phenotype previously characterized in the cecum of mice infected with *Salmonella* that is dependent on the microbiota

(Thiemann et al., 2017), but resulted in reduced colonization instead of an increase, as described in this study. Enhanced IFNγ, IL17A and GM-CSF production was associated with selecting commensal *E. coli* and was considered as hallmarks of intestinal inflammation (Kittana et al., 2018). Germ-free and SURV-experiment mice did not exhibit enhanced IL12/IL23p40 protein levels from B12 supplementation as observed in EPC-experiment mice. This suggests that the effects from cyanocobalamin are mediated through the changes to the gut microbiota; however, this cannot be the only factor contributing to pathogen exclusion because SURV-experiment mice supplemented B12 in excess were also more susceptible to *C. rodentium* pathogenesis.

4.5 Conclusion

In the present study, we show that cyanocobalamin induced-dysbiosis reduced key members in the Firmicute population, which brought on low-grade intestinal inflammation that ultimately enhanced *C. rodentium* colonization and pathogenesis. Excessive cobalamin levels in the gut alters mutualistic host-microbe interactions that would normally help to prevent pathogen colonization. In general, the cyanocobalamin-induced dysbiosis likely caused members belonging to *Clostridia vadinBB60* and *Lachnospiraceae* NK4A136 groups to be displaced. This in turn caused a dysbiosis that altered the IL12p40 signaling pathway in the colon and opened a niche for *C. rodentium* colonization. This provided a gut environment that promotes *C. rodentium* metabolism to be more virulent, as determined by the decrease in glucose enzyme activity and increases in virulent gene expression.

5 CHAPTER 5: GENERAL DISCUSSION

5.1 Symbiosis and stability

The symbiotic relationship between organisms and their host is dynamic. Symbiosis was first defined by Anton de Bary in 1879 as 'the living together of unlike organisms' and is now recognized as a central driver of evolution and health (Raina et al., 2018). Symbiosis is ingrained in every aspect of life, from the endosymbiosis of free-living cells to form mitochondria and chloroplast to nitrogen fixation in plants and energy harvesting in animals and insects (Dimijian, 2000; Raina et al., 2018). Symbiotic relationships between microbes and host began millions of years ago with the establishment of multicellularity. The multicellular organism provided a niche for the microbiota, forcing an evolutionary *tug-of-war* of symbiotic interactions that over millions of years would shape life as we know it. Microbes co-evolved with host and forced adaptations that made multicellularity possible, this includes cell adhesion properties, cell-cell communication and coordination, and programmed cell death (Grosberg & Strathmann, 2007). Although stochastic at first, a state of stability between the microbiota and host would eventually had to have occurred, ensuring the existence of both host and microbiota. The stability of symbiotic interactions can manifest in both positive and negative effects and are considered to lie on a continuum between parasitism (harmful), commensalism (neutral) and mutualism (beneficial) (Drew et al., 2021). Parasitic interactions led to the evolution of resistant host defences to exclude pathogenic microbes, whereas mutualism and commensalism are a product of defences that promote tolerance between host and microbe (Ayres, 2016). A host's ability to tolerate and control the microbes is a major part of the complex symbiotic interactions that governs the assembly and function of a stable microbiota. This is supported by the fact that a

significant degree of host specificity in microbial communities exist between species and across body sites (Jackrel et al., 2021; Mallott & Amato, 2021). The specificity in microbial community structure is a reflection of the environment provided by the host. In the case of the gastrointestinal (GI) tract, host diet and genetics shape microbial gut ecosystems, so any change to either would be reflected in a change to microbial community structure and activity. These host-microbe interactions are important when considering the contributions of the microbiota to host health, particularly in the context of a hosts ability to resist enteric infection (Forgie, Fouhse, et al., 2019). Studies carried out in this thesis focused on phytochemical and vitamin B12 as it relates to gut integrity because they both have the ability of directly modify the gut environment and are known to directly contribute to host health.

