PHYSICAL ACTIVITY AS A MODULATOR OF INTESTINAL HEALTH AND ITS IMPLICATIONS IN INFLAMMATORY BOWEL DISEASES

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Abstract

The interactions between humans, the environment, and intestinal microbiota form a tripartite relationship fundamental to the overall health of the host. Disruptions in this delicate balance between the microbiota and host immunity are implicated in various chronic diseases including inflammatory bowel disease (IBD). IBD encompassing ulcerative colitis and Crohn's disease are idiopathic, relapsing chronic inflammatory disorders of the intestinal tract with annual health care burdens of over $1.8 billion in Canada. There is no known cure for IBD, as so, novel therapeutic in its prevention and management are of great interest. Recently, physical activity (PA) has been proposed as a potential therapy in combating IBD. Here we show that higher aerobic fitness in humans is associated with increased bacterial diversity in the gut and higher abundances of butyrate, a type of short-chain fatty acid produced by resident bacteria with known anti-inflammatory properties. We confirm these findings in animal models showing that voluntary wheel running (VWR) in mice increases butyrate production. Additionally, VWR mice show increased microbial diversity, decreased expression of pro-inflammatory (TNF-α, TGF-β, and IFN-γ) and increased expression of anti-inflammatory (IL-10) cytokines suggestive of the potential to be primed against the damaging effects of chronic inflammation. These enhancements however were absent in a life-long model of mucin2 deficient ($MUC2^{-/-}$) murine colitis and VWR offered no protection in these mice against disease symptoms. Taken together, these suggest that the benefits of PA against IBD are preventative in nature and cannot reverse existing disease states like those found in IBD. We further showed that certain PA-derived changes in the intestines such as microbial community changes, upregulation of IL-10, and attenuation of IFN-γ are dependent on the amount of PA while reduction of anti-inflammatory cytokines TNF-α and TGF-β can occur even under low running conditions. In summary, we showed that PA can beneficially modulate the intestinal environment in healthy hosts, leading to a primed anti-inflammatory state likely effective in IBD management during remission.

Lay Summary

Physical activity (PA) is associated with a myriad of health benefits in a variety of diseases. The role of PA in inflammatory bowel diseases (IBD) however is not known. In this work we explore the relationship between PA and intestinal health by examining different components of the gut environment such as the microbial population and inflammatory signaling. We found that PA can beneficially alter the intestinal environment in healthy mice but does not reverse existing symptoms of IBD. We conclude that PA is a promising adjunctive therapy in prevention and reemission maintenance of IBD.

Preface

A version of Chapter 2 has been published and redistributed here under the Creative Commons Attribution License 4.0: Estaki M, Pither J, Baumeister P, Little JP, Gill SK, Ghosh S, Ahmadi-Vand Z, Marsden KR, Gibson DL. Cardiorespiratory fitness as a predictor of intestinal microbial diversity and distinct metagenomic functions. Microbiome. 8;4(1):42. Under the supervision of my committee members I designed the study, conducted lab experiments, collected and analyzed the data, and wrote the manuscript. Dr. Jonathan Little and his students Peter Baumesiter and Katelyn Marsden conducted VO2max tests on participants, collected dietary intake data as well as stool samples. Sandee K Gill performed GC analysis of fecal short-chain fatty acids. Zahra Ahmadi-Vand processed dietary data. Dr. Jason Pither supervised and provided continuous expert support with regards to community ecology analyses. Dr. Sanjoy Ghosh provided equipment and continuous support for GC analyses. Dr. Deanna L. Gibson was involved in supervision of all aspects of the project. All members provided critical review of the manuscript and editing.

Chapter 3 was conducted at UBCO under supervision of all committee members. I designed the study, conducted the animal experiments, carried lab work, analyzed the data, and wrote the version herein. Dr. Douglas Morck and Sandeep K. Gill were involved in animal care and tissue collection. Jacqueline Barnett and Dr. Anton Callaway were involved with RT-qPCR analyses. Jacquelin Barnett provided additional assistance with histopathological scoring. Candice C. Quin performed GC analyses under the supervision of Dr. Sanjoy Ghosh and assisted with amplicon preparations. Dr. Jason Pither acted as statistical analyses supervisor. Dr. Deanna L. Gibson was involved in supervision of all aspects of this project and provided critical review of the version presented here. A version of this chapter will be submitted for publication in mSystems journal under the title "*Wheel running modulates the intestinal microbiota, short-chain fatty acid production, and immunity in WT but not MUC2 deficient mice*".

Mehrbod Estaki, Anton Callaway, Jason Pither, Candice C. Quin, Jacquelin Barnett, Sandeep K. Gill, Douglas Morck, Sanjoy Ghosh, and Deanna L. Gibson

For Chapter 4, the animal experiments were carried out at Centre for Disease Modelling in UBC, Vancouver by animal technician Chuanbin Dai. Under supervision of my supervisory committee, I designed the experiment, collected tissues, carried out lab experiments, analyzed the data, and wrote the version herein. Dr. Anton Callaway assisted with RT-qPCR experiments. Dr. Deanna L. Gibson supervised the entirety of the project and provided critical review of the manuscript here. A portion of the data from this experiment has been published. Spielman LJ, Estaki M, Ghosh S, Gibson DL, Klegeris A. The effects of voluntary wheel running on neuroinflammatory status: Role of monocyte chemoattractant protein-1. Mol Cell Neurosci. 79:93-102. A version of this chapter will be submitted for publication to PLoS One journal under the title "*Low volume wheel running in mice elicits changes in the intestinal immune profiles without changes to the intestinal bacteriome"* Mehrbod Estaki, Chuanbin Dai, Anton Callaway, and Deanna L. Gibson

Work in Chapter 2 was approved by UBC Clinical Research Ethics Board under the certificate number H14-00482 (Fitness and gut microbiota). All animal works were conducted with approval from UBC Animal Care Committee under certificate number A17-0120 (Exercise and IBD).

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To my brother Sina who has taught me to never fear being me and only me. To my parents, thank you for every sacrifice you have ever made for the sake of me and Sina. Thank you for a lifetime of endless love and unwavering support. Everything I am I owe to you.

Last but certainly not least, thank you to my amazing partner Sydney who has taught me the true meaning of compassion, caring, and love. Thank you for putting up with me and carrying me through the finish line in these recent months. I look forward to many years of trying to show you my appreciation.

Dedication

I'd like to dedicate this work to my Maman Nahid, whose love knows no limits and continues to teach me to be a better person every day. Thank you for always reminding me to love.

Chapter 1: Introduction[1]

The overall goal of this thesis was to explore the role of physical activity (PA) in modulating intestinal health. In this chapter I first provide an overview of the functional anatomy of the intestinal tract and the role of their microbiota in health and disease, with emphasis on inflammatory bowel diseases. Next, I introduce our current understanding of factors that influence the intestinal microbiome, methods and limitations of microbiome studies, followed by a review of existing literature regarding the role of PA in intestinal health and the gut microbiome and highlight any knowledge gaps in the field. I then conclude by describing how this thesis attempts to fill in these gaps by outlining the specific goals and objectives of each chapter.

1.1 Anatomy of the Intestines

The human intestine or gut is a multilayered and multifunctional organ which holds tremendous influence over the host's health. The anatomy and function of the intestines varies from their proximal origin at the stomach through their distal point at the anus, however the overall ultrastructure of the tract remains relatively similar (Figure 1). The outer region of the intestine is primarily made up of two smooth muscle layers collectively referred to as muscularis externa, which is involved in transport of food down the intestinal tract via peristalsis. Innervating between these two muscle layers is a mesh-like network of neurons and ganglia referred to as the myenteric (or Auerbach's) plexus, a major component of the enteric nervous system (ENS). The myenteric plexus can act autonomically without communication with the central nervous system (CNS), such as during peristalsis of the colon, however normal functions require continuous bidirectional communication with the CNS, predominantly via the vagus

[1] Figure 1 is copied here with permission. The original image was published in: Estaki M, Quin C, Gibson DL (2015) "Diet And Dysbiosis" in Luigi Nibali and Brian Henderson (ed.), The Human Microbiota And Chronic Disease- Dysbioses As A Cause Of Human Pathology. Chapter 30, 1st Edition. Wiley Blackwell, 2015

nerve. The myenteric plexus transmits messages from the CNS to the intestines and is involved in various processes such as motor activity, absorption, vascular blood flow, and secretion of mucus and digestive juices by further signaling to other organs such as the gallbladder or pancreas (Yoo & Mazmanian, 2017). The next layer known as the submucosa is rich in blood and lymphatic vessels and is the main source of metabolic supply to the gut. The submucosa contains the other branch of the ENS referred to as the submucosal (or Meissner's) plexus which derives from extensions of the myenteric plexus. Neurons and ganglia from the submucosal plexus further extend through the next layer, the mucosal layer, forming a direct channel of communication with the intestinal epithelial cells (IECs). For example, neurotransmitters released by the ENS bind to goblet cells and can stimulate or suppress mucus secretion (Birchenough, Nyström, Johansson, & Hansson, 2016; Gustafsson et al., 2012) as well as regulate the rate by which these cells allow passage of antigens across the IEC to be sampled by the immune system (McDole et al., 2012). The mucosal layer is the inner most layer that houses more lymphocytes than the rest of the body and thus is considered a major component of the body's immune system. The mucosal region consists of another thin layer of muscle referred to as muscularis mucosa, lamina propria, epithelium, and finally the mucus layer lining the lumen wall which is in direct contact with the trillions of symbiotic microbes that reside within the intestinal tract. The single celled layer of the epithelium is made up of IECs which are the major constituents of the gut covering an estimated 300 m^2 folded into villi, microvilli, and crypts. One major difference between the small and large intestine is in the composition of this epithelial barrier. IECs in the small intestine can differentiate into seven cell lineages (enterocytes, Paneth cells, goblet cells, enteroendocrine cells, Tuft cells, M cells, or cup cells) whereas a healthy colon contains only enterocytes, goblet cells, and Tuft cells. The goblet cells secrete heavy glycosylated proteins known as mucin which form a thick two-tiered mucus layer covering the lumen of the large intestine or a single layer in the small intestine (Pullan *et al*., 1994). Regulation of mucus production involves highly complex interactions

between the IECs, immune cells, the intestinal bacteria, food metabolites, and the ENS (Sharkey & Savidge, 2014). Germ-free rodents have smaller and fewer number of goblet cells and display an overall thinner mucus layer compared to their conventional counterparts (Ishikawa et al., 1989; Szentkuti, Riedesel, Enss, Gaertner, & Von Engelhardt, 1990), a phenotype that can be rescued by introduction of bacteria or their byproducts (Petersson et al., 2011). In the small intestine where majority of nutritional uptake takes place, the mucosal layer is loose and porous to allow for food particles to reach the IEC. This means that the mucosal layer is penetrable by the microbes, however they are largely kept away from the IECs by antimicrobial peptides (AMPs) secreted into the apical side by Paneth cells (Ermund, Schütte, Johansson, Gustafsson, & Hansson, 2013). In the large intestine where uptake of water and sodium chloride occurs, the inner mucus layer is dense, firmly attached to the epithelium, and mainly impenetrable to the bacteria creating a relatively sterile environment. In contrast, the outer layer is loose and easily dislodged, therefore it is continuously regenerated (McGuckin, Lindén, Sutton, & Florin, 2011); this outer layer houses the majority of the commensal microbes of the colon. The mucus layer is made of several different types of mucins which are found in varying composition throughout the intestinal tract, however the predominant mucin across both small and the large intestine is the MUC2 type (Johansson & Hansson, 2016). Upon its release from the goblet cells, MUC2 mucin granulae are exposed to the increased pH and decreased calcum ion levels of the gut environment allowing water to rush in and causing massive expansion of these multimers (Ambort et al., 2012). This newly formed gel-like mucin sheet is attached to the epithelium as the inner mucus layer and replaces the overlaying layer by dislodging it further towards the lumen. This rapid and continuous process of mucus layer replacement is crucial to maintaining intestinal health with a new layer of mucus being produced approximately every hour (Johansson, 2012).

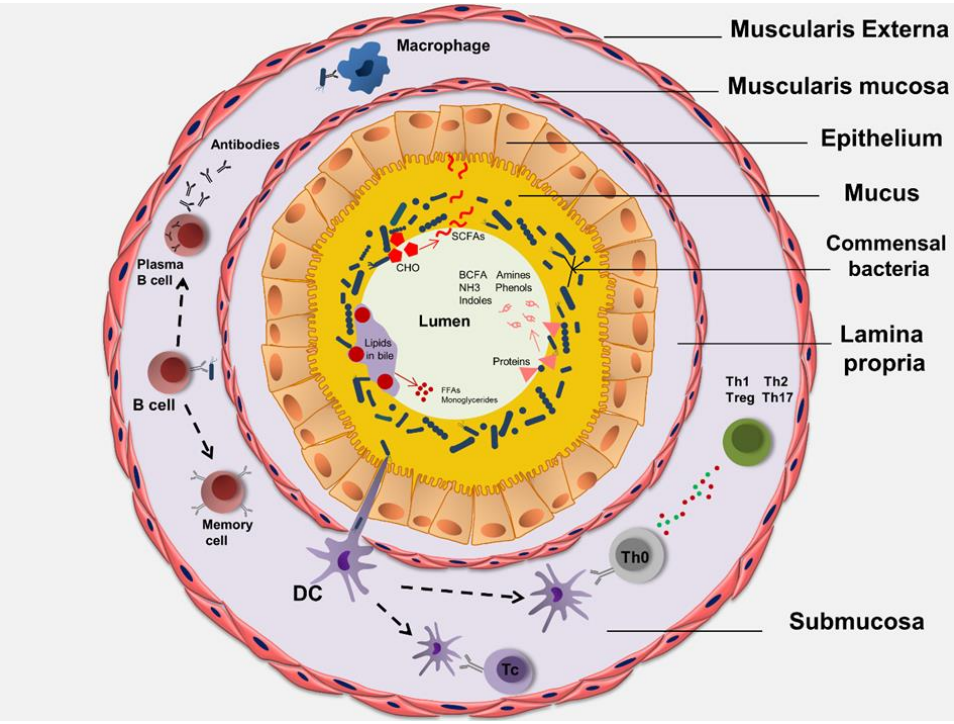

Figure 1. Functional anatomy of the intestine

The host cells, intestinal microbes, and the environment (i.e. food) form a complex tripartite relationship that is essential to human health. See text for details. SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids; NH3, ammonia; APC, antigen presenting cells; DC, dendritic cells

1.2 Intestinal immunity and homeostasis

In addition to acting as a physical barrier, IECs are also capable of mounting immunological responses which are essential in gut homeostasis. Pattern recognition receptors (PRRs) present on the IECs sense danger motifs referred to as microbial-associated molecular patterns (MAMPs) and respond by initiation of downstream inflammatory responses. PRRs including the toll-like receptors (TLRs) and NOD-like receptors (NLR) are particularly important as they can recognize different components of bacteria cells. Due to the large number of commensal bacteria in the lumen, surface TLRs such as TLR-2, TLR-4, and TLR-5 are primarily

expressed on the basolateral side of the epithelial layer in order to prevent improper activation of inflammatory responses (Oppong *et al*., 2013). Inappropriate translocation of commensal bacteria or intrusion of pathogens across the epithelium is detected by IECs and signifies a threat. In response, IECs initiate the innate immune system by recruiting highly phagocytic neutrophils which are the first line of defense in charge of killing the foreign bacteria. This is followed by arrival of larger phagocytic macrophages (type M1 and M2 associated with 'killing' and 'repair' phenotypes, respectively) which are also responsible for elimination of neutrophils to prevent secondary damage to the host. Another type of antigen presenting cell (APC), dendritic cells (DCs), now act as the bridge between the innate and adaptive immune system. DCs interact directly with goblet cells to allow for passage of luminal ligands across the epithelium (McDole *et al*., 2012). Dendrites extending from DCs then reach through the epithelium and continuously sample luminal ligands. The detection of MAMPs by DCs induces maturation of B cells and naïve T cells (Th0) into various effector T cells. B cells mature to immunoglobulin A (IgA) secreting effector cells which tag potential pathogens for removal while another subset become memory cells as part of the adaptive immune system. The Th0 differentiate to CD8+ presenting cytotoxic T cell (Tc) which induce apoptosis in infected cells, or CD4+ presenting T helper (Th) cells which promote various inflammatory responses by release of cytokines into the extracellular environment. Differentiation of Th0 cells into various Th subtypes depends on their exposure to specific cytokines. For example, Th0 differentiation in the presence of interleukin-(IL-)12 leads to Th1 subtypes involved in intracellular immunity and IL-4 leads to Th2 types involved in extracellular immunity. A combination of transforming growth factor beta (TGF-β), IL-6, IL-21, and IL-23 contribute to Th17 development which is associated with tissue inflammation, clearance of extracellular pathogens, and autoimmunity. Th0 cell differentiation into Th17 cells is also dependent on the presence of the transcription factor retinoic acid-related orphan receptors gamma (RORγ). Another type of effector cell, the regulatory T cells (Tregs) are activated under the presence of TGF-β. Tregs are immunosuppressive in that they inhibit T-

cell mediated actions towards the end of an immune response to prevent excessive inflammation. One important aspect of the intestinal environment is tolerance to commensal bacteria as to avoid immune activation in response to innocuous antigens (Rimoldi, 2005). The host can differentiate between commensal and pathogenic bacteria, while the commensal bacteria can develop resilience to host inflammation. While the exact mechanisms behind commensal vs. pathogen recognition is not fully understood, it involves elaborate and interconnected regulatory mechanisms. For example, commensal bacteria in the gut develop resistance to host AMPs (Cullen et al., 2015) to a much higher degree than pathogens, allowing them to survive the AMP-rich luminal environment. Secretory IgA in lumen bind to microbial cells, blocking their access to interact with the IECs, however they preferentially bind to pathogenic bacteria (Mantis, Rol, & Corthésy, 2011). Commensal bacteria produce metabolic byproducts such as short-chain fatty acids (SCFAs) that are beneficial to IECs and thus do not elicit inflammatory responses, while toxins produced by pathogenic bacteria are recognized by host cells and recruit strong pro-inflammatory responses. These SCFAs can also diffuse across the IEC and interact directly with the enteric nervous and immune system (Yoo & Mazmanian, 2017). Finally, the host can differentially regulate suppression of inflammatory cascades in response to commensal bacteria. In this regard, Tregs play an important role in suppressing overactive immune responses by production of IL-10 and TGF-β which in turn inhibit production of pro-inflammatory cytokines such tumor-necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and IL-1. IL-10 can further suppresses T-cell proliferation of both Th1 and Th2 cells by downregulating release of IL-12 and IL-4 by APCs, inhibit maturation of DCs, and limit the release of pro-inflammatory cytokines in mast cells (Taylor, Verhagen, Blaser, Akdis, & Akdis, 2006). IL-10 is also integral in preserving the mucosal integrity by preventing protein misfolding and ER stress in goblet cells (Hasnain et al., 2013). TGF-β on the other hand contributes to apoptosis of self-reactive clones, cell growth, and Th differentiation, making it an important component of intestinal healing during injury (Beck et al., 2003). Highlighting the importance of

these cytokines in intestinal homeostasis is the observation of chronic intestinal inflammation in mice with defective or complete absence of signaling in these cytokines (Gorelik & Flavell, 2000; Kühn, Löhler, Rennick, Rajewsky, & Müller, 1993). Due the fact that MAMPs vary across bacteria species and can recruit different T-cell mediated response by the host, the composition of the intestinal microbiota can significantly control the type of immune responses in the host.

1.3 Intestinal microbiota

Virtually every surface of the human body exposed to the external environment, including the intestines, is inhabited by a myriad of bacteria, archaea, viruses, fungi, and unicellular eukaryotes. This panoply of microorganisms that coexist within their host is referred to as the 'microbiota', while the catalogue of these taxa and their associated genes is referred to as the 'microbiome' (Ursell, Metcalf, Parfrey, & Knight, 2012). A growing body of evidence from recent years link disruptions of the intestinal microbiome, termed dysbiosis, in pathogenesis of various chronic inflammatory diseases such as type 2 diabetes, obesity, colorectal cancer, atherosclerosis, non-alcohol fatty liver disease, irritable bowel syndrome, and inflammatory bowel disease (IBD) [reviewed in (Y. K. Chan, Estaki, & Gibson, 2013)]. Most of our understanding of the intestinal microbiome comes from studies focusing only on the bacterial community, the bacteriome. This imbalanced perspective is due in part to lack of comprehensive databases and technical challenges with surveying other groups such as viruses, and the incorrect view that only bacteria significantly contribute to host health. The intestinal bacteriome is predominately (~ 95 %) composed of strict anaerobes residing within the oxygen-restricted mucosal layer, and to a much lesser extent, facultative anaerobes and aerobes. Starting from the stomach through to the colon, the number of bacterial cells increase exponentially moving from the proximal to the distal tract. The colon alone is estimated to harbor over 70 % of all the human microbial cells which are estimated to outnumber total human cells by 1.3:1 and weigh roughly ~ 0.3% of host's total mass (Sender, Fuchs, & Milo, 2016). More

impressively, over 2 million genes have been catalogued from the human microbiome to date (Qin *et al*., 2010), a number which vastly outnumbers the host's own ~19,000 protein-coding genes (Ezkurdia et al., 2014). Of the ~50 bacterial phyla discovered to date (P. D. Schloss & Handelsman, 2004), two dominate the human gut: the Bacteroidetes and the Firmicutes, while the remaining niches are colonized to a much lesser extend by Proteobacteria, Verucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria (Eckburg, 2005). It is generally thought that about 500 to 1,000 species of bacteria colonize a healthy human intestine (Xu & Gordon, 2003) with a high degree of individual variability in both diversity and composition. Bacterial composition also differs longitudinally along the intestinal tract corresponding to the primary function of each site, i.e., the small intestine is mainly involved in digestion and nutrient absorption whereas the large intestine is largely responsible for fermentation, water retention, and waste processing.

1.4 Microbiota in health and disease

The intestinal microbiome plays an important role in various physiological functions of the host such as digestion and absorption of nutrients from partially digested food, vitamin synthesis, production of SCFAs, immunomodulation, defense against pathogens, and even behavior (De Palma, Collins, Bercik, & Verdu, 2014). The resident bacteria act as a protective barrier against opportunistic pathogens by out-competing them for food and niche as well as excluding them physically by forming slime-like polymers known as biofilm (N. Kamada *et al*., 2012). They further induce the production of AMPs which have bactericidal properties similar to antibiotics, though they are less effective against the commensals themselves. During digestion, the intestinal bacteria are required for the breakdown of complex carbohydrates like starch and cellulose into SCFAs such as acetate, propionate, and butyrate which are the primary food source of the enterocytes (Bugaut, 1987). Butyrate in particular has been shown to possess therapeutic potential in IBD by enhancing IEC integrity and inhibiting inflammation by

upregulating Treg production. Up to 10% of the dietary energy in humans is estimated to derive from activities of their microbiota (McNeil, 1984). In addition, intestinal bacteria are essential in production of various vitamins such as vitamin K and certain B-vitamins that are otherwise not available to the host.

The microbiota's ability to train the immune system begins during the early developmental stages of the host and utilizes the immaturity of the neonate immune system. During these stages of life, skewed regulatory rather than inflammatory responses are favored in the host, thereby allowing the establishment of the microbiota (PrabhuDas et al., 2011). Continuous exposure to commensal MAMPs, such as TLR ligands, condition the gut IECs to become hypo-responsive to these interactions in later years (Chassin et al., 2010). As discussed previously, commensal bacterial can induce host tolerance to bacterial and food ligands by recruitment of Tregs. These actions however depend on bacterial lineages such that different species can invoke different immune responses. For example, polysaccharide A derived from *Bacteroides fragilis*, a prominent human symbiont was shown to induce IL-10 producing Tregs leading to reduced intestinal inflammation (Mazmanian, Round, & Kasper, 2008). Similarly, other groups of the human microbiota such as the Clostridiales, specifically within the IV and XIVa clusters have also been shown to recruit TGF-β-dependent regulation of Tregs (Atarashi et al., 2011). The activation of other regulatory pathways have also been observed, for example the segmented filamentous bacteria (SFB) and Cytophaga-Flavobacter-Bacteroidetes phyla appear to favor induction of Th17 cells under normal homeostatic conditions (Ivanov et al., 2008, 2009). Given the major influence of the intestinal microbiota on host immunity, there is great interest in characterizing a healthy microbiome and equally important ones associated with diseases.

Though attempts at identifying a healthy 'core microbiome' have been largely unsuccessful, a healthy gut microbiota is generally thought to consist of a highly diverse ecosystem of anaerobic microbes with high tolerance to physiological stresses. Dysbiosis refers

to a deviation away from this state, often resulting in low species diversity, fewer beneficial microbes, or the overabundance of pathobionts. Studies in mice lacking a microbiota (germ-free) have consistently shown exacerbated disease phenotypes such as inflamed intestines, reduced intestinal peristalsis, IEC morphology defects, decreased antibody production, and overall higher mortality rates in response to infectious insults and chemical models of murine colitis [reviewed in (Round & Mazmanian, 2009)].

1.5 Factors influencing the microbiota

The intestinal microbiota, much like any other living ecosystem, experiences fluctuations in growth and adaptations for survival. While this plasticity and innate ability to adapt to change is important for homeostasis, it is highly individualized and so prevents generalization of the effects of intrinsic and extrinsic factors that impose on the community's structure. So, while numerous factors have been identified with potential to alter the human microbiome, the exact nature of their influence is not consistent across the literature. In this section I briefly discuss some of these known factors.

Colonization of the intestinal microbiota begins rapidly at birth and thereafter until a stable microbiome is acquired by 2-4 years (Koenig *et al*., 2011). Factors with immediate consequences at birth include: mode of delivery, transfer of skin, exposure to vaginal and colonic bacteria, and presence of microbial populations in the environment from the father, doctors, nurses and the birthing area. Children delivered by Cesarean section have altered microbial diversity (Azad *et al*., 2013) and are at increased risked of various illnesses including asthma, obesity, allergies, and IBD (Neu & Rushing, 2011). Breast-feeding also plays an important role in the development of the infant immune system by delivery of not only essential nutrients but also beneficial microbes. The mother's breastmilk microbiota acts as a biological blue-print for establishing the infant's resident bacteria, with insufficient breastmilk exposure being associated with higher risk of auto-immune diseases in later years (Toscano, De Grandi,

Grossi, & Drago, 2017). The proper development of a child's immune system during the early developmental years is strongly attributed to the presence of a healthy microbiota and sufficient exposure to their biosphere. For example, children raised in farms have lower prevalence of various diseases such as hay fever, asthma, wheeze, and atopic sensitization compared to those raised in cities (Riedler *et al*., 2001; Von Ehrenstein *et al*., 2000).

The discovery of antibiotics is regarded as one of the greatest discoveries of the 20th century having saved millions of lives since their medicinal integration. Antibiotics are used to eradicate pathogens in the body, though their non-discriminatory mode of attack can concomitantly eradicate commensal bacteria leaving the gut vulnerable to colonization by other opportunistic species. It is generally thought that antibiotic-induced changes in the microbiota are normalized within weeks of cessation, however recent evidence in contrast to this dogma is emerging (Jakobsson *et al*., 2010; Jernberg, Löfmark, Edlund, & Jansson, 2010). In particular, antibiotics exposure during childhood has been associated with increased incidences of IBD in later years (Kronman, Zaoutis, Haynes, Feng, & Coffin, 2012), suggesting that dysbiosis may be one component of susceptibility to IBD. Changes in gut microbial populations are not only limited to antibiotic drugs. Increasing evidence points towards the involvement of nonantibiotic drugs such as antidiabetics, proton pump inhibitors, and nonsteroidal anti-inflammatory drugs, on gut microbiota [reviewed in (Maier et al., 2018)]. For example, a recent study testing the effects of 835 non-antibiotic drugs on commensal bacteria, found that 203 (24 %) of them could inhibit growth of some bacterial species (Maier et al., 2018). Strikingly, these species further displayed adaptation similar to bacterial resistance suggesting the potential risk of non-antibiotics in antibiotic resistance.

Perhaps the most influential environmental factor in microbiota dynamics is diet. The symbiotic relationship between the microbiota and host has co-evolved over many millennia and continues to evolve today. For example, the transition from hunter-gatherer lifestyle of the Paleolithic era to carbohydrate-rich farming style of the Neolithic period was accompanied by a

drastic phylogenic shift in oral microbes towards more cariogenic communities (Adler *et al*., 2013). Evidence suggests that long term dietary choices play an important role in shaping the intestinal ecology in humans [reviewed in (Y. K. Chan *et al*., 2013)]. For example, high red meat consumption is associated with a predominantly *Bacteroides* enriched microbiota compared to high *Prevotella* species dominance in vegetarians (Liszt *et al*., 2009). High fat diets common in North America are generally thought to promote dysbiosis through direct antimicrobial activity of bile secreted during high fat feeding (Islam *et al*., 2011). Further evidence suggests that the type of fat rather than total fat intake appears to be the key contributing factor in fat-induced dysbiosis (Ghosh *et al*., 2013). More recently, the therapeutic use of probiotics (living organisms) and prebiotics (food for probiotics) in promotion of localized and systemic health has become immensely popular. While generally positive results have been reported from the experimental use of probiotics in gastrointestinal diseases, no conclusive clinical trials support these claims. Furthermore, evidence for the use of probiotics in systemic diseases are even more variable and scarce.

While it is widely believed that the environment is mostly responsible for the composition of gut microbiota, recent evidence suggests host genetics may also play a part. For example, studies of monozygotic and dizygotic twins have shown more similar microbiota within monozygotic twins, specifically with certain taxa such as family Christensenellaceae, Ruminococcaceae, and Lachnospiraceae having higher hereditary components (Goodrich et al., 2014). The specific genes and pathways that may be involved in these compositional changes however remain unknown.

1.6 Current methods and challenges in studying the microbiome

Developments in sequencing technologies have rapidly evolved in the past 15 years leading to massive advances in our understanding of microbial populations across various biospheres including the human intestine. Prior to introduction of high-throughput sequencing

(HTS) technologies, studying microbial populations were primarily based on culture-dependent techniques. While helpful in studying the specific functions, traits, and behaviors of individual species or similar clades of microbes, these techniques were vastly limited in their ability to discover novel microbial populations, especially those not easily cultured in the lab. The use of HTS circumvents these limitations by amplifying and examining slow-evolving hypervariable regions of DNA which allow for high resolution classification of microbes without dependency on culturing methods. By far the most common regions targeted for microbial studies are the 16S ribosomal RNA (rRNA) for bacteria and archaea, the internal transcribed spacer (ITS) between the 16S and 23S rRNA region for fungi, and the 18S rRNA for eukaryotes. With growing power and reducing cost of genomic data, the application of HTS to existing branches of health sciences are becoming standard practice. For example, analyzing fecal bacteriome of IBD patients has been shown to be effective in predicting patients' response rates to infliximab therapy, a common anti-TNF-α therapy, with 87.5% and 79.1% accuracy for CD and UC, respectively (Zhou et al., 2018). Although vastly powerful, there remains several important challenges with HTS systems which require expertise considerations at various steps such as experimental design, wet lab techniques, and bioinformatics analyses. At the experimental design level, great care must be taken to avoid introduction of bias across experimental groups. For example, in animal models, identical mouse strains raised in different animal facilities, different rooms within the same facility, or even different cages within the same room can all lead to detectable differences in the intestinal microbiota (Kim et al., 2017). The inoculation of mice at birth is sensitive to the maternal microbiome, this is referred to as the 'maternal effect' and is a common confounding factor in microbiome studies when members of an experimental group all derive from the same litter (Goodrich et al., 2014). To mitigate these factors, when possible, researchers should randomize the allocation of littermates across experimental groups. Factors such as standard rodent food composition, water (tap vs. autoclaved vs. acidified), and exposure to chronic noise can also influence the microbiome and should be

considered in a study's design (Cui et al., 2018; Franklin & Ericsson, 2017; Wolf et al., 2014), especially if comparison to previous reports are desired. Sample collection and storage methods also can heavily influence HTS data (Gorzelak et al., 2015). For example, the microbial populations of the intestinal tissues can drastically change even within the same tissue, i.e. proximal vs. distal colon (Sekirov, Russell, Antunes, & Finlay, 2010a), thus the exact location of sampling sites should be reported for meaningful comparisons. In human fecal samples, the bacterial population of the outer region differs than the internal region (Gorzelak et al., 2015) and so should also be considered. Further bias is introduced during DNA extraction and amplification arising from differences in molecular techniques, reagent contamination, and human error. Collectively, these and other factors during the amplification and sequencing processes lead to the phenomenon referred to as 'batch effect' which is discussed in detail in Chapter 5. Another important point of consideration is the choice of primers to target various specific regions of the 16S rRNA (Tremblay et al., 2015). For example, primers spanning the V3-V4 region of the 16S rRNA can detect important bacteria within the vaginal microbiota such as *Gardnerella vaginalis*, *Bifidobacterium bifidum*, and *Chlamydia trachomatis*, while the V1-V2 region is unable to detect these within the same samples (Graspeuntner, Loeper, Künzel, Baines, & Rupp, 2018). These and many other factors highlight the need for thorough and careful considerations of various components of experimental designs prior to sequencing, however, other challenges also exist following sequencing. Numerous bioinformatics pipelines have been developed for processing of HTS data which are often made available as open-source, task-specific, stand-alone programs, and/or offered within larger software suites such as QIIME (Caporaso et al., 2010) and mothur (Patrick D Schloss et al., 2009). These tools are often designed to primarily deal with specific challenges within particular niches of HTS data analysis and so are typically benchmarked within those types of experiments. While this may produce favorable results in certain experiments, their inappropriate use across other types of data may produce erroneous or unmeaningful results. For example, the accuracy of predictions

of the functional traits of microbial communities using an extended ancestral-state reconstruction algorithm (Langille et al., 2013) is heavily dependent on the completeness of the reference databases. This means that in well characterized samples such as mouse and human microbiomes, such algorithms have higher predictive power compared to poorly characterizes environments such as deep ocean waters. As with experimental design, the choice of bioinformatic steps also holds tremendous potential for introduction of bias that often leads to apparent inconsistencies across similar experiments (also discussed in further detail in Chapter 5). While general patterns in microbiome data, especially those with large effect size remain consistent across studies, identification and reproduction of subtle changes in individual taxa remains a challenge. This is largely due to lack of standardization in this field, however these obstacles are rapidly being resolved with advances in the field. Another inherent limitation of studying microbial communities using HTS technologies is that the observed taxon abundances are compositional in nature and only offer estimates of relative abundances. This means that the true abundance changes of one taxa in a community influences the observed estimates of relative abundances of all other taxon. This may lead investigators to erroneously report changes in other taxa even though the true abundance of these groups were not altered. For a comprehensive review of this topic the reader is referred to the work in (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017) and for statistical tools designed to deal with compositional dataset see Fernandes *et al.* (2014). Further, compositional data offer no information regarding the microbial loads of samples, meaning that the inter-sample differences in cell density of observed communities, which hold biologically relevant information, are not considered. Recently, the adjunct use of flow-cytometry with HTS has been proposed as one approach in estimating the absolute quantities of microbial taxa instead of relative abundances (Props et al., 2017; Vandeputte et al., 2017). Using this quantitative approach, Vandeputte *et al.* (2017) showed that reduced community richness, a well-documented observation in patients with Crohn's disease, is significantly underestimated using relative abundances.

1.7 Inflammatory bowel disease

Inflammatory bowel disease (IBD), encompassing UC and CD, are idiopathic, relapsing chronic inflammatory disorders of the gastrointestinal tract (GIT). Inflammation associated with CD can affect any part of the GIT while UC is local to the colon. Canada has amongst the highest incidence of IBD in the world with over an estimated 250,000 -or 1 in 150- diagnosed cases leading to annual health care costs of over $1.8 billion (Crohn's and Colitis Canada, 2008). In Europe, over 2 million people suffer from the disease (Ng et al., 2018). While exact prevalence of IBD across the globe, especially in developing countries, is not known, in developed countries it is estimated to affect over 0.3% of the population (Ng et al., 2018). Once considered a disease of Westernized countries, IBD incidences are rapidly rising globally, including in developing countries in Asia and Africa (M'Koma, 2013). India, once thought to be generally free of IBD has now amongst the highest incidence and prevalence rates in Asian countries (Kedia & Ahuja, 2017), though incidence in North America are still magnitudes of order higher. Approximately 25 % of patients with IBD are diagnosed prior to 20 years of age (Baldassano & Piccoli, 1999) with 18% before the age of 10 (Abramson et al., 2010). A combination of genetic, immunological, and environmental factors are implicated in the pathogenesis of IBD, however its etiology remains unknown. Genome-wide association studies of IBD patients have identified over 230 disease related loci (de Lange et al., 2017) though they account only for a fraction of the expected heritability of IBD suggesting numerous undiscovered genes. These loci represent a wide range of phenotypic traits including epithelial barrier integrity, phagocyte defects, T and B cell differentiation signaling, microbe recognition, and mucosal homeostasis to name a few (de Lange et al., 2017). In Crohn's disease the most common gene variation occurs in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) involved in cytoplasmic sensing of microbial products, followed by IL-23 receptors involved in mediating Th17 and Natural killer cell responses (Cho & Brant, 2011). The

UC-associated risk loci are predominantly related to maintenance of epithelial barrier and antigen pattern recognition (e.g. TLRs) (Sarlos et al., 2014). Amongst IBD patients, defects in function of IL-10 and its receptors are also common (Tremelling et al., 2007), prompting the study of IL-$10^{-/-}$ mice which develop spontaneous colitis under the presence of intestinal microbiota. The environment undoubtedly plays a crucial role in IBD pathogenesis, with factors such as smoking, air pollution, nutrition, exercise, stress, drugs, and psychological elements all being implicated (Loftus, 2004). These factors, albeit in varying modes and degrees, can induced improper immunological responses by the host leading to increased susceptibility to disease onset. Among these, smoking has the most replicated relationship across all epidemiological studies of IBD. Smoking increases the risk of CD while unexplainably protects against UC development and is associated with lower relapse rates (Birrenbach & Böcker, 2004). Clinical symptoms of IBD may vary depending on patient and disease severity but often include abdominal pain, diarrhea, weight loss, and gastrointestinal bleeding. There is no known cure for IBD and symptoms are either targeted pharmaceutically with amino-salicylates, corticosteroids, immunomodulators, antibiotics, and biological therapies or through dietary interventions such as specific nutritional management and elemental diets. In CD pediatric populations, exclusive enteral nutrition (EEN), which is defined as a complete provision of nutritional requirements through liquid formula for 6-8 weeks, is regarded as efficacious as corticosteroid therapy and is the first-choice treatment for inducing remission in this group (Assa & Shamir, 2017). The EEN intervention however, appears to be less effective in adult CD when compared to corticosteroids (Wall, Day, & Gearry, 2013).These therapies can be financially burdensome, temporarily efficacious, and have undesirable side-effects. As so, novel therapies in prevention, management, and treatment of IBD is increasing in demand. In this regard, PA has emerged as one possible solution.

1.8 Animal models of IBD

Animal models are crucial in dissecting the mechanisms underlying IBD and have proven invaluable in discovery of novel therapeutic interventions. There are at least 66 different animal models of IBD reported to date [reviewed in (Mizoguchi, 2012)] with majority of them developed in mice. Each model provides unique insights into various aspects of the disease; however, no single model fully captures the complexity of IBD in humans. Animal IBD models can be categorized into one of four groups: congenial mutants, adoptive cell-transfer, genetically engineered, or chemically-induced models. This section will briefly discuss the latter two groups of these models as these make up the most common types of murine IBD.

Chemical incitants are considered fast, effective, and economic strategies to induce intestinal injury and inflammation in mice. Amongst these, the most commonly used chemical is dextran sulfate sodium (DSS) and to a lesser extent, 2,4,6-trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, and azoxymethane are also used. Chemically-induced models of colitis primarily represent acute disease states and are important in studying innate responses to intestinal injury. As cessation of the toxic chemical such as DSS and TNBS leads to spontaneous reversal of clinical symptoms, these models can also be used in studying intestinal tissue recovery and repair. Administration of 1-5% DSS in drinking water for 5-9 days in mice is commonly used to induce Th1 and Th2 mediated colitis, however a more chronic disease state can also be produced by repeated intervals of DSS administration and cessation. DSS colitis is caused by direct disruption of the intestinal epithelial barrier and subsequent exposure of luminal antigens to the lamina propria. Interestingly however, unlike other models of murine IBD, the enteric bacteria contribute to the suppression of acute colitis in this model as germ-free mice have been shown to develop lethal colitis (Kitajima, Morimoto, Sagara, Shimizu, & Ikeda, 2001). DSS colitis is characterized by ulcers, loss of epithelial crypts, and infiltration of granulocytes, resembling human UC and can occur in the absence of T cells mediating adaptive immunity

(Kiesler, Fuss, & Strober, 2015). A hallmark of DSS inflammation is increased levels of TNF-α as well as IL-6 and IL-1a which are also implicated in human IBD (Gkouskou, Deligianni, Tsatsanis, & Eliopoulos, 2014). One important limitation of this model is that disease severity is strongly influenced by the total volume of the chemical toxin ingested. As mice drink DSS water *ad libitum*, variability in drinking habits can significantly alter disease outcomes. This is especially problematic in experimental models such as VWR where water intake can be significantly influenced by total energy expenditure.