5.2 Symbiosis in gut integrity

The mammalian GI tract must breakdown food into absorbable components and at the same time, prevent the external environment and microbes from compromising host physiology. All multicellular life had to evolve specialized mechanisms at the interface between host tissue and the external environment to maintain homeostasis and protect themselves against microorganisms and toxins. This was accomplished in part by the development of cellular adhesion properties, such as tight junction proteins, which not only provided a means to develop specialized structures, but also enhanced cellular coordination and communication (Zihni et al., 2016). Epithelial cells that line the GI tract are highly specialized, from Paneth and Mast cells that guide immune defences like antimicrobial peptides to goblet cells that produce and secrete mucus (Dupont et al., 2014; Johansson et al., 2013), all aspects of the gut are a product of the co-evolution between host and microbe. Lastly the ability of controlled cell death, known as

apoptosis, plays a vital role in the gut as it is a dynamic organ with a high cell turnover rate. Together these aspects are the general categories to consider when evaluating symbiosis in GI integrity in health and disease.

Any breakdown in cellular adhesion, communication and apoptosis is directly associated to disease and physiological changes in the host. For this reason, the gut microbiota has been notoriously associated with various health outcomes because it is constantly shaped by the ecosystem the host provides, of which host diet and physiology have the greatest influence. Dysbiosis is characterized by an over-presentation of pro-inflammatory organisms and loss of beneficial ones. Although causation remains poorly defined, the general consensus is that the symbiotic interactions between host, environment, and microbe become disjointed, and normal cellular communication is obstructed. This is followed by the expansion of pathobionts and other inflammatory factors that further aggravates digestion and absorption of nutrients that are vital to support host defenses (Forgie et al., 2020). The hypothesis is that this leads to a collapse in the symbiotic interactions between host and microbe. The microbiota provides a living barrier to exclude pathogenic and other foreign bacteria, but this can only function properly if the ecosystem as a whole is stable and cooperative. Nurturing commensal and mutualistic relationships between microbe and host would therefore provide the resident microbial population a competitive advantage over new and pathogenic colonizers of the gut.

5.3 Applying colonization resistance in gut ecology

In our studies, we focused on the first few days post-infection to characterize the collapse in the gut ecosystem that alters infection severity. We show that infection severity can change in response to diet-induced modifications to microbial networks. Specifically, we found that the

antimicrobial and mucus-altering properties of phytochemicals and collapse in vitamin B12 sharing among gut microbes leads to a dysbiosis and increased pathogen colonization. As outlined in Chapter 1, numerous genetic factors help to control intestinal integrity and a host's ability to resist infection. Although genetics can predispose a host to infection (Casanova & Abel, 2013), diet and symbiosis between the host and microbiota is suggested as more important than host genotype in microbial assembly in the gut (Carmody et al., 2015). Colonization resistance has been used to describe the protection that the intrinsic microbiota offers against pathogens (Kreuzer & Hardt, 2020). The main mechanisms of colonization resistance are by niche occupancy and the competition and sharing of nutrient between microbes. Other factors include; inhibitory metabolites that alter growth, antagonism where microbial bacteriocins and colicins kill other related strains, and bacteriophages that have the ability to reduce the fitness of a particular bacterial strain in the gut (Kreuzer & Hardt, 2020). The purpose of understanding how diet-host-microbe interactions contribute to colonisation resistance will help improve food-based strategies to prevent and treat gut diseases caused enteropathogenic bacteria.

5.4 Translating research into human nutrition

Symbiotic interactions in the gut have provided a new perspective in our ability to understand how nutrition contributes to health. Intestinal integrity can be enhanced by nurturing the gut ecosystem with a diet that promotes beneficial and commensal interactions. When optimized, the microbiota would provide colonization resistance and the host would promote commensalism of pathobionts and pathogens, thereby strengthening host intestinal integrity. The proper function of the intestine is in the balance between the negative and positive interactions that occur between microbes and host. Therefore, a dysbiosis index could be implemented into

personalized nutrition, but would require a precise understanding of the symbiotic relationships of the gut with defined markers to measure. For example, a marker could range from the displacement of the sensitive Firmicute populations like in each study (Chapter 2, 3 and 4) to the increased glucosidase activity of an avirulent *C. rodentium* population as shown in chapter 4 and supporting studies (Anhê et al., 2020; Sanchez et al., 2018). Other markers could include microbial metabolites which would help explain how microbes utilize nutrients from host diet. Characterizing these interactions with measurable markers provides a means to help guide personalized diet programs and therapies, particularly as it pertains to gut integrity and preventative healthcare strategies.