In genetically engineered models, the gene(s) of interest are manipulated to either be knocked out (KO), impaired, or continuously overexpressed. In contrast to chemically-induced colitis, these models typically represent a chronic state of inflammation, with the underlying disease presence right from birth, though visible clinical symptoms may not manifest until several months of page. Amongst these, the IL-10$^{-/-}$ model is perhaps the most widely used KO model which is associated with impairment of the adaptive immune system. As discussed previously, IL-10 is a regulatory cytokine largely produced by Tregs and is a major player in downregulation of Th1 cytokines thus preventing accumulation of pro-inflammatory events leading to chronic intestinal inflammation and injury. Under conventional environments, IL-10$^{-/-}$ mice develop spontaneous colitis at around 3 months of age (Kühn et al., 1993). In this model the enteric microbiota and microbial antigens drive the disease through TLR-dependent signaling, as evident by the absence of colitis in IL-10$^{-/-}$ mice raised under germ-free conditions (Sellon et al., 1998a). The main limitation of this model in relation to studying PA, is that anti-inflammatory events through IL-10 regulation, such as previously described with PA (Packer, Hoffman-Goetz, & Ward, 2010), may be prohibited from displaying their full therapeutic potential. Another common genetic model of mouse colitis involves targeting the secretory MUC2 protein. As the predominant component of the colonic mucosa, MUC2 is integral in maintaining epithelial integrity and preventing direct interaction of microbial antigens with the IEC. The inflammation in this model is associated with intestinal lymphocytes, IL-1β, and TNF-α

expression (Van der Sluis et al., 2006) which can be detected as early as one months of age. Colitis in MUC2$^{-/-}$ animals manifests primarily in the distal colon and involves both the innate and adaptive immune system. Further, the mucosal defense factor resistin-like molecule-beta (RELM-β) has been shown to be involved in the development of this model by inducing dysbiosis (Morampudi et al., 2016). Importantly, the severity of colitis in this model is dependent on regulation of IL-10, as MUC2$^{-/-}$ + IL-10$^{-/-}$ double KO mice are shown to have exaggerated colitis symptoms compared to either individual KO models alone (van der Sluis et al., 2008). This model then provides an appropriate environment to study the potential effects of PA on IBD through both downregulation of proinflammatory cytokines as well as upregulation of key anti-inflammatory cytokine such as IL-10.

1.9 Physical activity and IBD

PA is defined as “any bodily movement produced by the contraction of skeletal muscles that increases energy expenditure above a basal level”, whereas exercise refers to “a subcategory of physical activity that is planned, structured, repetitive, and purposive in the sense that the improvement or maintenance of one or more components of physical fitness is the objective” (Booth, Roberts, & Laye, 2012a). While there are no exercise guidelines for IBD patients, PA is recommended to IBD patients to combat secondary complications such as loss of bone mineral density, psychological stress management, and weight loss, however its protective role in IBD pathogenesis is not known (Hashash & Binion, 2017). Recent epidemiological studies suggested a link between PA and reduced risk of IBD onset in the pre-illness period (Hlavaty *et al*., 2013; Khalili *et al*., 2013; Melinder *et al*., 2015), however these studies are unable to disentangle this relationship from the possibility that physical inactivity instead, is associated with higher IBD risk. In addition, most IBD patients are generally less active than healthy controls due to severity and sociocultural complications of disease symptoms (D. Chan, Robbins, Rogers, Clark, & Poullis, 2014; Narula & Fedorak, 2008; Wiroth

et al., 2005). A limited number of studies have shown the potential of PA to modestly reduce severity of chemically-induced colitis (J. M. Allen *et al*., 2017; Bilski *et al*., 2015; Marc D Cook *et al*., 2013a; Saxena *et al*., 2012). Interestingly, the mode, intensity, and volume of PA appears to be important in these models. For example, Cook *et al*. showed that four weeks of voluntary wheel running (VWR) in mice prior to DSS-induction had no protective effects on clinical diseases scores, but did reduce expression of pro-inflammatory cytokines TNF-α and IL-6. Interestingly, they observed that forced treadmill running (FTR) for the same duration, exacerbated clinical symptoms leading to increased morbidity and mortality. In contrast, Saxena et. al 2012, showed that FTR in mice showed no change in clinical symptoms of DSS-induced colitis but reduced inflammatory cytokines such as TNF-α, IL-6, and IL1-β. In rats, FTR did not protect against TNBS-induced colitis, however was associated with increased tissue healing following insult cessation (Bilski *et al*., 2015). While these studies clearly differ significantly in various aspects such as mode of PA, strains and sex of animals, and experimental model of colitis, one often underappreciated factor is the variance in total volume of PA. For example, the volume of physical movement in VWR models far exceeds those in FTR with mice moving 2-8 km/night on a voluntary basis. In contrast, a typical treadmill training program in mice [as used in (M D Cook *et al*., 2015)] consists of 40 min/day x 5 d/week of running at a speed of 8-12 meters/min which sums to ~ 400 meters/day. In addition, forced running has been shown to induce stress in mice (Moraska, Deak, Spencer, Roth, & Fleshner, 2000) leading to physiological adaptations much different than those associated with VWR. In a follow-up study to Cook *et al*. it was shown that VWR and FTR had differentially altered gut microbiota in mice (Jacob M Allen *et al*., 2015) suggesting a possible link between the microbiota composition and IBD severity in these experiments.

1.10 Microbiota in IBD

Evidence in recent years implicate a pivotal role of the intestinal microbiota in pathology of IBD though causality has not been established. Patients with IBD show significant decreased microbial diversity, especially in the dominant Firmicutes and Bacteroidetes phyla, and increased abundances of pathobionts (Manichanh, 2006). A twin study of IBD cohorts showed that UC patients house higher levels of pathobionts from the Actinobacteria and Proteobacteria phyla compared to their healthy siblings (Lepage *et al*., 2011). IBD patients show higher levels of pro-inflammatory cytokines in response to commensal bacteria alluding to a hyper sensitive and less tolerogenic gut phenotype (Nobuhiko Kamada *et al*., 2008). One hallmark feature of IBD pathology is the disruption of the intestinal epithelium layer. In ileal CD the production of key AMPs is decreased allowing bacteria to come in direct contact with the epithelium and prompt inflammatory responses. Left unchecked, this leads to further break down of the epithelial layer, increased bacterial translocation, and further exaggeration of inflammatory responses by the host (Wehkamp, 2004; Wehkamp *et al*., 2005). Interestingly, in colonic CD and UC the reverse is true where the atypical presence of Paneth cells in the colon leads to excessive production of AMPs leading to reduced abundances of commensal bacteria (Shanahan, Carroll, & Gulati, 2014). In this scenario, foreign opportunistic bacteria can now colonize the newly available niches and employ immunogenic responses which ultimately lead to barrier dysfunction. In light of these and other findings linking the microbiota to intestinal diseases, novel therapies that aim to manipulate the intestinal microbes have been advocated as novel tools in the fight against IBD.

1.11 Physical activity and microbiota

Recently, the potential role of PA as a modulator of human microbiota has gained popularity. This concept was first explored by Matsumoto *et al*. in 2008 whereby they reported changes in cecal microbiota of rats following VWR and accompanying increase in butyrate

abundance (Matsumoto *et al*., 2008). Using gel electrophoresis profiling of the bacterial communities, they showed that these changes were attributed to changes in the gut bacteria. Not until the popularization of high-throughput sequencing (HTS) technologies however, did this topic advance in a significant manner. In 2014, Evans *et al*. using HTS showed that 5 weeks of VWR in mice indeed correlated with significant shifts in gut bacterial composition, often shifting towards a high Firmicutes to Bacteroidetes ratio (Evans *et al*., 2014). Not long after, Hsu *et al*. further highlighted the crucial role of the microbiota in physical work when they showed germ-free mice exhibiting significantly worsened exercise performance in a swimming model of PA (Hsu *et al*., 2015). Perhaps even more interesting was the finding that when littermates of these germ-free mice were inoculated with single species of bacteria, they showed significant improvements in their endurance capacity. These differences were attributed, in part, to modulation of the mice's anti-oxidant system activity. Since then, several other studies in rodents have emerged, though not all report similar findings. For example, Lamourerux *et al*. found only limited changes in VWR mice (Lamoureux, Grandy, & Langille, 2017), while Zhang *et al*. showed no significant changes in mice with access to free-wheels throughout their whole lives (C. Zhang *et al*., 2013). Others showed that PA-induced changes of the microbiome are dependent on the background diet (Evans *et al*., 2014) and age of the animals (Mika *et al*., 2015). Our current knowledge of PA and microbiota in humans is currently limited to a handful of studies, with only one providing evidence with regards to causality (Jacob M. Allen *et al*., 2018). Clarke *et al*. first showed that the fecal bacteriome of elite rugby players was more similar to each other than population-matched non-athletes (Clarke *et al*., 2014). They showed increased diversity richness and abundances of genus *Akkermansia* in elite athletes. Work from our group (shown in Chapter 2) further showed that changes in bacterial community richness are positively correlated with cardiorespiratory fitness and that these changes are true regardless of sex, age, body mass index (BMI), or dietary patterns (Estaki *et al*., 2016). Most recently, Allen *et al*. showed that a six weeks endurance exercise training program elicited

beneficial changes in microbiota of lean, but not obese females and that these changes were reversed with exercise cessation (Jacob M. Allen *et al*., 2018).

1.12 Thesis outline and objectives

In this section, I will introduce the research questions initially proposed in this thesis with regards to the knowledge gap of the field at the time of their conception. The overall questions posed by this work was whether PA can alter the intestinal microbiota and whether such changes are beneficial to the host, in the context of intestinal inflammation.

At the time of this project's inception, no prior studies in humans, and only one in animals had been reported regarding the role of PA and microbiota. Therefore, in Chapter 2 we designed a simple cross-sectional study to explore structural microbial patterns amongst humans with varying cardiorespiratory fitness levels using HTS. A secondary objective of this study was to confirm earlier observations by Matsumoto *et al*. that showed higher levels of the SCFA butyrate following wheel running in rats. We reasoned that if the microbiome of fit individuals did indeed align with known traits of a healthy community (as discussed earlier), this would warrant further investigation in the use of PA as a novel strategy to mitigate primary complications of dysbiosis-associated diseases such as IBD.

Having established an association between aerobic fitness and gut health from our first cross-sectional study, we set out to test the hypothesis that PA can protect against IBD by altering the microbiome. While other reports had surfaced suggesting a protective role of VWR in chemically-induced models of murine colitis (Bilski, Brzozowski, Mazur-Bialy, Sliwowski, & Brzozowski, 2014; Marc D Cook *et al*., 2013b; Saxena *et al*., 2012), the role of the microbiome remained to be elucidated. In Chapter 3 we aimed to investigate two primary objectives: 1) establish causality in the microbial patterns observed from our human study, and 2) determine whether such changes are protective against a spontaneous model of murine colitis.

In Chapter 4 we make a case for explaining discrepancies reported across the literature on the effects of PA and intestinal bacteria. We introduce a model of low-volume wheel running in mice by which we attempted to reveal whether the volume of physical work affects the observed changes in the colonic bacteriome.

Finally, in Chapter 5 I synthesize the findings from all previous chapters, present overall conclusions drawn from these studies, and offer a prospectus for future studies.

Chapter 2: Aerobic fitness and microbiota in humans[2]

2.1 Background

The interactions between humans, their environment, and intestinal microbiota form a tripartite relationship that is fundamental to the physiological homeostasis and overall health of the host (Sekirov, Russell, Antunes, & Finlay, 2010b). The human intestinal microbiota aid their host in several important biological functions such as: digestion, absorption, stimulating immune responses, and protection against enteropathogens, to name a few. The bacteria break down partially digested complex carbohydrates via fermentation and produce short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate as by-products. These SCFAs act as the primary food source of the colonocytes which consume up to 10% of the dietary energy expenditure in humans. In particular, butyrate has been shown to play a critical role in overall gut homeostasis and health (Leonel & Alvarez-Leite, 2012). Lasting disturbances in the microbial community composition, termed dysbiosis, can have deleterious health effects in the host [reviewed in (Y. K. Chan *et al*., 2013)]. Gut microbiome diversity has emerged as a candidate indicator of overall host health. Low community richness has been correlated with metabolic markers such as adiposity, insulin resistance, and overall inflammatory phenotypes (Le Chatelier *et al*., 2013), as well as gastrointestinal (GI) conditions such as inflammatory bowel disease (Ott *et al*., 2004), *Clostridium-difficile* infection (Chang *et al*., 2008), colorectal cancer (Ahn *et al*., 2013), and irritable bowel syndrome (Giamarellos-Bourboulis *et al*., 2015). As a result, considerable research in recent years has focused on understanding and developing strategies to promote overall GI health via community manipulation in attempt to resolve dysbiosis-associated diseases.

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[2] A version of Chapter 2 has been published. Estaki M, Pither J, Baumeister P, Little JP, Gill SK, Ghosh S, Ahmadi-Vand Z, Marsden KR, Gibson DL. Cardiorespiratory fitness as a predictor of intestinal microbial diversity and distinct metagenomic functions. Microbiome. 8;4(1):42

Various extrinsic variables such as stress, probiotic and antibiotics use, alcohol consumption, and diet have been identified as factors that can instigate changes in the microbiome (Sekirov et al., 2010b; Sommer & Bäckhed, 2013). The link between physical activity and gut microbiota however is currently not well understood. Matsumoto *et al*. first identified increases in butyrate levels in cecum of physically active rats which they suggested was a result of compositional changes in butyrate-producing bacteria (Matsumoto *et al*., 2008). Evans *et al*. explored the effects of voluntary wheel running in mice fed low or high fat diets and found that microbial communities clustered based on both diet and physical activity (Evans *et al*., 2014). Allen *et al*. further showed that the mode of physical activity, for example forced treadmill running versus volunteer wheel running differently altered the microbiota (Jacob M Allen *et al*., 2015). Recently, Clarke *et al*. also found clustering of bacterial communities between professional rugby players and high/low body mass index (BMI) controls (Clarke *et al*., 2014). They further identified increases in bacterial community richness in these elite athletes compared to both control groups. In their study however, extreme dietary differences, especially high protein intakes amongst the athletes, confounded interpretations regarding the specific role of physical activity and microbial changes.

To better isolate how physical fitness may moderate microbial diversity, we analyzed the fecal microbiota of individuals with varied fitness levels with comparable diets. We used peak oxygen uptake (VO_2peak), the gold standard of cardiorespiratory fitness (CRF), as an indicator of physical fitness. We asked the questions a) does taxonomical richness vary with CRF alone, b) do variations in CRF drive changes of specific taxa in a predictable manner, and c) do such changes significantly influence the microbiota's contribution to functional pathways? We show that VO_2peak, independent of diet, correlates with increased microbial diversity and production of fecal butyrate amongst physically fit participants.

2.2 Methods

Study design - Healthy young adults between 18 and 35 years old were recruited. Exclusion criteria included: antibiotic treatment within the previous 6 months, current drug utilization, or active acute or chronic diseases. All participants were verbally interviewed on their dietary habits and CRF was determined using a peak oxygen uptake (VO_2peak) cycle test. Participants were then provided a stool collection kit with instructions and were asked to provide a sample within a week following their lab visit.

Ethics, consent, and permissions - This study was conducted according to the Declaration of Helsinki guidelines and all procedures were approved by University of British Columbia Clinical Research Ethics Board.

Nutritional data collection - Nutritional data, including supplements, was collected by means of a 24 hr dietary recall interview and assessed by a research nutritionist using FoodWorks nutrient analysis software (version 16.0). Food items described by participants that were not available in the software were manually added as needed. On average over 100 food categories per participant was produced by the FoodWorks software. A manual screening was applied to select categories of interest based on *a priori* interest and existing literature showing a significant interaction between those categories and intestinal microbiota. The selected 24 food categories data are available in the uploaded metadata mapping file.

Cardiorespiratory fitness testing - Participants initially completed a physical activity readiness questionnaire (PAR-Q) to rule out any contraindications to vigorous exercise. A continuous incremental ramp maximal exercise test on an electronically braked cycle ergometer (Lode Excalibur, the Netherlands) was used to determine peak oxygen uptake (VO_2peak) and peak power output (Wpeak). Expired gas was collected continuously by a metabolic cart (Parvomedics TrueOne 2400, Salt Lake City, Utah, USA) calibrated with gases of known concentration. The test started at 50 Watts and increased by 30 Watts/min. The test was

terminated upon volitional exhaustion or when revolutions per minute fell below 50. VO_2peak was defined as the highest 30-sec average for VO_2 (in ml/kg/min). Criteria for achieving VO_2peak were: i) respiratory exchange ratio >1.15; ii) plateau in VO_2; iii) reaching age-predicted HRpeak (220-age); and/or iv) volitional exhaustion. Following VO_2peak assessment, participants were categorized to either low (LOW), average (AVG), or high (HI) fitness based on their sex and age according to a modified Heyward normal VO_2max reference chart (Table 1).

Stool collection and storage - Participants were provided with a home stool collection kit including a sterile 120 ml polypropylene container (Starplex, Etobicoke, Ontario), sterile tongue depressor and gloves, and an ice box. Participants were instructed to avoid alcohol for 3 days prior to stool collection. Stool samples were immediately stored in the participant's freezer overnight and transported on ice to the lab and stored in -80 ºC until further analysis. Frozen portions from the inner area of the samples were scrapped using sterile razor blades for DNA extraction and short-chain fatty acids analysis.

LOW AVG HI

VO2max (mL•kg^{-1}•min^{-1}) Classifications for Women

Age (years)	Poor	Fair	Good	Excellent	Superior
20 - 29	≤ 35	36 - 39	40 - 43	44 - 49	50+
30 - 39	≤ 33	34 - 36	37 - 40	41 - 45	46+
40 - 49	≤ 31	32 - 34	35 - 38	39 - 44	45+
50 - 59	≤ 24	25 - 28	29 - 30	31 - 34	35+
60 - 69	≤ 25	26 - 28	29 - 31	32 - 35	36+
70 - 79	≤ 23	24 - 26	27 - 29	30 - 35	36+

VO2max (mL•kg^{-1}•min^{-1}) Classifications for Men

Age (years)	Poor	Fair	Good	Excellent	Superior
20 - 29	≤ 41	42 - 45	46 - 50	51 - 55	56+
30 - 39	≤ 40	41 - 43	44 - 47	48 - 53	54+
40 - 49	≤ 37	38 - 41	42 - 45	46 - 52	53+
50 - 59	≤ 34	35 - 37	38 - 42	43 - 49	50+
60 - 69	≤ 30	31 - 34	35 - 38	39 - 45	46+
70 - 79	≤ 27	28 - 30	31 - 35	36 - 41	42+

Source:
V. H. Heyward, Advanced Fitness Assessment and Exercise Prescription, Fifth Edition, 2006, Champaign, IL: Human Kinetics.
Original Source:
The Cooper Institute for Aerobics Research, The Physical Fitness Specialist Manual. Dallas, TX. 2005.

Table 1. Heyward's 2006 normal VO2max reference chart

Subjects characterized as “Superior” or “Excellent” according to the Heyward classification were grouped under the “HI” group, “Fair” and “Good” subjects were placed into the “AVG” group, and “Poor” was renamed to “LO”.

Short-chain fatty acids analysis - SCFA (acetic, propionic, heptanoic, valeric, caproic, and butyric acid) were analyzed from the feces by gas chromatography (GC) as described previously (Brown *et al*., 2016). In brief, ~ 50 mg of stool was homogenized with isopropyl alcohol, containing 2-ethylbutyric acid at 0.01% v/v as internal standard, at 30 Hz for 13 minutes using metal beads. Homogenates were centrifuged twice, and the cleared supernatant was injected to Trace 1300 Gas Chromatograph, equipped with Flame-ionization detector, with

AI1310 auto sampler (Thermo Fisher Scientific) in splitless mode. Data was processed using Chromeleon 7 software. An aliquot of 50 mg of stool was freeze dried to measure the dry weight, and measurements are expressed as mass % (g of SCFA per g of dry weight stool).

High-throughput sequencing - DNA was extracted from feces using QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions following 3 x 30s of homogenization using metal beads on a Retsch MixerMill MM 400 homogenizer. Metagenomic sequencing libraries were prepared according to the Illumina MiSeq system instructions. In brief, the V3 and V4 region of the 16S bacterial rRNA gene was amplified using recommended primers (Klindworth *et al*., 2013) (IDT, Vancouver, Canada): Forward 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and Reverse 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, which create amplicons of ~460 bp. Amplicons were cleaned using AMPure XP beads step then adapters and dual-index barcodes (Nextera XT) were attached to the amplicons to facilitate multiplex sequencing. After another clean-up step, libraries were validated on an agarose gel, quantified, normalized, and sent to The Applied Genomic Core (TAGC) facility at the University of Alberta (Edmonton, Canada) for sequencing using the Illumina MiSeq platform. The resulting ~ 16,000,000 paired-end reads were merged using PEAR software (J. Zhang, Kobert, Flouri, & Stamatakis, 2014) and screened to exclude sequences containing one or more base calls with a Phred score < 20. Rarefaction curves demonstrated that sufficient sampling depth had been reached amongst all samples (Figure 2). All sequence reads and associated metadata file are available from the Sequence Read Archive (accession number#: SRP068480).

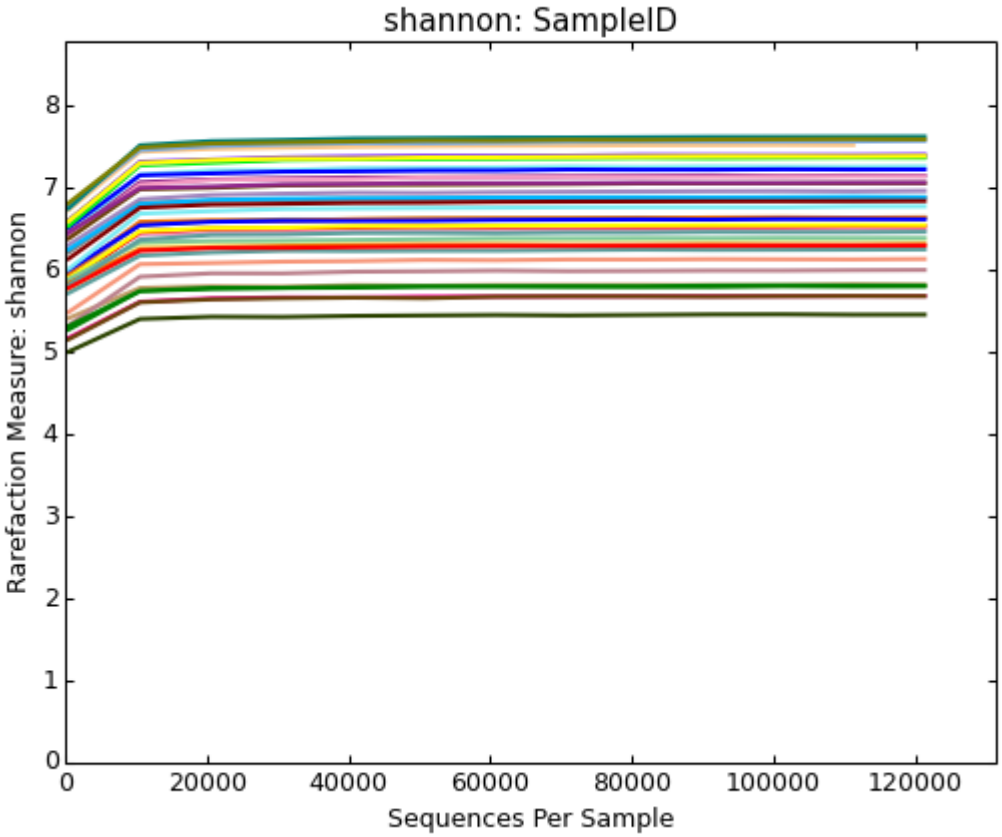

Figure 2. Sampling depth rarefaction curves

Rarefaction curves of all subjects at 97% similarity levels shown as a function of Shannon diversity index and number of sequence tags sampled.

Bioinformatics - Bioinformatics analyses on the demultiplexed paired reads were conducted using QIIME 1.8.0 software suites (Caporaso *et al*., 2010). Reads were clustered at 97% identity using the *uclust* method into operational taxa units (OTUs) then aligned to the most recent available version (2013/08) of Greengenes bacterial database (McDonald *et al*., 2012). Singleton and doubletons were removed and the produced OTU table was normalized using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille *et al*., 2013) to adjust for different 16S rRNA gene copy numbers. Uneven variance as a result of differential sample sequencing depth was stabilized using the cumulative sum scaling (CSS) method of metagenomeSeq package in R. Alpha diversity indexes, rarefaction curves, OTU tables, and distance metrics were also generated using QIIME.

Statistical analysis - All statistical analyses were performed using R version 3.2.0 unless stated otherwise. All data and annotated R scripts, which include detailed description of all statistical analyses used, methods for model variable selection, and checking of model assumptions are available publicly at https://osf.io/js86c/.

The groups' age and VO_2peak data were tested for normality using Shapiro-Wilk test and a one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test used to compare mean differences amongst groups. Kruskal-Wallis non-parametric test was used for comparing BMI as this dataset failed normality tests even after several transformation attempts. For comparison of dietary intake amongst groups, a permutational multivariate ANOVA (PERMANOVA) with 999 random permutations was used. Due to the inherent high variability of dietary data we further searched for dietary patterns amongst groups by looking at a principal component analysis (PCA) plot of participants' dietary scores using the *ggbiplot* package.

To facilitate comparisons with previous work, we first compared average alpha diversity among the three fitness categories using a one-way ANOVA, followed by a Tukey's multiple-comparison. To simultaneously evaluate the role of CRF alongside other potential predictors of alpha diversity (sex, age, BMI, and dietary components), we performed a multiple regression

analysis. Given our comparatively low sample size (*n* = 39), and the general rule that multiple regressions should include at least 10 observations per predictor variable, we first screened potential predictors that were continuous variables using a Spearman correlation matrix. Those that showed a significant correlation with alpha diversity were retained for entry in the multiple regression model. Multicollinearity was checked using the variable inflation factor (VIF) index with a maximum cut off score of 10.

Microbial communities in fecal samples were ordinated using the Bray-Curtis, weighted and unweighted UniFrac distance metrics. Principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity metric was made using *cmdscale* function of '*stats'* package in R, while PCoA based on the weighted and unweighted UniFrac distances were made using EMPeror tool (Vázquez-Baeza, Pirrung, Gonzalez, & Knight, 2013). Redundancy analysis (RDA), implemented using the '*vegan'* package version 2.2-1 in R, was used to assess variation in microbial composition in relation to the constraining variables: sex, age, BMI, and dietary components. Abundance data at each taxonomical resolution (phyla, class, order, family, and genus) were first Hellinger-transformed. Variable selection in RDA was implemented using the *ordistep* function using both forward and backward stepwise inclusion. Predictors selected by this method at each classification level are presented in Table 6. To identify genera that significantly contributed to total variance we evaluated Spearman correlations between the transformed genera abundance data and the first 2 RDA axes. OTUs with a significant correlation coefficient (evaluated at Bonferroni adjusted alpha level) were drawn on the RDA plots with type II scaling. Classification of relative abundance data according to the previously described enterotypes (Arumugam *et al*., 2011) was carried out using the Calinski-Harabasz (CH) index as described online (http://enterotype.embl.de/enterotypes.html).

The normalized genus abundance OTU table was used to predict the microbiome's metagenomic functions using PICRUSt's extended ancestral-state reconstruction approach. A new abundance matrix of predicted functional categories based on the Kyoto Encyclopedia of

Genes and Genomes (KEGG) database was created. We constructed a biplot from the output of a principal components analysis (PCA) of functional categories data and visually assessed clustering patterns based on CRF groupings. Next, to isolate the influence of specific predictor variables, an RDA was also performed using these functional categories as response variables and the same variables and selection methods previously described.

Similarly, to determine the role of our exploratory variables in explaining variance in fecal SCFAs, an RDA was performed using SCFAs abundance data as the response variables.

2.3 Results

Diet was not a confounding factor across fitness groups - Twenty-two males and nineteen females participated in the study. Two female participants were removed from sequencing analysis due to technical errors. Table 2 represents a summary of the 39 participants' characteristics and dietary intake. Age distribution was similar across all groups. The LOW group had a marginally higher BMI (25.5, SD 3.9) compared to the AVG (23.5, SD .5) and HI (22.8, SD 1.5) groups, however the difference was not statistically significant. BMI of AVG and HI groups fall within the 'normal weight' range (18.5 - 24.9) as defined by Health Canada, while the LOW group is marginally above the 'overweight' threshold of 25. The results of the PERMANOVA (Table 3) showed no main differences (permutation P= 0.56) across any nutritional classes based on fitness groups. PCA plot (Figure 3) of dietary patterns amongst the different fitness groups also showed no distinct clusters, further supporting comparable dietary patterns amongst fitness groups.

	LOW (n=14)		AVG (n=12)		HI (n=13)	
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Age (years)	25.5 (3.3)	25.5 (23-27.8)	24.3 (3.7)	24.5 (21.8-26)	26.2 (5.5)	28 (21-31)
BMI (kg/m²)	25.5 (3.9)	24.9 (23.2-27.8)	23.5 (.5)	23.4 (22.1-23.8)	22.8 (1.5)	22.4 (21.9-24)
VO_2peak	33 (4.8)*	33.3 (30.7-26.3)	41.9 (4.3)*	41.2 (38.5-44.2)	54.8 (5.6)*	52.4 (51.3-60.9)
Dietary components						
Energy (kcal)	2477.5 (1168.4)	2119.5 (1537.2-3565)	2230 (605.4)	2092 (1793-2561)	2458.3 (668.3)	2647 (2060-2714)
Protein (g)	128.7 (88.5)	104.8 (55.4-182.7)	110.2 (53.7)	90 (80-134.6)	111.2 (49.7)	97.5 (84-127.2)
Carbohydrate(g)	278.9 (97.5)	294.7 (201.7-347.6)	245.2 (90.4)	245.2 (182.1-275.2)	276.9 (80.2)	268.5 (248.3-310.8)
Fat (g)	95.4 (61.9)	74.1 (46.5-121.3)	95.8 (29.1)	85.6 (78.4-113.6)	105.3 (41.1)	111.9 (84.2-131.30
Saturated Fat (g)	37.7 (30)	25.2 (16.9-62.2)	32 (29.1)	31.2 (26.5-34.5)	31.6 (14.7)	32.6 (21.2-36)
MUFA (g)	30.7 (19.9)	27.6 (14.1-36.9)	35 (14.4)	34.9 (27.6-40.8)	38.6 (16.5)	36.4 (28.5-46.9)
PUFA (g)	15 (6.8)	15.1 (9.3-20)	20.2 (11.7)	17.9 (11.1-26.7)	23.3 (10.7)	22.6 (15.4-28.2)
Trans fat (mg)	730 (960)	358(28.5-89.3)	580 (440)	552 (243.7-896.8)	500 (530)	407 (87-501)
Omega 3 (mg)	2260 (1470)	1958 (-1166-3068)	2990 (2320)	1958 (1307-4779)	3110 (3600)	1535 (1200-1942)
Omega 6 (mg)	1790 (3320)	418 (28.8-1624)	1010 (1040)	438 (283.3-1672)	3820 (4250)	2477 (198-4951)
Sugar (g)	96.7 (59.1)	68.9 (54.8-134.2)	83.2 (43.9)	80.2 (67-95.5)	103.6 (38.4)	97.4 (81.7-121.7)
Fiber (g)	28.4 (11.7)	22.5 (20.2-34.7)	31.3 (30.2)	23.2 (17.3-29.4)	36.5 (20.2)	28.8 (24.2-40.2)
Cholesterol (mg)	358 (348.7)	263.6 (59.4-453.4)	346.5 (194.6)	288.6 (196.1-466.1)	443.1 (269.3)	442.6 (186.3-638.6)
Butyrate (mg)	470 (740)	212.5 (39.8-578)	690 (690)	573.5 (283.5-929)	480 (470)	366 (194-518)

Table 2. Summary of group characteristics and dietary intake

* denotes a significant (Bonferroni adjusted P<0.01) pair-wise difference amongst the other two groups. Macronutrients amongst groups were compared by PERMANOVA as described in the text.

	df	SS	MS	F-Model	Pr
Fitness	2	0.053	0.027	0.7	0.56
Residuals	36	1.38	0.038		
Total	38	1.43			

Table 3. Summary of PERMANOVA for dietary intake across fitness groups

df, degrees of freedom; SS, sum of squares; MS, mean of squares; Pr, Permutation based P value

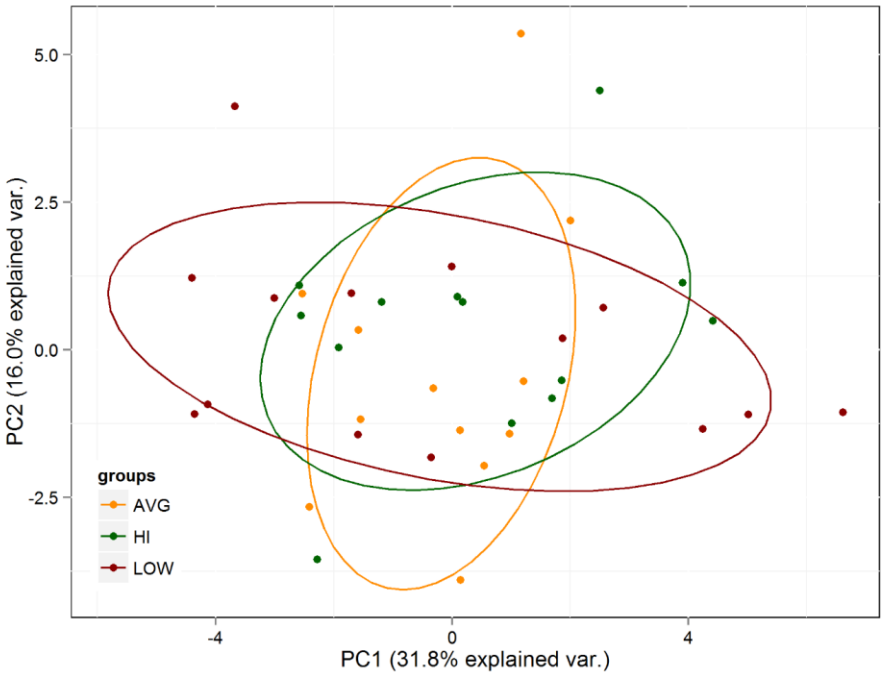

Figure 3. Dietary patterns amongst fitness groups

Scores of the two first components of the principal component analysis of dietary data for all 39 subjects are presented. Each circle represents one participant; colored based on their CRF fitness levels. A lack of distinct clustering amongst groups suggests comparable dietary patterns amongst groups.

CRF is correlated with increased microbial diversity - Species diversity of each participant (alpha diversity) was determined using several indexes: species richness (SR), chao1, Shannon, Simpson, and Faith's phylogenetic diversity. As all the alpha diversity indexes were highly correlated (Figure 5) and produced qualitatively identical results, SR was chosen as a proxy in the regression model. After screening of potential predictors via Spearman correlation analysis, 3 variables were included in the multiple regression model: VO_2peak, sex, and relative fat intake. Of these, only VO_2peak was a significant predictor of alpha diversity (Table 4), with SR significantly (P = 0.011) associated with increasing VO_2peak (R_{adj}^2=0.204, coefficient estimate = 5.36; t = 2.17) (Figure 4).

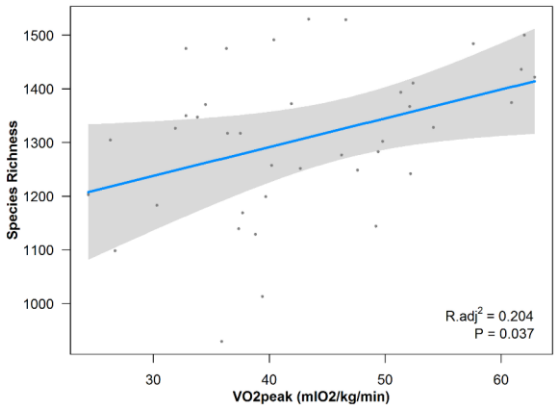

Figure 4. Correlation between VO2peak and Species Richness

Result of a multiple regression model showing a significant association between VO2peak and SR when holding all other variables constant. Shaded area represents 95% confidence intervals.

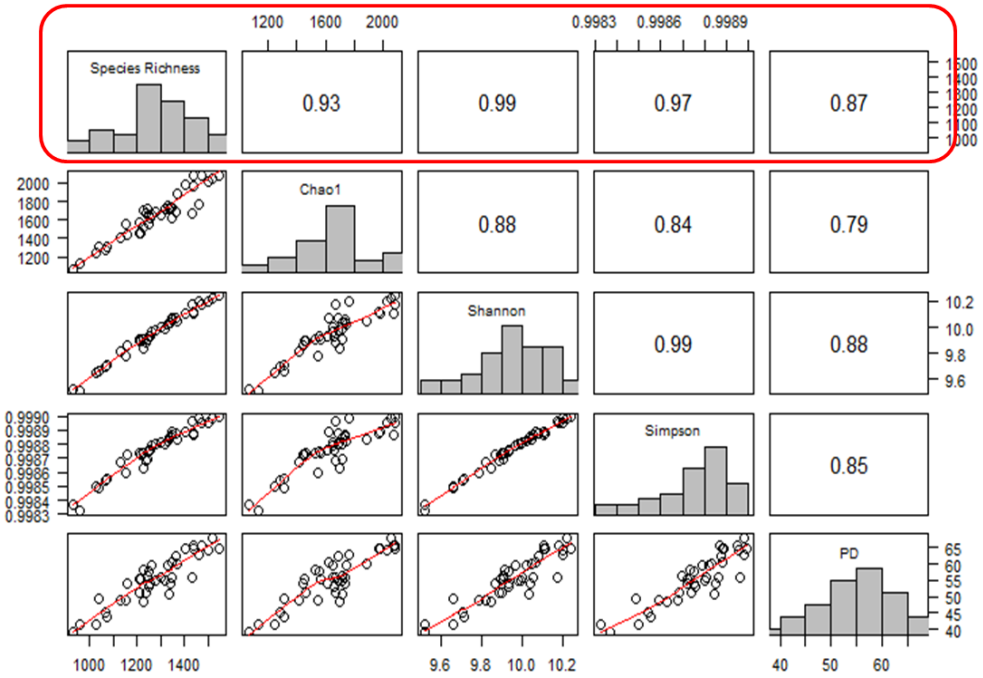

Figure 5. Correlation between alpha diversity matrices

A correlation matrix using Spearman's r showing strong correlation between all alpha diversity matrices used. Species Richness (S) was thus used as a proxy for the response variable in the multiple regression model.

Variables	Unstandardized Coefficients		Standardized Coefficients	t	P
	B	Std. Error	Beta		
VO2peak	5.36	2.47	0.37	2.17	0.037*
Relative fat intake	432.46	250.10	0.26	1.72	0.094
Sex ♂	24.70	51.23	0.08	7.54	0.63
Model adjusted $R^2 = 0.20$		P-value=0.01		*denotes statistical significance	

Table 4. Multiple regression results of SR

Result of multiple regression test showing VO2peak as the only significant variable in predicting species richness (SR). The B coefficient represents the amount of change in SR along its 95% confidence intervals per unit change of VO2peak (ml/kg/min). The standardized coefficients show VO2peak as the strongest variable to influence SR variability.

CRF levels do not promote distinct clustering of beta diversity data - Overall, 14 phyla and 207 genera were represented across all participants (Table 5). The HI group showed the highest diversity composed of 186 genera compared to 161 and 143 in the AVG and LOW groups, respectively. PCoA plots constructed using Bray-Curtis (Figure 6), weighted and unweighted UniFrac dissimilarity indices (Figure 7) did not show group clustering based on fitness levels. Clustering of our dataset based on the CH index favored a 2 cluster partitioning (Figure 8) rather than the proposed 3 enterotypes (Arumugam *et al*., 2011).

	LOW (n=14)	AVG (n=12)	HI (n=13)	Total (n=39)
Phylum	13	12	11	14
Class	28	25	24	31
Order	43	42	43	52
Family	74	78	87	100
Genus	143	161	186	207

Table 5. Taxa representation among subjects

The HI group show the highest diversity at the genus and family level, followed by AVG then LO groups.

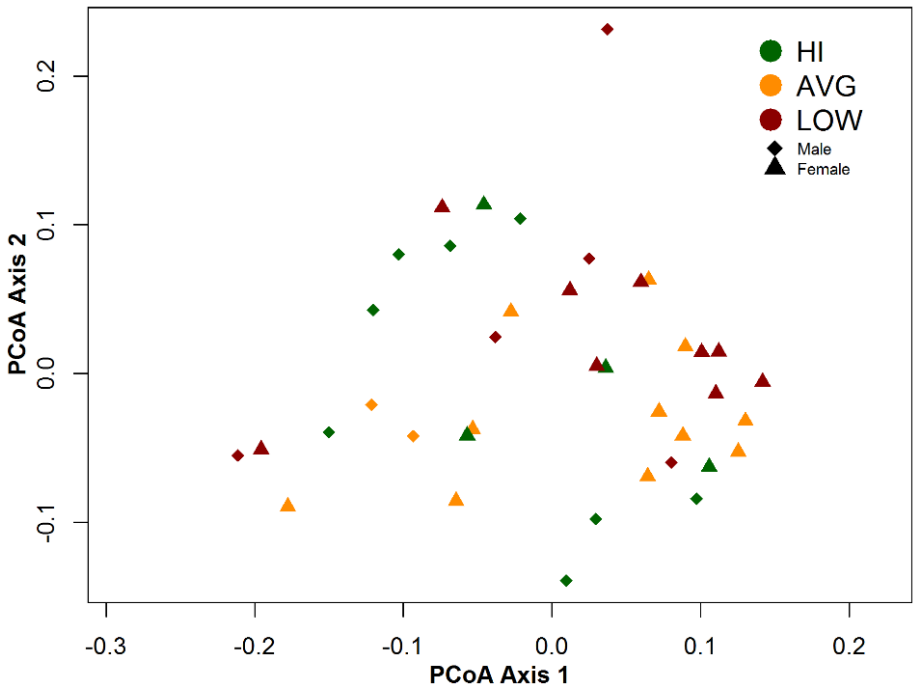

Figure 6. Beta diversity amongst fitness groups

PCoA plot of genus abundance data based on Bray-Curtis dissimilarity measure show no clear clustering when grouped according to CRF levels.