5.5 Limitations and future studies

Studies included in this thesis are based on a communicable infectious disease challenge model in mice to infer dietary influence on intestinal integrity. Since microbial communities are individualized and variable between species, extrapolating these studies in mice to understand human nutrition can only provide insight into the conserved diet-microbe-host interactions among mammals. Additionally, the high degree of functional redundancy between taxonomically distinct microorganisms makes it difficult to single out any one microbe in the pursuit of causation (Louca et al., 2018). Although the full mechanisms remain unknown, these studies demonstrate that diet induced dysbiosis from phytochemical and vitamin B12 consumption can alter infectious disease tolerance and resistance. In effect, dysbiosis increased GI inflammation making mice more susceptible to *C. rodentium* colonization. Although studies support the inflammatory contributions of a gut dysbiosis to host's ability to resist infection, there are still some limitations that are worth considering in future studies.

In all three studies, the bacterial microbiome was profiled using the 16S rRNA gene amplicon method optimized for bacteria. The focus on using extraction, sequencing and analysis methods specific for bacteria left an unexplored portion of the microbiota that could contribute to the overall syntrophic interactions with host. This includes the archaea, fungi, protist, and viruses that are considered integral to symbiotic ecosystem, but like bacteria their contribution to health remain poorly understood. Initial microbiome studies have focused on bacteria because they are considered to outnumber eukaryotes and archaea in the colon of humans by 2-3 orders of magnitude (Sender et al., 2016). It has become clear that non-bacterial organisms of the microbiota can contribute to health and disease (Matijašić et al., 2020), but taking a multi-kingdom approach provides a new set of challenges to move from correlation to causation in host-microbe interactions. Similar to the findings covered in this thesis, most amplicon-based studies of the microbiota rely on relative abundances in microbial signatures to make correlative interpretations on host physiology or pathology. Although this can provide a means to compare intra-kingdom differences in microbiota community structure, it fails when applied to inter-kingdom interactions studies where absolute quantification is necessary (C. Rao et al., 2021). This is partly due to the fact that the relative abundance between two groups can change even when the absolute quantification of one remains constant. To overcome this, a genetic spike-in method to appropriately quantify the absolute abundance of bacteria, fungi, and archaea simultaneously can and has been adopted (Tkacz et al., 2018). The genetic spike-in method requires the addition of synthetic DNA fragments that contain universal primer binding sites specific for prokaryotes, eukaryotes and fungi at known concentrations. Absolute quantification of the entire microbiota will allow for more accurate interpretations of the interactions among microbes and their host as they relate to diet and health.

Diet-induced dysbiosis from both vitamin B12 and phytochemicals seem to be characterized by a change in the Firmicutes population. Most of the microbiota has yet to be cultured, so our ability to assess their dynamics in the gut ecosystem has been limited to 16S rRNA amplicon sequencing or metagenomic studies. The large amount of information gathered from the various sequencing technologies requires annotated databases for appropriate taxonomic classification. Tailored DNA extractions have been optimized for the difficult to lyse endospore-forming Firmicutes; however, it still wasn't enough to get an the appropriate detection in metagenomic datasets due to insufficient databases and analysis methods (Filippidou et al., 2015). A large portion of the uncultured species of the microbiota belong to the genetically diverse group of Firmicutes and have yet to be properly annotated and cultured (Almeida et al., 2019), so databases to date, and the ones used in this study, may underrepresent this dynamic group. In addition, most of the identifiable Firmicute species of the gut ecosystem are in low abundance so the standard sequencing and analysis methods may not completely capture the Firmicute species in microbiome data as they may be below the detection limit of the sequencing method. Future work should focus towards isolating and culturing Firmicute species in order to improve databases and conduct follow-up gnotobiotic studies with defined communities to confirm their contributions to the gut ecosystem.