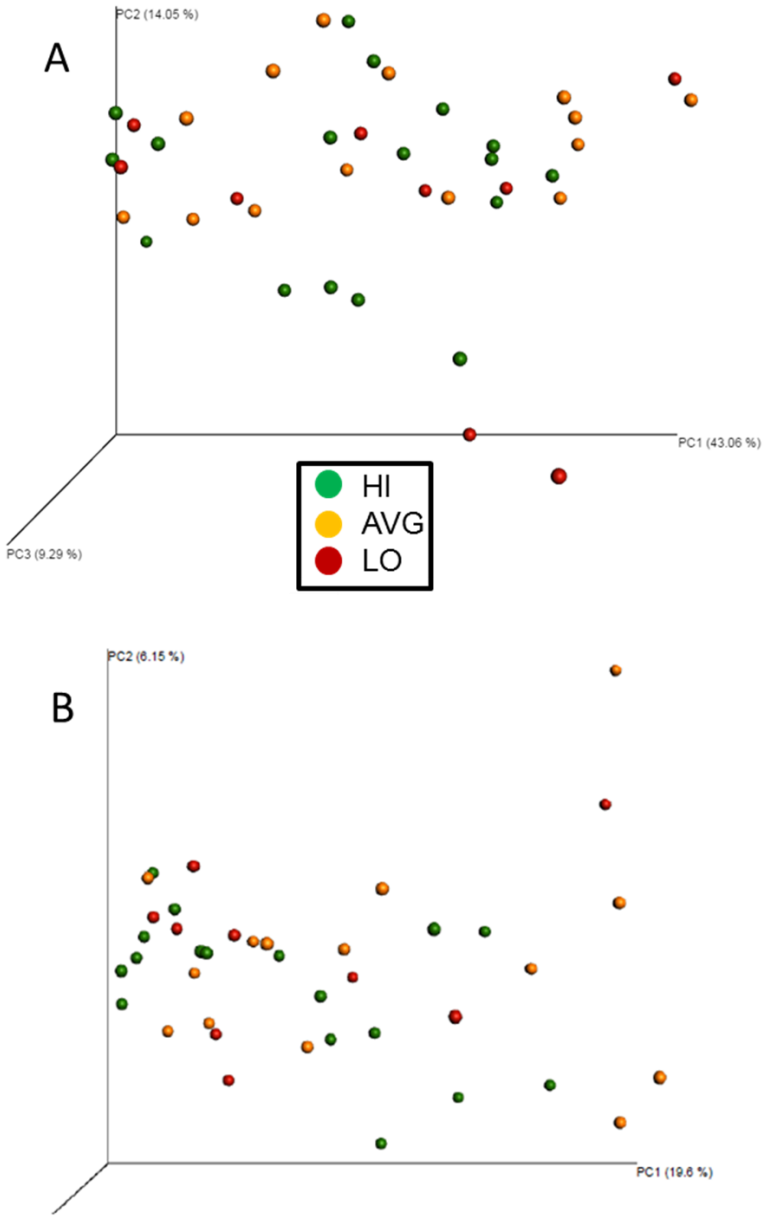

Figure 7. Beta diversity amongst fitness groups

Three dimensional PCoA plots of genus abundance data transformed with weighted (A) and unweighted (B) UniFrac dissimilarity matrices show no clear clustering based on CRF levels.

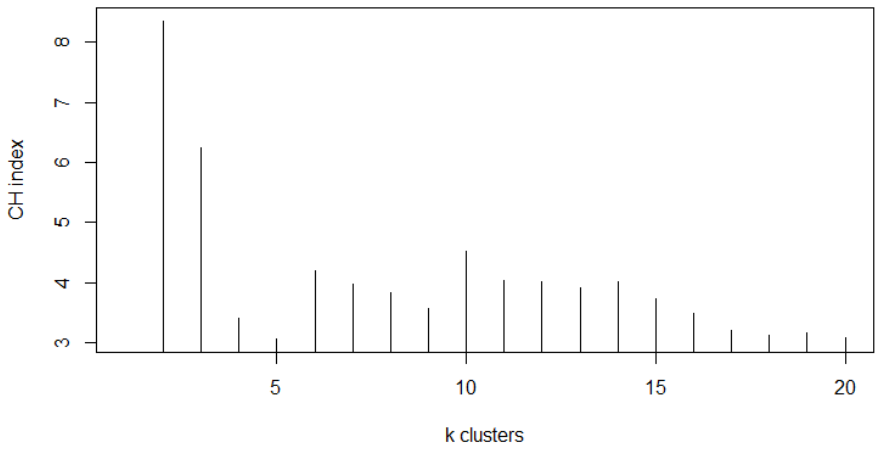

Figure 8. Optimal clustering selection of bacterial data

The number of optimal clustering of all data was determined using the Calinski-Harabasz (CH) index. Optimal number of clusters did not identify the classical 3 enterotypes but rather favored a 2-cluster partitioning.

Protein intake and age but not CRF constrain beta diversity - The global RDA model which included sex, age, and protein as explanatory variables was significant (P = 0.005) as assessed by Monte Carlo Permutation Procedure (MCPP) (1000 permutations) and yielded an R_{adj}^2 of 0.053. Though small, this value is typical of RDA analyses of highly diverse assemblages (Wu *et al*., 2011). Overall, 12.7% of the variance was attributed to these explanatory variables. The first and second axes (Figure 9) accounted for 7.9% and 2.3% of the variation, respectively. The RDA indicated that VO_2peak did not significantly constrain beta diversity at any taxonomic resolution, whereas total protein intake was significant at each resolution tested (Table 6). In addition, age, sex, and the omega6-omega3 ratio (n6:n3) were also marginally significant constraining variables, though only at specific taxa resolutions. In Figure 9B, we highlight 19 genera that were significantly correlated with one or both of the first two RDA axes. Amongst those, *Bacteroides* was strongly associated with protein intake along RDA2 while *Odoribacter*, *Rikenellaceae*, *Oscillospira*, and an unclassified *RF39* were most strongly correlated with age

along RDA1. Other genera strongly aligned with RDA1, but not strongly correlated with any explanatory variables, included *Blautia*, and unclassified genera from *Lachnospiraceae*, *Christiensenelliaceae*, *Ruminococcaceae*, and *Clostridiales*.

	Variables included	**R_{adj}^2**	**anova.P**
Phyla	Protein	0.061	0.011
Class	Protein+Age+Sex	0.088	0.008
Order	Protein+Age	0.050	0.010
Family	Protein+Age+Sex+n6:n3	0.104	0.003
Genus	Protein+Age+Sex	0.052	0.005
Predicted metagenomic function	VO2peak+Sex+Fiber+Sugar	0.055	0.063
Short-chain fatty acids	VO2peak+Sex+Age+Carb	0.102	0.001

Table 6. Predictor variables included in the RDA models

A manual pre-screening of dietary variables based on existing literature and categories of interest was initially carried. Next, a combination of 'both' forward and backward stepwise inclusion selection method using vegan's *ordistep* function was used on the remaining 23 variables plus VO_2peak, Sex, BMI, and Age.

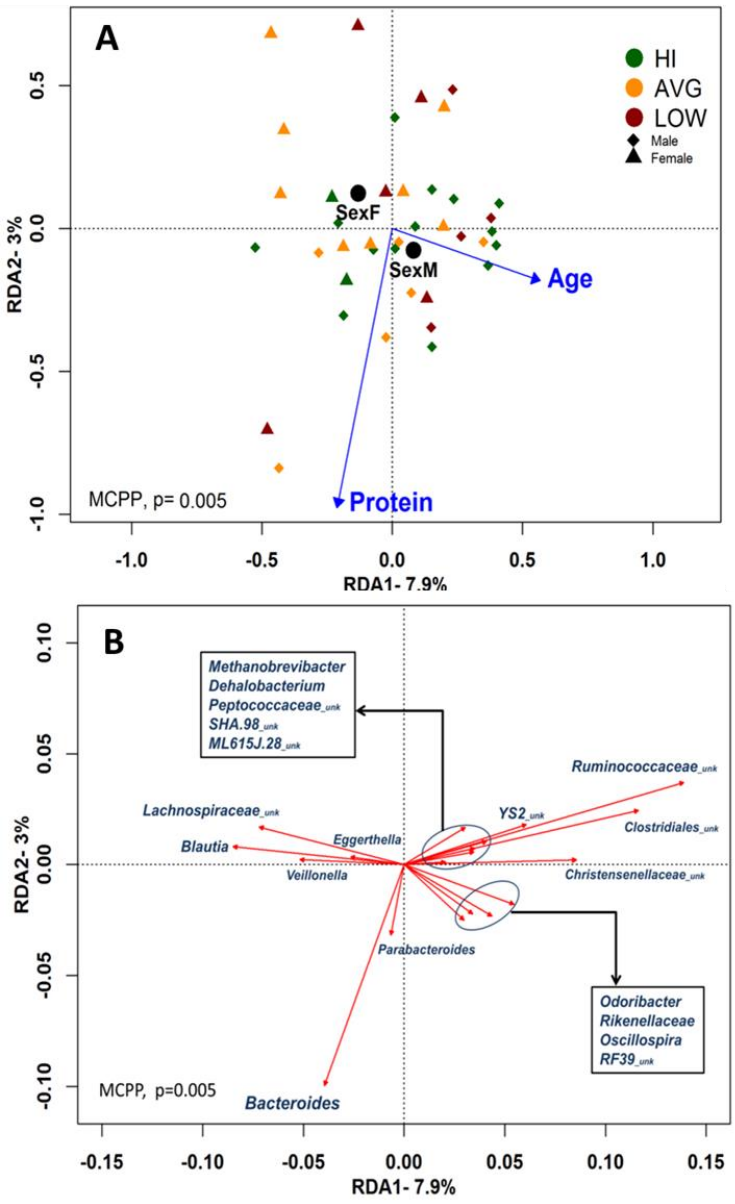

Figure 9. Bacterial RDA correlation biplots constrained by selected explanatory variables

The sites and explanatory variables (A) and genera (B) plots are presented separately for clarity, however they are derived from the same RDA model, note the difference in axes scales. RDA1 and RDA2 representing over 10% of the constrained variance in beta diversity are plotted. The global model's p-value was calculated using the Monte Carlo Permutation Procedure (MCPP). In plot A subjects are color coded according to their CRF levels for illustrative purposes only as groupings were not included in the model. Black circles represent centroids for the categorical variable Sex.

CRF is associated with distinct microbiome functions rather than specific bacterial taxa - Bacterial phylogeny is sufficiently linked to their functional capabilities and can be used to computationally predict the functional composition of the community metagenome (Langille *et al*., 2013). Since CRF did not constrain variation in bacterial composition, we explored whether the functions of the microbiome were associated with CRF. Similar to the beta diversity analyses, no clear group clustering emerged based on CRF classification alone (Figure 10). The RDA however, showed that VO_2peak, sex, fiber, and sugar intake collectively significantly constrained variation in functional categories (MCPP P=0.063, R_{adj}^2=0.055) (Table 6). Overall, 15.5% of the total variation was accounted for by these explanatory variables, of which 11% and 2.2% were accounted by the first and second axes, respectively (Figure 11). Of the 274 functional categories observed across all participants we identified 65 significant categories. The RDA plots illustrate a pattern of VO_2peak and fiber intake constraining variation amongst participants with high CRF levels. VO_2peak was most strongly correlated with sporulation, bacterial motility proteins including proteins involved in flagella assembly, and chemotaxis while negatively correlated with lipopolysaccharide (LPS) biosynthesis and LPS biosynthesis proteins. Total sugar intake was strongly correlated with the transporters, ABC transporters, and transcription factors while inversely associated with membrane & intracellular structural molecules and pores ion channels. Sex of participants did not play a significant role in any of the described parameters. Given the importance of SCFAs in gut health, we had *a priori* interest in 'fatty acid biosynthesis' despite its exclusion from the RDA selection process. We found VO_2peak to be positively correlated (P=0.046, Spearman's rho=0.322) with fatty acid biosynthesis (Figure 12). Thus, to understand which SCFAs correlated with VO_2peak we quantified fecal SCFAs via GC.

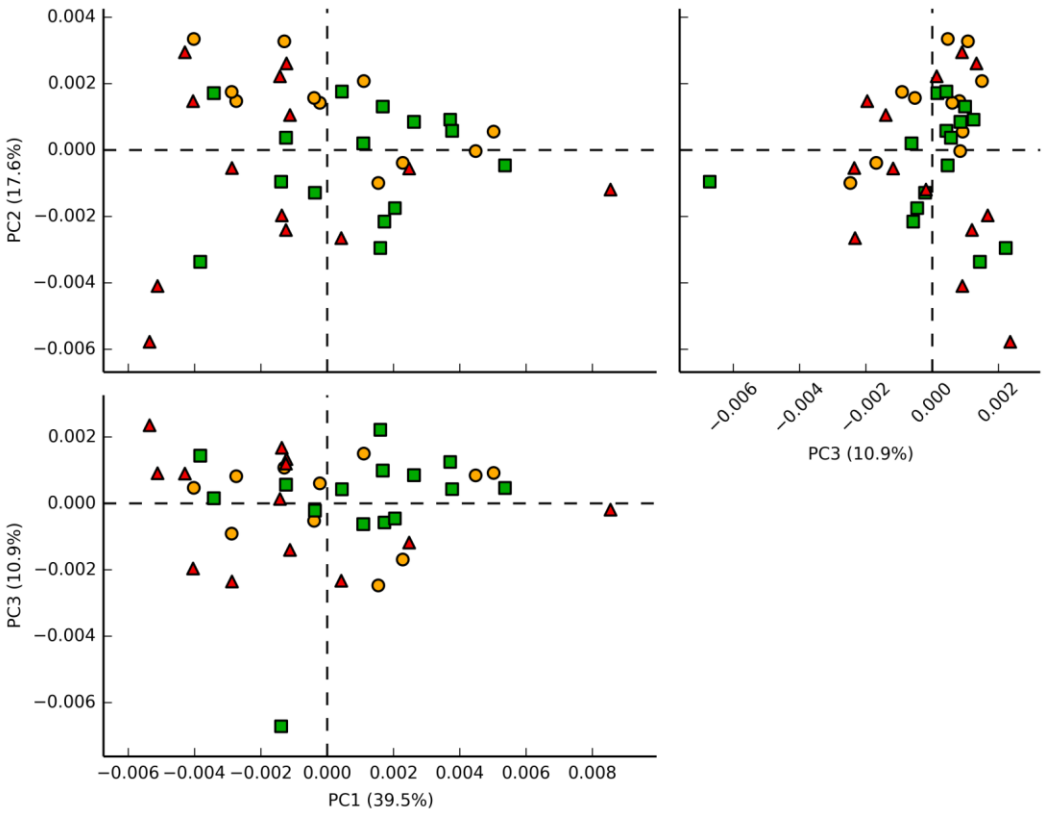

Figure 10. Ordination of predicted metagenomic functions data

PCA plot of centered functional category abundance data showing no clear clustering of groups based on their CRF levels. Plots were created using STAMP (Statistical Analysis of Metagenomic Profiles) tool.

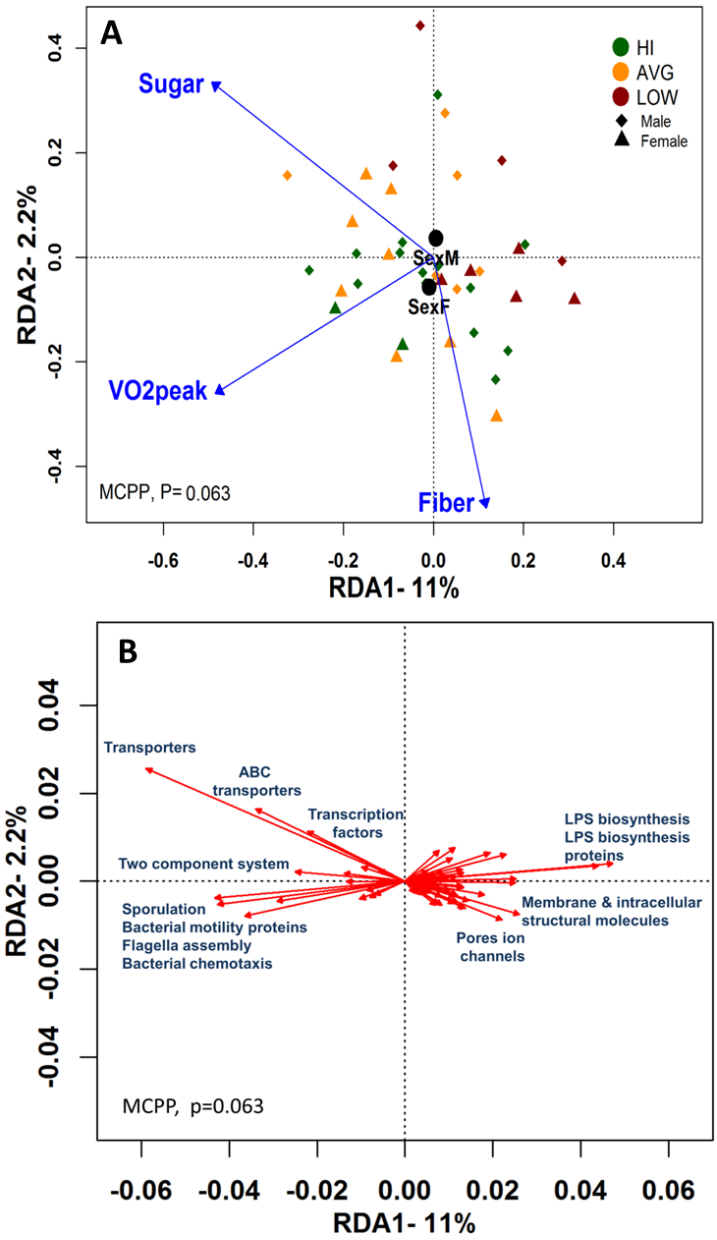

Figure 11. RDA correlation biplots of predicted functions constrained by explanatory variables

The sites and explanatory variables (A) and genera (B) plots are presented separately for clarity however they are derived from the same RDA model, note the difference in axes scales. RDA1 and RDA2 representing over 13% of the constraint variance in the data are plotted. The global model's p-value was calculated using the Monte Carlo Permutation Procedure (MCPP). In plot A subjects are color coded according to their CRF for illustrative purposes only as groupings were not included in the model. Black circles represent centroids for the categorical variable Sex.

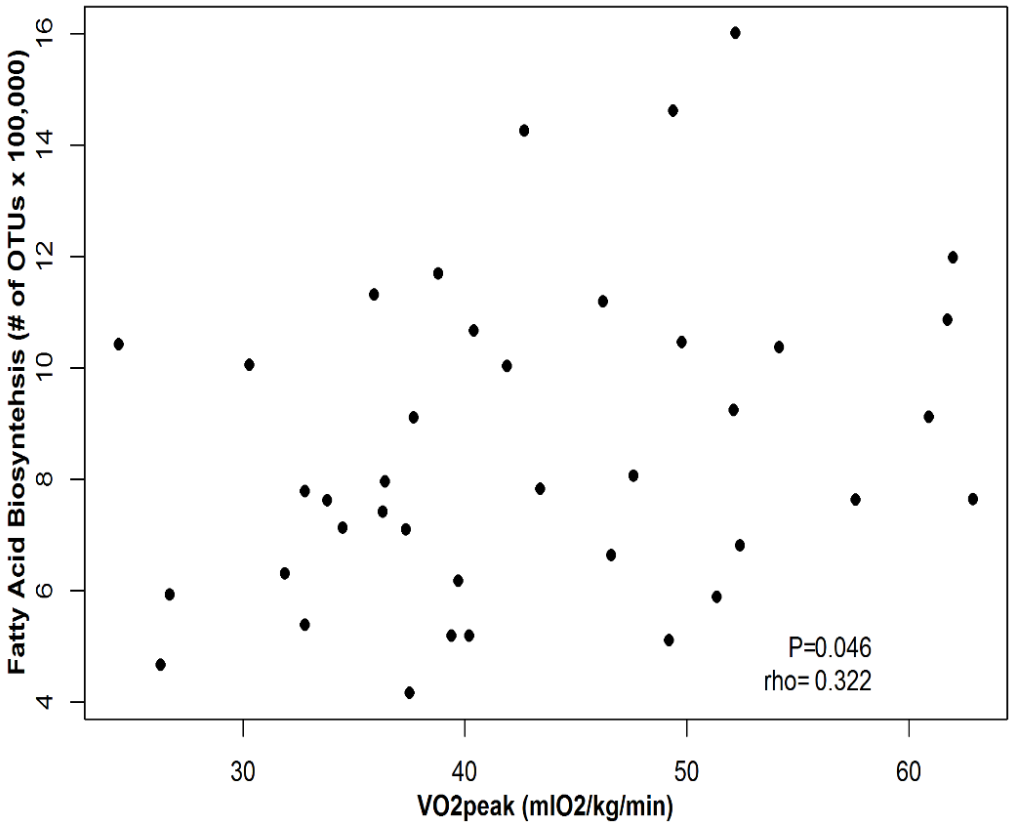

Figure 12. Correlation between VO2peak and fatty acid biosynthesis

Spearman correlation plot showing a positive correlation between VO2peak and the functional category 'fatty acid biosynthesis'. rho, Spearman's correlation coefficient.

CRF is positively correlated with fecal butyric acid - Figure 13 represents the RDA triplot corresponding to fecal SCFAs as constrained by our exploratory variables. The global model selected sex, age, carbohydrate intake, and VO_2peak as significant (MCPP $P = 0.001$) constraining variables yielding an R_{adj}^2 of 0.102. Overall, 30.1% of the constrained variation could be explained by these factors of which 17.9% and 11.9% were accounted for by RDA1 and RDA2, respectively. Along RDA1, age was strongly positively correlated with valeric acid and to a lesser degree with hepatonoic and caproic acid; both which were strongly inversely correlated with carbohydrate intake. Along RDA2, VO_2peak was strongly correlated with butyric acid which is represented mainly across HI and AVG fitness participants. Proprionic and acetic acid on the other hand were inversely correlated to VO_2peak and were represented across an area with more LOW fitness participants. Sex of the participants as represented by centroids on the triplot did not play a major role in observed variance.

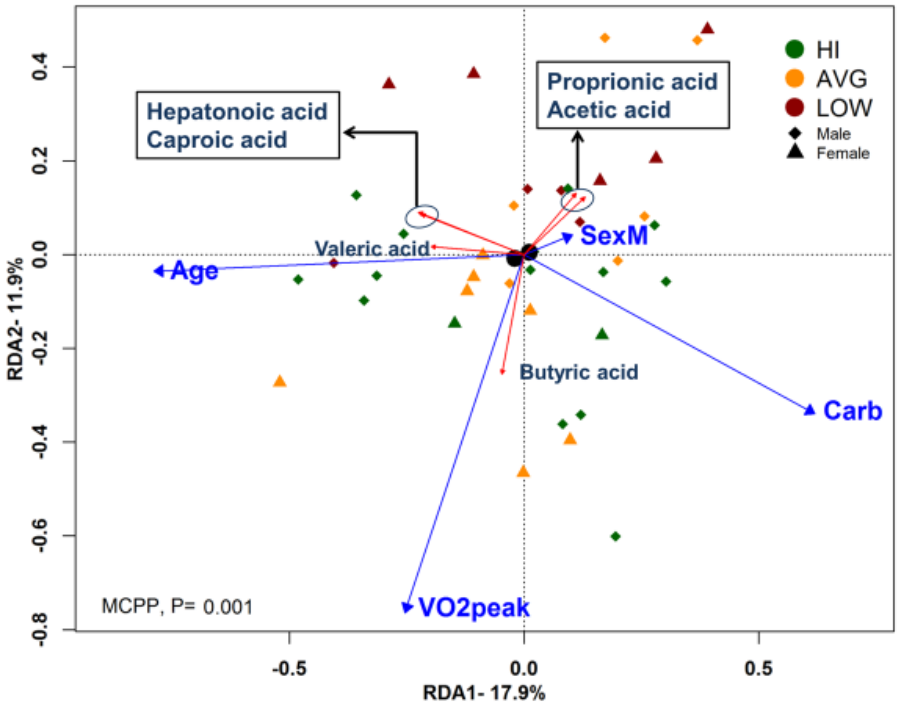

Figure 13. RDA correlation triplots of SCFAs abundance constrained by selected explanatory variables

RDA1 and RDA2 representing over 29% of the constraint variance in SCFA data are plotted. Subjects are color coded according to their CRF for illustrative purposes only as groupings were not included in the model. Black circles represent centroids for the categorical variable Sex. The global model's p-value was calculated using the Monte Carlo Permutation Procedure (MCPP).

2.4 Discussion

CRF is considered a better predictor of mortality than clinical variables as well as established risk factors such as smoking, diabetes, and hypertension (Kodama, 2009; Myers *et al*., 2002). Its role as a possible indicator of intestinal microbial diversity however, has not been investigated. Our regression model showed that ~ 20% of variation in gut bacterial alpha

diversity could be explained by VO_2peak alone; in fact, VO_2peak stood as the only variable that significantly contributed to increased alpha diversity. The primary findings from this study suggest that CRF is an excellent predictor of gut microbial diversity in healthy humans, outperforming several other variables including sex, age, BMI, and dietary components. While no specific bacterial taxa abundance could be predicted in relation to CRF levels, the overall function of the microbiome in high CRF individuals seems to favor an increase in chemotaxis related genes and decreased LPS biosynthetic pathways. In addition, a strong positive correlation was observed between fitness levels and fecal butyric acid, a SCFA associated with gut health (Leonel & Alvarez-Leite, 2012).

A recent study by Clarke *et al*. showed increased gut community richness amongst professional rugby players compared to sedentary BMI-matched and non-matched populations (Clarke *et al*., 2014). Due to extreme dietary differences amongst their groups however, the contribution of physical fitness could not be isolated from possible diet-driven influences. For example, it has been shown that increased species richness as a result of voluntary wheel running in mice is only robust under high-fat but not low-fat feeding conditions (Evans *et al*., 2014), highlighting the importance of the background diet. In our study we minimized the potential influence of diet as a confounding factor by examining LOW, AVG, and HI fitness participants with no significant differences in a comprehensive number of dietary variables. In addition, we quantify fitness using VO_2peak, a measure of capacity for aerobic work and the gold standard of CRF. In their study, Clarke *et al.* highlighted the importance of protein intake by showing its positive correlation with alpha diversity. Interestingly, the magnitude of this correlation was comparable to our correlation coefficient between VO_2peak and alpha diversity in the absence of a correlation between protein intake and alpha diversity. This may suggest that the reported correlation between protein and alpha diversity may have been a secondary product of increased CRF amongst the elite athletes. The mechanisms by which physical activity may promote a rich bacterial community are not known but likely involve a combination

of intrinsic and extrinsic factors. For example, physically active individuals are more likely to be exposed to their environmental biosphere and follow an overall healthy lifestyle and as so harbor a richer microbiota. Simultaneously, intrinsic adaptations to endurance training can lead to changes in the GI tract, for example: decreased blood flow, tissue hypoxia, and increased transit and absorptive capacity (Gisolfi, 2000; Rosa *et al*., 2005). These and other potential adaptation mechanisms such as change in gut pH are likely to create an environmental setting allowing for richer community diversity.

Beta diversity analysis of our cohort did not show distinct clustering of bacterial communities based on fitness categories. This contrasts with previous reports (Evans *et al*., 2014), which showed distinct clustering resulting from wheel running in mice, as well as those by Clarke *et al*. who showed clustering of rugby players' microbiota (Clarke *et al*., 2014). In addition to extreme dietary differences, several mechanisms may explain these discrepancies. Community clustering amongst cohabited animals or the 'cage-effect' is known to show high community structure concordance (Lees *et al*., 2014; McCafferty *et al*., 2013), it is therefore plausible that this phenomena extends to humans. As team members are likely to spend extended periods of time together on and off the field, there is an increased likelihood of microbial exchange leading to distinct similar bacterial profiles. Participants in the current study on the other hand did not belong to a common organization and did not show any detectable dietary differences. Other components of fitness not accounted for in the current study such as anaerobic capacity and resistance muscle training may also influence community composition, though to date no existing work has examined these parameters in relation to gut microbiota.

Total protein intake was consistently seen as a significant contributor to beta diversity at each taxonomic rank tested, while sex and age were only influential beyond the phyla level. Unlike dietary carbohydrates and fats, which are commonly studied, the role of protein in the context of intestinal microbiota is considerably less understood. Protein-rich diets have been associated with prevalence of *Bacteroides* genus (Wu *et al*., 2011). Echoing this, results from

our RDA analysis showed a strong correlation between protein intake and *Bacteroides* without bias towards any specific fitness groups. Excessive fermentation of dietary protein in the GI tract is generally considered detrimental due to the production of toxic by-products such as amines, phenols, indoles, thiols, and ammonia (Macfarlane & Macfarlane, 2012; Rist, Weiss, Eklund, & Mosenthin, 2013). Further research however is needed to determine the synthesis kinetics and clinical consequence of these by-products during increased nutritional status and metabolic demands such as during prolonged exercise training. The RDA results further showed significant contribution of members of the *Ruminococcaceae* and *Lachnospiraceae*, two of the most abundant families in gut environments (Jalanka-Tuovinen *et al*., 2011), in explaining community diversity. These plant degraders persist in fibrolytic gut communities and are considered an important component of a healthy gut, while their depletion has been observed in IBD patients (Frank *et al*., 2007; Fujimoto *et al*., 2013). *Ruminococcaceae* and *Bacteroides* were anticorrelated, likely reflecting the persistence of these groups in plant carbohydrate- versus protein-rich gut environments, respectively. Interestingly, an unclassified member of the *Christensenellaceae* family was seen significantly correlated with age; this was true despite the limited range of our participants' age (18-35 years). Though there is limited published work regarding its role, a recent study identified *Christensenellaceae* as the most heritable member of the gut microbiota and highlighted their role in promoting a lean phenotype (Goodrich *et al*., 2014).

An increase in CRF demands various phenotypic and metabolic adaptations by the host which subsequently may require adaptation by the commensal bacteria. The results of our RDA showed that despite VO_2peak not constraining beta diversity in a predictable manner, it is however a driving force in changing the metagenomic functions of the microbiome. Functional categories most strongly correlated with VO_2peak were related to bacterial motility (categories: bacterial motility proteins, flagella assembly, and bacterial chemotaxis), sporulation, and to a lesser extend the two-component system which enables bacterial communities to sense and

respond to environmental factors. One possible mechanism behind these associations may derive from the observation that butyrate, which was more abundant amongst fit participants, can modulate neutrophil chemotaxis (Bocker *et al*., 2003; Vinolo *et al*., 2011) . VO_2peak was inversely correlated with LPS biosynthesis and LPS biosynthesis proteins which were more aligned amongst less fit participants. LPS is a major component of the cell wall of Gram-negative bacteria and is considered an endotoxin when present in the blood. By binding to extracellular toll-like receptor 4 (TLR4) found on of many cell types, LPS elicits strong inflammatory responses that may be detrimental to the host. Continuous low-level translocation of LPS into circulation can induce chronic low-level inflammatory states that are associated with development of obesity and other metabolic syndromes (Monteiro & Azevedo, 2010). These inflammatory states are thought to derive, to some extend from inflammatory responses to blood LPS which is elevated in sedentary humans (Lira *et al*., 2010). Exercise training attenuates inflammation in part by reducing elevated blood LPS (Lira *et al*., 2010). The inverse relationship between VO_2peak and LPS biosynthesis pathways observed in the current study therefore extends previous research, suggesting a beneficial consequence of increased physical activity to derive from decreased LPS biosynthesis. The findings here suggest that the gut microbiota adapt to metabolic demands of a physically active lifestyle, anchored around a set of physiological functions.

Production of SCFAs is the primary result of carbohydrate fermentation under anaerobic conditions in the gut. Butyric acid or butyrate is the most commonly studied of these SCFAs regarding intestinal health. As the primary food source of colonocytes, butyrate plays an important role in gut homeostasis and health. It has been shown to possess anti-cancer and anti-inflammatory properties (Hamer *et al*., 2007), and be involved in gut motility (Hurst, Kendig, Murthy, & Grider, 2014; Scheppach, 1994) energy expenditure (Gao *et al*., 2009), intestinal permeability (Kanauchi *et al*., 1999), and appetite control (Sleeth, Thompson, Ford, Zac-Varghese, & Frost, 2010), while a decrease in butyrate levels has been suggested in etiology of

ulcerative colitis (Kumari, 2013). We observed a strong positive correlation between VO_2peak and fecal butyrate levels, which could not be accounted for by ingested dietary butyrate or its substrate, fiber. This suggests that the microbial profiles of physically fit individuals favor butyrate producing taxa leading to increased fecal butyrate. This is in accordance with Matsumoto *et al*. (2008) who observed increases in butyrate levels in cecum of rats exposed to 5 weeks of wheel running (Matsumoto *et al*., 2008).

The primary findings from this study suggest that aerobic fitness may predict gut microbial diversity in healthy humans and that dietary protein plays a significant role in microbial community composition. We further observed that adaptation of the microbiota to demands of increasing physical fitness is anchored around a set of functional cores rather than specific bacterial groups. In particular, the microbiome profile of fit individuals favors butyrate production, a common indicator of gut health. Overall, our findings are consistent with a role for physical activity in promoting gut intestinal health via associated changes in the microbial community composition. These findings warrant further research in the use of aerobic exercise prescription as an adjuvant therapy in prevention and treatment of dysbiosis-associated diseases.

Chapter 3: Effects of PA on gut health in murine colitis

3.1 Background

Inflammatory bowel diseases (IBD) encompassing Crohn's disease (CD) and Ulcerative colitis (UC) are idiopathic, relapsing chronic diseases characterized by chronic inflammation of the gastrointestinal tract. While pathology varies between UC and CD, both burden patients with common debilitating clinical symptoms such as diarrhea, rectal bleeding, abdominal pain, and weight loss. The etiology of IBD is not known, however a combination of genetic, immunological, and environmental factors is implicated in its development. Most recently, the contribution of the intestinal microbiota in IBD pathogenesis has risen as an active area of research (Sheehan, Moran, & Shanahan, 2015). This hypothesis is primarily driven by observations that IBD patients have reduced gut microbial diversity (Harris & Chang, 2018) and are more likely to have been prescribed antibiotics in the 2-5 years preceding diagnosis (Shaw, Blanchard, & Bernstein, 2011). In animal models, this is further supported by findings that mice genetically predisposed to colitis (IL-$10^{-/-}$) are resistant to disease onset while kept under germ-free conditions (Sellon et al., 1998b).

The human intestinal tract is continuously exposed to the trillions of microbes residing within the mucosal layer of the lumen. Under homeostatic conditions, these microbes are tolerated by the host as they provide essential functions such as digestion of complex carbohydrates, protection against enteric pathogens, and production of beneficial short-chain fatty acids (SCFAs), to name a few. Separating the luminal microbes from the intestinal epithelial cells (IEC) is a mucosal bilayer largely composed of the highly glycosylated protein mucin 2 (MUC2). In the colon, the loosely structured outer mucus layer allows for colonization of microbes in a nutrient-rich environment, while the dense inner layer segregates them from the IEC (Faderl, Noti, Corazza, & Mueller, 2015). To colonize the mucosal layer, microbes must tolerate its specific biological conditions such as absence of oxygen, alkaline pH, and

physiological temperatures. In this sense, fluctuations in mucosal health can dictate community dynamics of the microbiota (Faderl *et al*., 2015). Conversely, mucus phenotypes can also be influenced by microbial composition (Rodríguez-Piñeiro & Johansson, 2015). Inflamed tissues of UC patients often display structural defects or thinning of the mucosal barrier (Fyderek *et al*., 2009) that lead to heightened exposure of microbes to host cells. In a healthy gut, a small portion of luminal microbes inevitably infiltrate the inner mucosal layer and come in direct contact with the IEC. These microbes are generally eliminated by the host innate immune system without development of immunological memory. For example, antimicrobial peptides (AMPs) secreted by the specialized intestinal Paneth cells may impose their microbicidal actions directly on the luminal side while antigen-presenting cells recruit activation of pro-inflammatory cascades to eliminate such invading cells in the submucosa. In an unhealthy gut with a compromised mucosal layer, the excessive exposure of microbial antigens to the host cells prompts a chronic state of inflammation and apoptosis leading to further loss of IEC integrity and thus further exposure and injury. MUC2 deficient mice ($MUC2^{-/-}$) or those with missense mutation impairing the release of MUC2, develop spontaneous colitis (Van der Sluis *et al*., 2006). Interestingly, symptoms severity and timing in $MUC2^{-/-}$ littermates varies across animal facilities (personal observation), further suggesting the role of microbes in this model.

With incidences of IBD rising globally, there is an increasing demand for novel therapeutics. Physical activity (PA) has been proposed as both a primary and an adjunct therapy for prevention and treatment of various chronic diseases (Booth, Roberts, & Laye, 2012b) with IBD having been recently marked as a new candidate (Bilski *et al*., 2016). Studies of PA in rodents have shown ameliorated symptoms of chemically-induced colitis (Bilski *et al*., 2015; Marc D Cook *et al*., 2013b; Saxena *et al*., 2012; Szalai *et al*., 2014) that appear to be dependent on the colitis model and type of PA. These studies however, only assess the role of PA as a preventive measure leading up to induction of acute colitis via a chemical toxin. As so the potential benefits of PA succeeding disease onset is not known.

In this study we aimed to advance this knowledge gap by utilizing the MUC2$^{-/-}$ model of chronic colitis. In our facility, MUC2$^{-/-}$ mice are born with an underlying predisposition to intestinal inflammation that show rapid progression of disease symptoms from an early age. MUC2 $^{-/-}$ mice generally display clinical symptoms of colitis following weaning (~ 1 month) and histological analysis as early as two months of age indicates a moderate-level colitis, reaching high-severity by 4 months (Morampudi *et al*., 2016). We hypothesized that introduction of MUC2$^{-/-}$ mice to VWR would reduce the severity and delay the onset of disease symptoms. Having recently shown a significant correlation between aerobic fitness and overall microbial diversity (Estaki *et al*., 2016) we hypothesized further that these PA-associated protections would be mediated through changes of intestinal microbiota and their metabolites.

3.2 Methods

Animals - All procedures involving the care and handling of the mice were approved by the UBC Committee on Animal Care, under the guidelines of the Canadian Council on the Use of Laboratory Animals. Four-week-old male C57BL/5 mice were purchased from Charles River (Vancouver, CA) and kept under specific pathogen-free conditions. MUC2$^{-/-}$ mice, generated also on a C57BL/5 genetic background, were bred inhouse in our facility with the founding colonies having been kindly donated by Dr. Bruce Vallance from the Child and Family Research Institute (UBC Vancouver). All animals were housed in a temperature-controlled room (22 $\pm$ 2°C) on a 12h light/dark cycle with access to acidified water and irradiated food (PicoLab Rodent Diet 20-5053, Quebec, CA) *ad libitum.* At 5 weeks of age, using a random number generator, animals were assigned to individual cages under one of four groups (n=8) for 6 weeks: wild-type mice with access to a free running wheel (VWR) or a locked wheel (SED), and MUC2$^{-/-}$ mice with access to free wheel (MVWR) or a locked wheel (MSED). Due to technical issues with the running wheels, a second cohort of animals were purchased and assigned to VWR. We chose to start wheel running immediately following weaning at 5 weeks of age, which

in MUC2$^{-/-}$ animals is delayed compared to WT, in attempt to maximize their PA prior to disease progression into a severe state. In our hand these animals begin to develop severe clinical symptoms of colitis around 3 months of age, which may overmine any protective effects of PA. The running wheels (Columbus Instruments, dimeter 10.16 cm, width 5.1 cm) recorded total number of revolutions at 1 hr intervals for the duration of the experiment. Body weights, food, and water intake were measured weekly at approximately at the same time during the light cycle. Food weight measurements consisted of subtracting the week's remaining pellets on the cage lids and bottoms from that week's starting weight.

Tissue collection - For fecal sample collection, mice were kept in isolation in a sterile and DNA-zap treated containers until defecation. Fecal pellets were then snap-frozen in liquid nitrogen then stored in -80 °C until further analysis. Fecal samples were collected on day 1 immediately following assignment to individual cages, and again on the final experiment day immediately preceding tissue collection. Whole blood was collected via intracardial puncture while under isoflurane inhalation, followed by termination by cervical dislocation. The cecum was isolated, its content removed, and tissue frozen in liquid nitrogen. Colon tissues were collected as follows: starting from distal end, three ~1.5 cm sections were collected with the most distal section being fixed in formalin for histological staining, the middle section was stored in RNAlater (Theromo Fisher Scientific) for RNA extraction, and the most proximal section was snap-frozen in liquid nitrogen for use in DNA extraction. All frozen samples were then stored in -80 °C until further use.

Clinical and Histopathological Scoring - Disease progression in MUC2$^{-/-}$ animals was assessed based on an in-house clinical symptom scoring system (Appendix A). Briefly, each animal was graded weekly based on the following: observed behavior from a distance, stool/rectal bleeding, stool consistency, weight loss, and hydration, with each parameter being assigned a score of 0-4. Humane endpoint was set as a total accumulative score of ≥12, rectal

prolapse, or a weight loss of >20% body weight for 2 consecutive days. No animals reached humane end-point in this study.

For histopathological scoring, colon cross-sections were fixed in 10% neutral-buffered formalin at 4°C overnight, washed 3 times with phosphate buffered saline (PBS, pH 7.4), transferred to 70% ethanol and sent for paraffin-embedded sectioning and hematoxylin and eosin (H&E) staining at Wax-it Histology Services (Vancouver, Canada). Tissue slides were coded throughout the microscopy analyses and investigators blinded to the groupings. H&E stained sections were viewed under 200x magnification on an Olympus IX81 microscope and the full image stitched together using MetaMorph® software. Stitched images were imported into ImageJ (Schneider, Rasband, & Eliceiri, 2012) (v. 1.51r) for scoring. Disease severity in colonic cross sections from the MUC2$^{-/-}$ animals were assessed using a previously described scoring system (Bergstrom *et al*., 2010). In brief, averaged scores were quantified using the following criteria:

1. *Edema*, as compared to a healthy WT control: 0=no change; 1=mild (<10%); 2=moderate (10-40%); 3=profound (>40%)
2. *Epithelial hyperplasia*, average height of crypts as a percentage above the height of a healthy control where 0=no change; 1=1–50%; 2=51–100%; 3=>100%
3. *Epithelial integrity*, shedding and shape of the epithelial layer as compared to healthy control where: 0=no change; 1=<10 epithelial cells shedding per lesion; 2=11–20 epithelial cells shedding per lesion; 3=epithelial ulceration; 4=epithelial ulceration with severe crypt destruction
4. *Cell infiltration*, presence of immune cells in submucosa: 0=none; 1=mild (2-43); 2=moderate (44-86); 3=severe (87-217).

The maximum score resulting from this system is 13.