The gut ecosystem is dynamically linked to host diet and metabolism. Although several unique effects on the host gut environment likely exist from various dietary components, the experimental chapters of this thesis focused solely on the consumption of phytochemicals and vitamin B12. It has become clear that the effects from diet, or for that manner any environmental assault to the gut is context dependent and requires an understanding of all dietary components together as they contribute to health outcomes. Chapters 3 and 4 were dedicated to understanding

the ecological effects of phytochemicals on the microbiota that could alter a host ability to resist infection. We tested the impact of phytochemicals in the context of a purified high and low-fat diet; however, under different dietary conditions we may not get the same effect. The fact that different sources of protein, carbohydrates and fats can have unique effects to the microbiota makes it difficult to draw conclusions based off only one dietary background. Using different diet backgrounds will alter the gut environment in different ways and microbes and their host may respond differently under these circumstances. Similarly, the vitamin B12 study in chapter 5 was only conducted using the standard chow diet (2020SX Envigo), therefore it would be useful to expand these dietary studies to include other diet backgrounds. By establishing a well characterized database of how microbes interact to one dietary component under various diet-gut environment conditions will help to elucidate causation in the microbe-diet-host paradigm.

Chapter 3 and 4 provide the basic framework that phytochemicals have on mucus formation and microbial assembly in the gut. Since phytochemicals are antimicrobial in nature, they can have direct effects on gut community structure; however, we suggest that they directly impact mucus formation which increases the activity of mucolytic bacteria species in the gut. Phytochemical-induce dysbiosis may manifest in various ways but in the case of enteric pathogens may be detrimental as it encourages a favourable gut environment for pathogens to flourish. It is worth noting that the antimicrobial properties of ingested phytochemicals could be optimized alone or in combination with other therapies to directly target enteric pathogens but this still comes with the risk of antimicrobial resistance (Willing et al., 2018b). Developing a database of purified concentrates of monomeric and polymeric phytochemicals will allow for a large-scale *in vivo* and *in vitro* experiments to characterize their direct effects on gut microbes. The use of crude polyphenolic extracts in the studied diets leaves unanswered questions of which

type of phytochemical is responsible for the antimicrobial and mucus altering properties in the gut. Future studies should consider testing individual isolates alone and in combination to appropriately classify their properties on gut ecology. Many challenges exist in the extraction and purification of phytochemicals which is the main limiting factor that prevented us from directly studying purified extracts, including getting large purified quantities to use in animal trials. Various methods from solvents to microwave and ultrasonic-assisted extractions technique and high-speed counter current chromatography, also known as centrifugal partitioning, may help in batch extractions of large quantities of phytochemicals needed for such studies (Hubert et al., 2013; Sridhar et al., 2021; Vinatoru et al., 2017). Isolating various phytochemicals and conducting controlled dose studies in animals with gnotobiotic (defined) and conventional microbiotas will help to elucidate the microbial interactions that alter gut health.

Chapter 5 focused on vitamin B12 supplementation in excess and how it contributes to microbial activity. Since microbial communities are well-known to modify and utilize vitamin B12 for their own metabolism (Degnan, Taga, et al., 2014), our study aimed to stop mutual or competitive interaction among microbes in the gut for cobalamin. These mutual and competitive interactions are thought to help maintain the symbiotic ecosystem of the gut and in the case of over supplementing vitamin B12, we found that this changed the microbiota, along with the ability of the host to protect against and resist a natural pathogen challenge. Although we tested two different doses and types of vitamin B12 (MeCbl and CNCbl at 10 and 40 ug/ml drinking water), we found that both induced similar host response of IL12p40 protein levels in colon tissue. Future studies should focus on doses lower than 10 ug/ml in drinking water to determine the threshold dose of cobalamin that initiates a change in gut ecology. It is only in this context that the changes in the microbiota can be well characterized to provide a causative understanding

of the microbial interactions impacting a host's ability to resist infection. More studies are warranted and should focus on dose and type under different context to establish a better understanding of the syntrophic cobalamin sharing networks of the gut.

In summary, controlling the microbiota with targeted dietary interventions may prove to be a vital therapeutic option in preventative healthcare. The mounting concerns over a dysbiosis has pointed towards nutrition, the microbiota and breakdown in host coordination as a causal factor towards disease susceptibility; however, the direct cause and effect are difficult to tease out. Future nutritional research requires well-controlled studies of a nutrient on its own and in combination with other components that stress and protect the gut. Using a multi-omics and an extensive host phenotyping approach will help unravel the complex interactions between GI integrity, microbiota and health.

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