Reverse Transcriptase-qPCR - The mRNA gene expression for tumor-necrosis factor alpha (TNFα), interferon-gamma (IFNγ), resistin-like molecule beta (Relm-β), regenerating islet-derived protein 3 (RegIII-γ), transforming growth factor beta (TGF-β), chemokine C-X-C motif ligand 9 (Cxcl9), and claudin 10 (Cldn10) were measured in colon tissues. Total RNA was purified from tissues using Qiagen RNEasy kits (Qiagen) according to the manufacturer's instructions with an additional initial bead beating step (3x30 seconds, 30 Hz) on a Retsch MixerMill MM 400 homogenizer. Next, cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) in 10 µl reactions. The RNA and cDNA products' purity and quantity were assessed by a NanoDrop spectrophotometer (Thermo Scientific). The cDNA products were normalized to ~ 40 ng/µl with DNAse free sterile water prior to qPCR reactions.

A total of 10 µl RT-qPCR reactions consisted of: 0.2 µl of each forward and reverse primers (10mM), 5 µl of Sso Fast Eva Green Supermix (Bio-Rad), 3.6 µl DNAse free water, and 1 µl of cDNA template. Reactions were run in triplicates using the Bio-Rad CFX96 Touch thermocycler and analyzed using Bio-Rad CFX Maestro software 1.1 (v4.1). The median quantitation cycle (Cq) value from each sample was used to calculate the $2^{-\Delta\Delta Ct}$ based on the reference gene TATA box binding protein (Tbp). A list of all the primer sets, their melting temperature, efficiencies, and detailed thermocycler protocol used in this study are described in Appendix B.

Short-chain fatty acids - SCFAs (acetic, propionic, heptanoic, valeric, caproic, and butyric acid) were analyzed from cecal tissues by gas chromatography (GC) as described previously in Chapter 2. Half of the cecal tissue was freeze dried to measure the dry weight, and measurements are expressed as µmol/g dry weight (d.w).

DNA extraction and 16S rRNA amplicon preparation - DNA was extracted from fecal samples using the QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions following 3 x 30 s of beat beating. The QIAmp PowerFecal kit was used to extract colonic DNA which utilizes garnet bead beating instead of metal beads which yields a higher ratio of bacteria:host final DNA concentration. Amplicon libraries were prepared according to the

Illumina16S Metagenomic Sequencing Library Preparation manual. In brief, the V3-V4 hypervariable region of the 16S bacterial rRNA gene was amplified using recommended degenerate primer sets 341F: CCTACGGGNGGCWGCAG, and 805R GACTACHVGGGTATCTAATCC, which create an amplicon of ~460 bp. Amplicons were purified using AMPure XP beads and adapters and dual-index barcodes (Nextera XT) were attached to the amplicons to facilitate multiplex sequencing. Following another clean-up step, libraries were quality controlled on an Experion automated electrophoresis system (Bio-Rad), and sent to The Applied Genomic Core (TAGC) facility at the University of Alberta (Edmonton, Canada) where they were normalized using fluorometric method (Qubit, Thermo Fisher Scientific) and sequenced using the Illumina MiSeq platform with a V3 reagent kits allowing for 2 x 300 bp cycles.

Bioinformatics - All bioinformatics processes were performed within the QIIME2 platform (Caporaso *et al*., 2010) using the various build-in wrappers described below. All used software packages, versions, and parameters are available under the 'provenance' section of the QIIME2 feature-table artifact which will be made available online. This file can be viewed locally on a browser by drag and dropping the file onto https://view.qiime2.org/. Paired-end sequences obtained from the sequencing machines underwent quality-filtering, dereplication, chimera removal, denoising, and merging using the DADA2 (Callahan *et al*., 2016) plugin with default settings. The output of this process is a feature table of amplicon sequence variants (ASV) that is a higher resolution analogue of traditional observational taxonomic unit (OTU) tables. A Naïve Bayes classifier that was trained on the specific region targeted by our primer sets using the most recent available version of the Greengenes (13_8) was used to assign taxonomy at the genus level. For analyses encompassing phylogenetic information, a phylogenetic tree was constructed using a SATé-enabled phylogenetic placement (SEPP) technique as implemented in the q2-fragment-insertion plugin (Janssen *et al*., 2018) with default settings. To predict the functional repertoire and phenotype of the microbiome, we used BugBase (Ward *et al*., 2017)

which utilizes PICRUSt's (Langille *et al*., 2013) extended ancestral-state reconstruction algorithm for metagenome composition prediction. As these tools require features to be classified against Greengenes taxonomy, we used QIIME2's VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) plugin to pick closed-reference OTUs from our denoised feature table at 97% similarity threshold against the 97% Greengenes OTUS database.

Statistical Analysis - All statistical analyses were performed using R version 3.5.1unless stated otherwise. During week 3, the VWR animals were exposed to 72 hrs of interruptions in their light:dark cycle due to an electrical malfunction in the room. The exact nature of this interruption is not known but likely consisted of irregular or an overall lack of dark cycle. Though short in duration, this may have affected the nature of wheel running between these groups, and as such further analyses were conducted in a 4 groups x 1 level factorial design rather than a 2 x 2 design.

Wheel running - Wheel running data was first analyzed across the 6 weeks time using linear mixed-effects regression (LMER) using the *lme4* package (Bates, Mächler, Bolker, & Walker, 2014) with individual animals set as the random effect. Homoscedasticity and linearity of the models were assessed using diagnostic plots of the residuals. The total distance ran between groups was compared using a Kruskal-Wallis non-parametric test as the data did not meet the assumptions of normal distribution.

Body weights and food/water intake - To account for natural differences in starting body weights, total weight gained relative to starting body weights was calculated at each week. Body weights, food and water intake comparisons across the 6 weeks were assessed using repeated measures utilizing LMER as before with 'week' also added as a random effect to the model to account for possible sources of variation due to different animal handlers and weight-scales used during the experiment. A Tukey HSD post-hoc test with the Benjamini-Hochberg (BH) P adjustment method was used when an overall significance (set as $P<0.05$) in the models were detected.

Clinical and Histopathological Scoring - Clinical and histopathological scores in MUC2$^{-/-}$ animals were compared using a cumulative link model (CLM) with a logit link using the *ordinal* package. This proportional odds type test is more appropriate for ordinal data type than classic linear regressions. For clinical scores, the model included week and group as the fixed effects, and individual animals as the random effects. For histopathological scores, the total average score of the MSED and MVWR groups were compared without a random effect variable.

Colon mRNA gene expression - Colonic mRNA gene profiles were first explored using an ordination method to visualize overall group profiles. The Euclidean distances of Hellinger-transformed relative gene expression values were ordinated onto a principle component analysis (PCA) plot. Group differences were assessed using a permutational multivariate analysis of variance PERMANOVA test in the *vegan* package, and pairwise differences calculated using *pairwiseAdonis* with BH adjustment for multiple testing. For differential abundance testing of each cytokine, a multivariable generalized linear model (GLM) test was carried out using the *mvabund* package (Wang, Naumann, Wright, & Warton, 2012). This fits separate GLMs to each cytokine while accounting for the inter-correlation amongst them and adjusting for multiple testing. The negative binomial distribution assumption was selected for the model and the mean-variance plot was used to assess the model fit. A Kruskal-Wallis post-hoc test was carried on individual genes when significance was detected in the overall model. Pairwise comparisons across groups was carried out using Conover's test for multiple comparisons within the *PMCMRplus* package.

Short-chain fatty acids - As with the cytokine analyses concentrations of various cecal SCFA were assessed using a multi-GLM test. With the global model showing a significant group effect, post-hoc tests were carried out on individual SCFA identified as significant from the univariate results from the global model.

Microbial Analysis - Community structural patterns of fecal bacteria across samples (β diversity) were explored in QIIME2 by calculating the Bray-Curtis and weighted-UniFrac distances across samples and plotting them onto a PCoA space using Emperor interactive graphic tool (Vázquez-Baeza *et al*., 2017). To reveal possible group differences, a PERMANOVA (Anderson, 2001) test was conducted across all groups and time-points using a rarefied feature-table at a depth of 9930. Pairwise testing was then followed using a Kruskal-Wallis test with a BH adjustment to control for false discovery rate (FDR). In addition, a generalized Hotelling's test on centered log ratio (clr)-transformed feature tables was used to compare the average microbiome composition between paired samples, as implemented in the *GHT* package (Zhao, Zhan, Guthrie, Mitchell, & Larson, 2018). This test has the advantage of accounting for the paired relationship of microbiome samples across time and does not require rarefying as it works with relative abundance data.

The overall within sample diversity (α diversity) was calculated based on the species richness, Shannon index, and Faith's phylogenetic diversity (PD) indexes, which capture the overall richness, evenness, and phylogenetic diversity of the communities, respectively. For each group, the difference between a sample's week 6 and week 0 diversity score was calculated and used to determine whether the change differed from zero (Wilcoxon test) and other groups (ANOVA plus Tukey HSD).

Differential abundance testing between individual taxa was performed using *ALDEX2* package (Fernandes *et al*., 2014). This approach also utilizes clr-transformation and has been shown to be superior to stand-alone multi-glms approaches in reducing false-positives (Weiss et al., 2017), and further appropriately accounts for the compositional nature of these types of datasets which is absent in stand-alone GLM methods. To reduce noise in the test, low abundant taxa were filtered based on the requirement that the average abundance of each taxa across all samples must be > 1.

BugBase was used to determine high-level phenotypes of bacterial communities based on the following default traits: Gram negative vs. Gram positive, biofilm forming, mobile element containing, oxidative stress tolerance, pathogenic potential, and oxygen utilizing. Pre- and post-treatment differences in relative abundances of these elements were tested in each group using a Kruskal-Wallis test with BH adjustment of P values to control FDR.

Boxplots in figures show the median plus IQR range. Whiskers in bars represent 1.5 x IQR range as per R base default settings.

3.3 Results

Wheel running - One animal from each group unexplainably did not run on the wheels, as so they were excluded from the analyses. The WT group ran an average (SD) of 46.6 (18.4) km in total throughout the 6 weeks, while the MUC2$^{-/-}$ animals ran slightly less at 40.7 km (21.5) which correspond to ~ 1.3 and 1.1 km/day, respectively. While the WT showed generally higher levels of wheel running, especially during the first three weeks, the differences were not statistically significant (Figure 14A) likely due to the highly variable nature of running data.

Bodyweights, food and water intake - Weight gain was not significantly different across activity levels, however as expected MUC2$^{-/-}$ mice gained less weight throughout the 6 weeks (Figure 14B). The mean (± SE) total weight gain of each group was: SED 33.12 ± 2.66 %, VWR 26.98 ± 2.14%, MSED 19.18 ± 1.96%, and MVWR 19.59 ± 2.99% grams relative to their starting body weights. By the final week, there was a trend towards VWR animals having gained less total weight compared to their SED counterpart, however this did not reach statistical significance. Food intake was not statistically different between groups across the 6 weeks (Figure 14C). MUC2$^{-/-}$ mice drank significantly more water than WT animals (Beta coefficient (B): 5.4, $P<0.001$) throughout the 6 weeks. Wheel running was associated with increased water intake in WT (B: 5.3; $P<0.01$) and to a lesser extend in MUC2$^{-/-}$ mice (B:1.9; $P<0.86$) (Figure 14D)

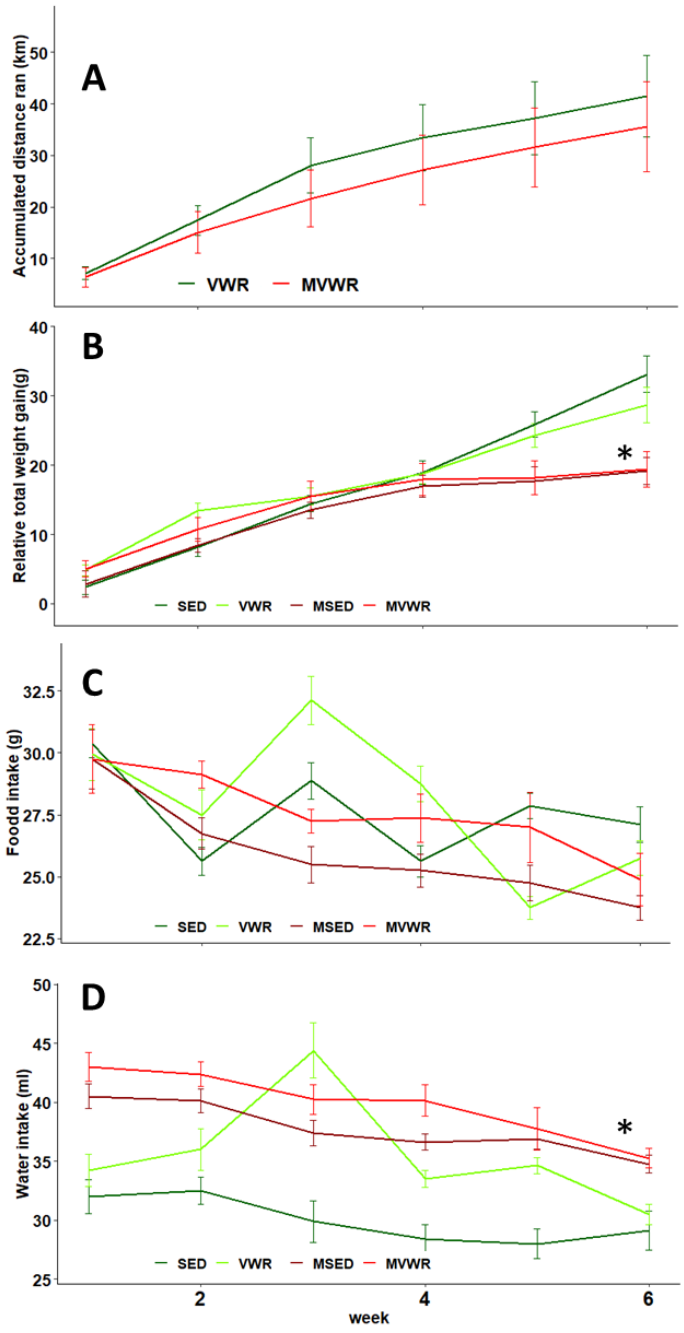

Figure 14. Weight gain, activity, food, and water intake

Longitudinal measurements of A) total accumulated distance ran, B) relative weight gain compared to starting weight, C) total weekly food intake, and D) total weekly water intake. Linear mixed models were used with week and animals set as random effects. There were no significant effects of wheel running in any of the parameters. * indicates a significant ($P<0.05$) main effect between phenotypes.

Histopathological and clinical scores

The result of the overall CML showed a modest but significant difference in clinical scores between MVWR and MSED groups (B: -1.67; P<0.01) across the 6 weeks, implying reduced disease symptoms in the running animals. However, post-hoc tests carried out at each week showed no significant difference between groups, though the difference between groups (B: -2.0, P=0.063) appeared to be increasing gradually through week 6 (Figure 15A). Histopathological scores based on H&E sections showed no differences between groups (Figure 15B).

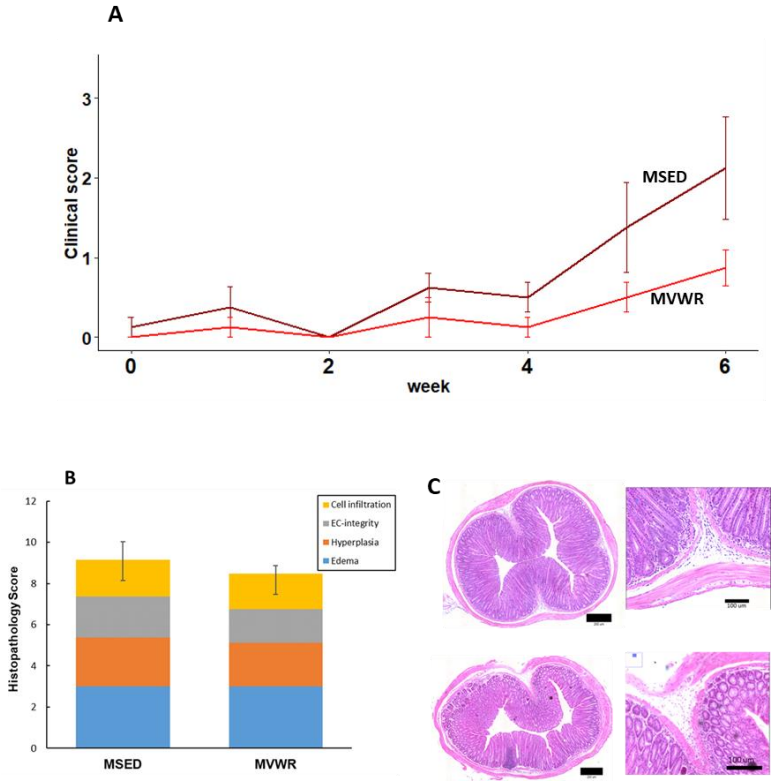

Figure 15. Assessment of colitis symptoms

Comparison of A) clinical scores across 6 weeks, and B) histopathological scores in MUC2$^{-/-}$ animals. C) Representative colon images of H&E stained sections from MSED (top row) and MVWR (bottom row) mice. No significant differences were observed between groups in either measurements. Values are means ±SE

Short-chain fatty acids - The results of the global GLM indicated a significant group effect (Test statistic: 14.83, P<0.01) while the univariate tests showed significant differences in acetate, propionate, butyrate, and valerate across groups. Post-hoc analyses on these SCFAs as well as total SCFA were carried out with results shown in Figure 16, and Table 7. Total SCFA concentration was significantly higher in VWR mice than all other groups, while SED mice had similar total SCFA compared to both MUC2$^{-/-}$ groups. VWR also had significantly higher total acetate and butyrate than all the other groups and higher propionate than SED. Overall, the major difference between MUC2$^{-/-}$ and WT animals was the significantly reduced levels of butyrate in MUC2$^{-/-}$ mice and inversely, higher levels of propionate, valerate, caproate, and heptanoate were similar across all groups. In terms of relative abundance, the main differences between MUC and WT was the higher propionate and lower butyrate proportions in MUC2$^{-/-}$ animals. Importantly, the proportion of butyrate in VWR mice (~12 %) was significantly higher than those in SED (~7.9 %).

Pairs	Total SCFA	Acetate	Propionate	Butyrate	Valerate
SED-VWR	↓	↓	↓	↓	**n.s**
MSED-MVWR	**n.s**	**n.s**	**n.s**	**n.s**	**n.s**
SED-MSED	**n.s**	**n.s**	↓	↓	**n.s**
VWR-MVWR	↑	↓	↓	↓	**n.s**

Table 7. Pairwise comparison results of SCFAs

N.s, not significant; arrows represent a statistically significant (adjusted P<0.05) different between the corresponding pair. The direction of the arrow refers to the level of the first group relative to the second.

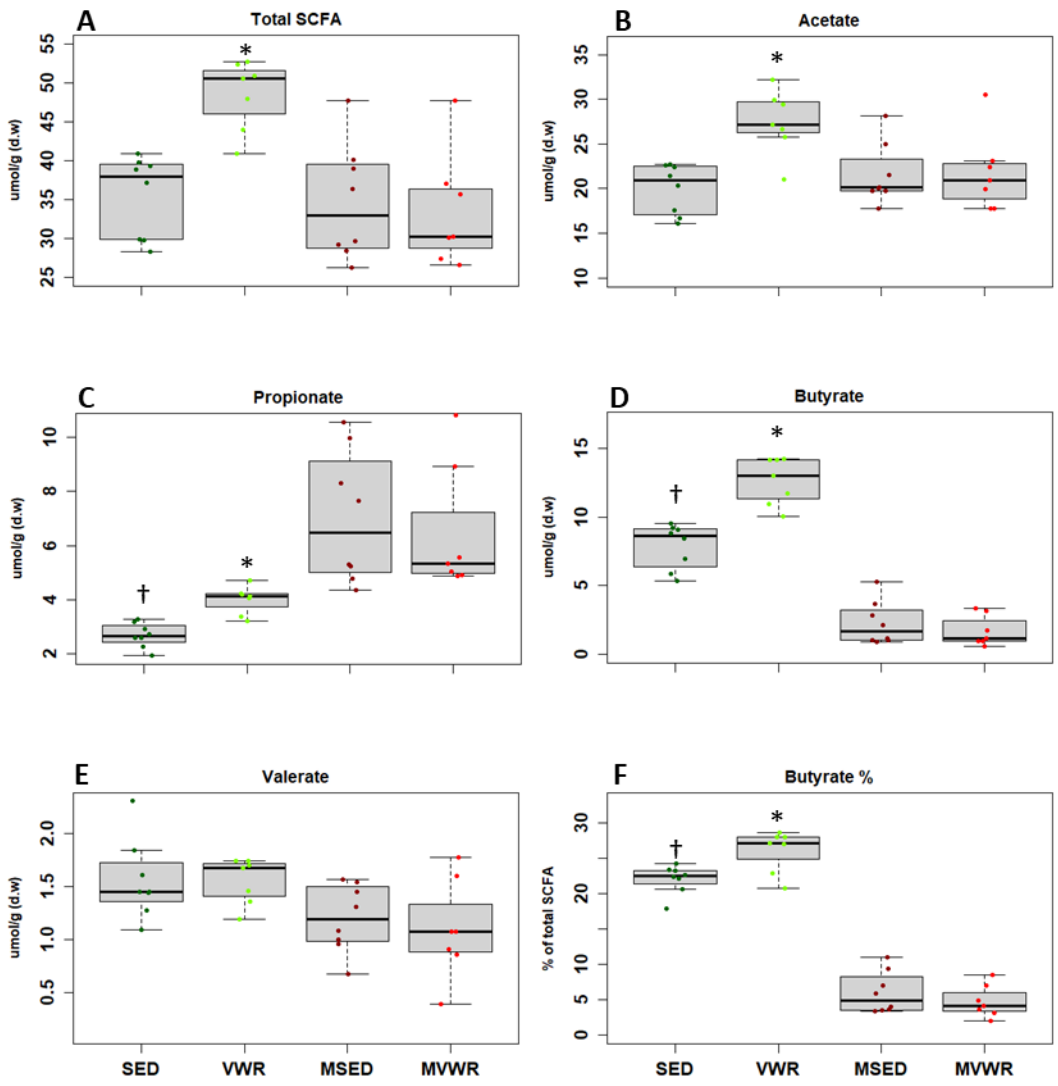

Figure 16. Cecal SCFAs composition

Cecal tissues analysed for SCFAs composition using gas chromatography. The *bottom* and *top of boxes* are the first and third quartiles, the middle band inside the boxes is the median, the *whiskers* contain the upper and lower 1.5 interquartile range (IQR). ***** denotes significantly different (adjusted P<0.05) than all other groups. **†** different compared to their $MUC2^{-/-}$ counterpart. SCFA, short-chain fatty acid

Colon mRNA gene expression - The exploratory PCA plot showed clear separation of the WT vs. MUC2 animals along the first principal component (PC1) axis which accounted for 41.7% of the variation. Further clustering between the SED and VWR groups but not between MSED and MVWR was observed along PC2, which accounted for an additional 17.7% of the variance. The result of the PERMANOVA test confirmed these observations revealing a clear separation amongst groups (F, 10.513; P <0.01). The pairwise comparison test shows a statistically significant separation between all pairs except between MSED and MVWR. The global multi-GLM model showed a significant difference (adjusted P = 0.001) across groups with the univariate tests showing a significant difference in all genes across groups. The results of the pairwise comparison of each cytokine between relevant groups is shown in Table 8. Notably, VWR mice had significantly lower TNF-α, TGF-β, IFN-γ, and RegIII-γ compared to SED mice (Figure 17); no changes were detected between MVWR and MSED animals. $MUC2^{-/-}$ animals had increased concentrations of IL-10, RELM-β, CXCL9, RegIII-γ, and TNF-α compared to their WT counterparts.

Pairs	RELM-β	TGF-β	CLDN10	CXCL9	IFN-γ	RegIII-γ	TNF-α
SED-VWR	**n.s**	↑	**n.s**	**n.s**	↑	↑	↑
MSED-MVWR	**n.s**	**n.s**	**n.s**	**n.s**	**n.s**	**n.s**	**n.s**
SED-MSED	↓	**n.s**	**n.s**	↓	**n.s**	↓	↓
VWR-MVWR	↓	**n.s**	↓	↓	↑	↓	↓

Table 8. Results of group pairwise comparison of colonic mRNA gene expression

N.s, not significant; arrows represent a statistically significant (adjusted P<0.05) different between the corresponding pair. The direction of the arrow refers to the level of the first group relative to the second.

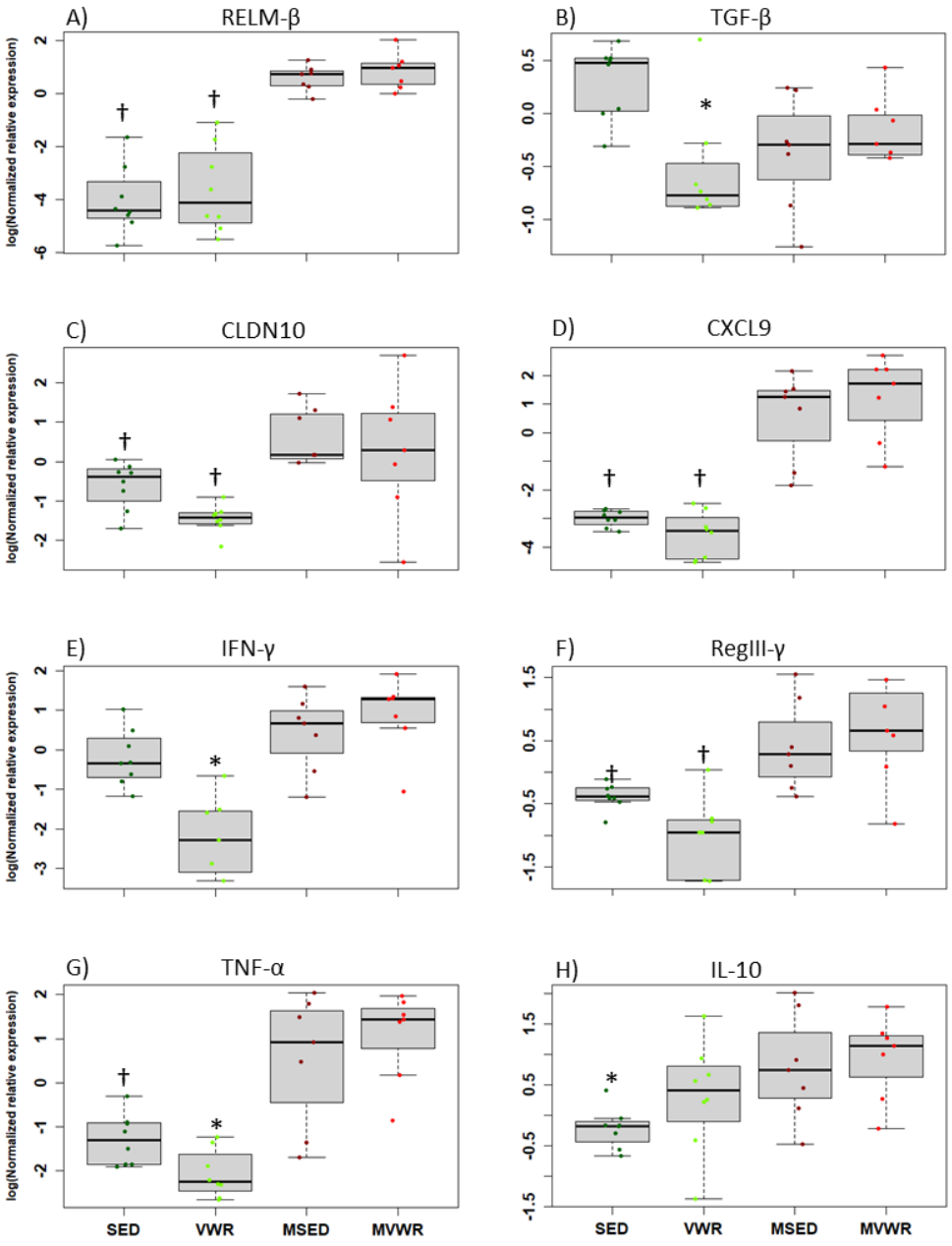

Figure 17. Colonic mRNA gene expression

The relative mRNA gene expression of selected pro- and-anti-inflammatory mediators in colon. * denotes significantly different (adjusted P<0.05) than all other groups. † different compared to their MUC2$^{-/-}$ counterpart.

Bacterial community analysis. Beta diversity: The PERMANOVA test showed a significant difference between MUC2$^{-/-}$ and WT animals across the Bray-Curtis dissimilarity (Pseudo F, 38.8; P <0.001) and weighted-UniFrac (Pseudo F, 105.5; P<0.001) distances, corresponding to clear clustering observed between these groups on the PCoA plots (Figure 18). Importantly however, in both matrices there was a significant (Bray-Curtis Pseudo-F 3.99, p<0.01; weighted-UniFrac Pseudo-F 5.742, p<0.05) distance between SED and VWR animals prior to treatment assignment (Figure 19). This fact strongly suggests the presence of a batch effect in our experiment which is likely explained by the fact that the VWR animals were purchased at different times compared to the other groups and their microbiome sequenced on a separate MiSeq run. As batch-effects are a well-known issue in short-read sequencing experiments (W. W. Bin Goh, Wang, & Wong, 2017), differences across groups are then likely confounded by this. Therefore, in all proceeding analysis examining the role of wheel running, groups are only compared to themselves across time rather than across groups. Pairwise analysis of each group comparing their week 0 to week 6 profiles showed no differences in MUC2$^{-/-}$ animals (Figure 20), however significant differences were observed in WT animals. A significant shift in community structure of VWR animals based on the Bray-Curtis distances was observed by week 6 (Pseudo F, 2.38; P<0.01) (Figure 19). SED animals also showed a significant, albeit to a much lesser degree, shift at week 6 (Pseudo F, 1.88; P<0.01), suggesting a slight change in overall structure of the microbiome as a function of time in WT animals. Differences based on the weighted-UniFrac phylogenetic distances (Figure 19) however showed a significant shift in VWR but not SED animals by week 6 (Pseudo F, 8.87, P<0.01). Changes in community structures were also analyzed using the more sensitive GHT test which accounts for shifts in each community's profile using a paired design, rather than comparing the group centroids. Results from the GHT agreed with the PERMANOVA results showing a significant shift in microbiome of SED and VWR (P<0.05 and P<0.01, respectively) but not in MSED and MVWR groups.

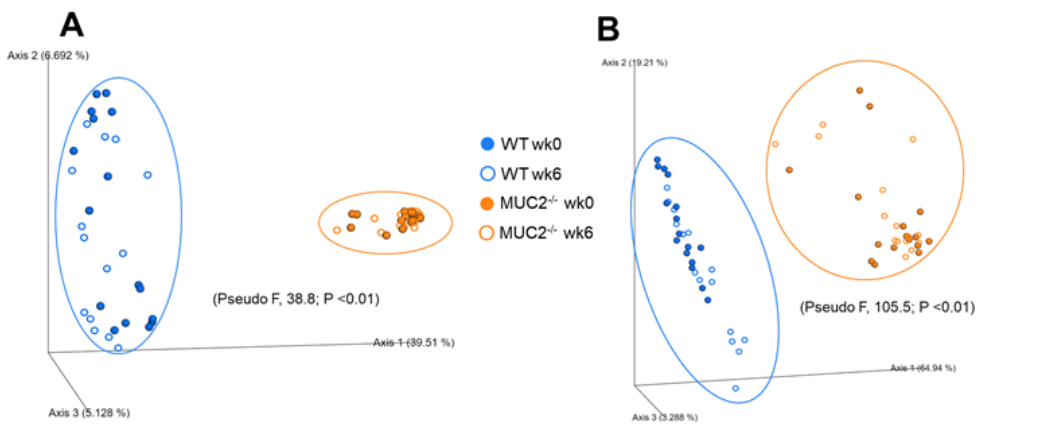

Figure 18. Fecal bacterial profiles colored by phenotypes

PCoA plots of A) Bray-Curtis dissimilarity and B) weighted UniFrac distances showing a significant separation of communities by phenotype across time. No significant separation is present as a function of time.

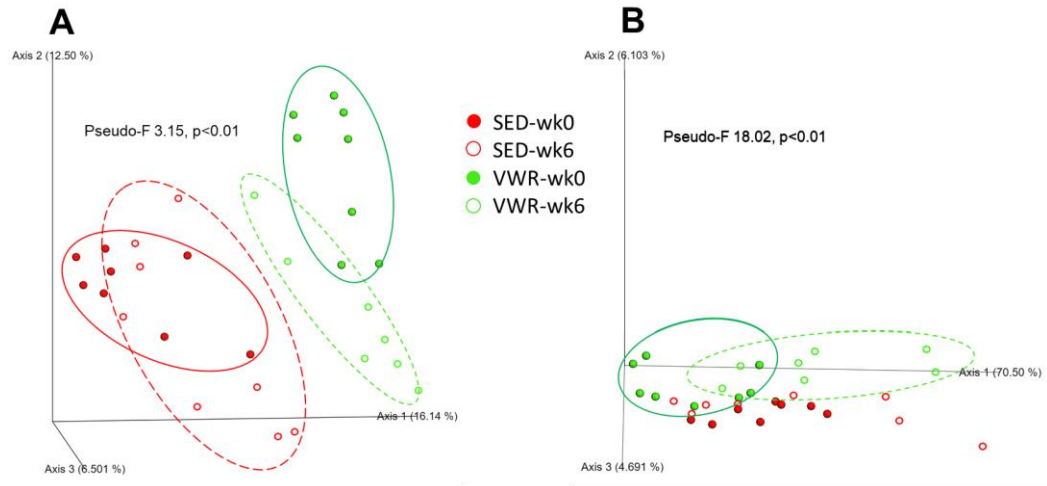

Figure 19. WT mice bacterial profiles colored by activity

PCoA plot of A) Bray Curtis Dissimilarity and B) weighted-UniFrac phylogenetic distances in WT animals. VWR animals show a significant shift across time in both distance matrices while SED animals show a smaller but significant shift in the Bray-Curtis dissimilarity plot only. Pseudo-F statistic and Permutation P values correspond to the global model. See text for details regarding pairwise differences.

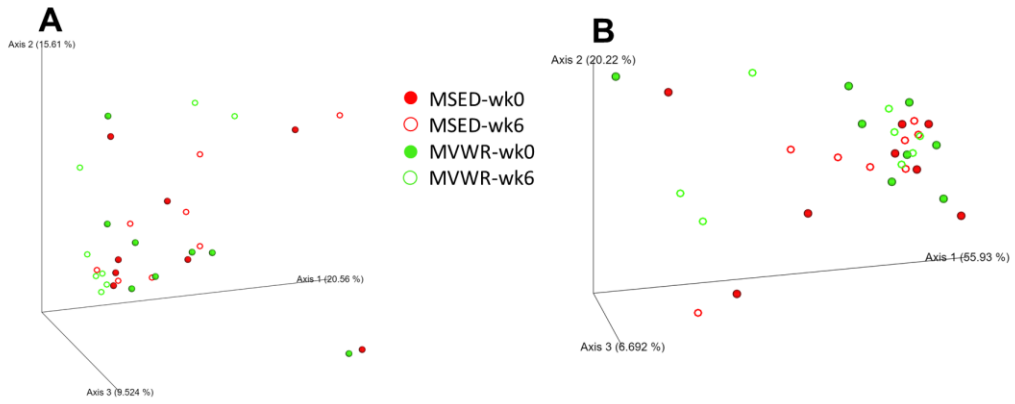

Figure 20. MUC2$^{-/-}$ mice bacterial profiles colored by activity

A plot of A) Bray-Curtis dissimilarity distances, B) weighted-UniFrac distances in MUC2$^{-/-}$ animals. No significant separation was identified as a function of treatment or time.

Univariate differential abundance testing comparing week 0 and week 6 from each group showed changes in both VWR and MVWR but not in SED and MSED groups. In MVWR mice, the relative abundance of only one taxa, an unclassified genus belonging to the Bacteroidales order, was lower by 18.7 folds from ~1.2 to 0.07 % total relative abundance. In the VWR group, the only detectable change by week 6 was the lower relative abundance of two taxa, both belonging to the family Ruminococcaceae. These were the species *Anaerotruncus colihominis* which was reduced from 4.4 to 2.02% relative abundance, and an unclassified genus which was modestly lower from 5.5 to 4.3% by week 6.

Alpha diversity - WT mice had significantly higher ($P < 0.001$) overall diversity than MUC2$^{-/-}$ animals in all examined diversity indexes: observed species (H test statistic: 12.2), Shannon (H: 27.8), and Faith's PD (H: 32.7) (Figure 21D). No significant change in any diversity measures was detected in any group after 6 weeks (Figure 21A-C). The average change of observed species over time in VWR group however was significantly higher than that of SED (difference ~262, $P < 0.05$).

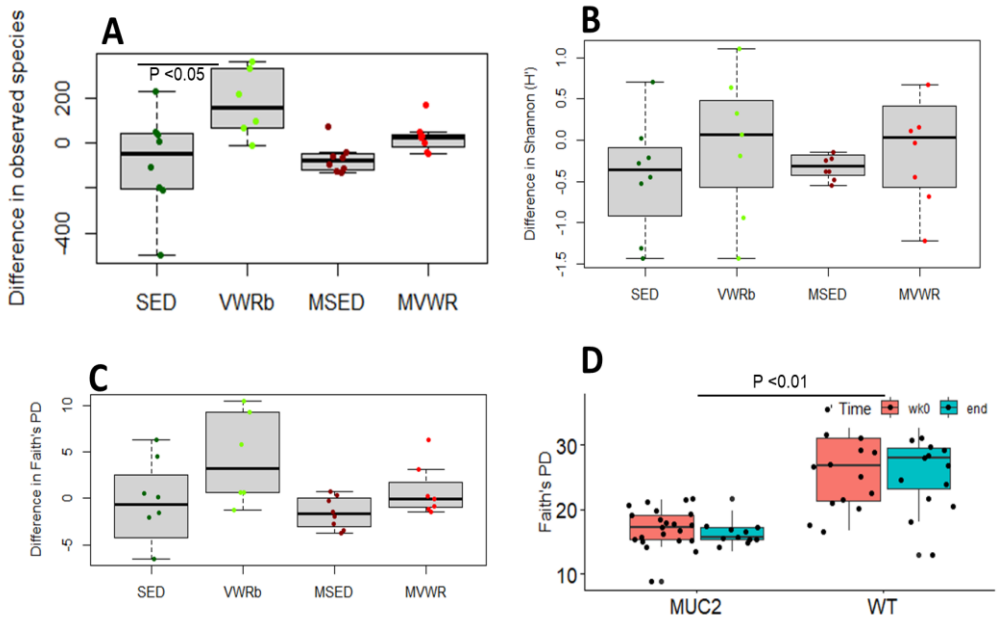

Figure 21. Change is alpha diversity indexes

The plots show the differences in diversity scores between week 6 and week 0 in A) Observed species, B) Shannon (H'), and Faith's PD. Group differences were compared to the null hypothesis of zero change as well as to other groups. D) Faith's PD differences in MUC2$^{-/-}$ and WT animals regardless of wheel running.

Predicted phenotypic traits - BugBase's prediction of each community's phenotypic traits suggest major differences between WT and MUC2$^{-/-}$ animals (Table 9). Bacterial communities in MUC2$^{-/-}$ mice were composed of significantly higher abundances of Gram negative, aerobic, and facultative anaerobic bacteria with a higher potential for biofilm formation. Their communities also housed less bacteria with mobile elements and had an overall lowered tolerance for oxidative stress. At week 6, only VWR mice showed significant changes in their bacterial phenotypes compared to week 0. Their communities showed lower average abundances of mobile-containing (~12 %, P<0.05) and Gram-positive (~14 %, P<0.001) bacteria (Figure 22).

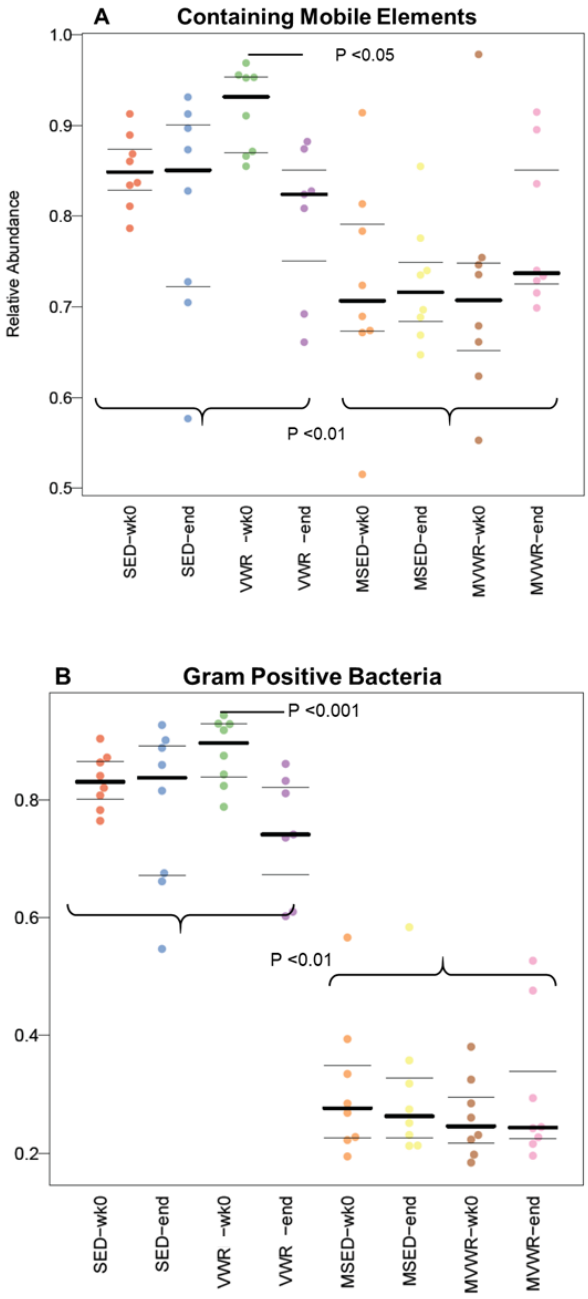

Figure 22. BugBase's predicted phenotypic traits

BugBase was used to predict the relative abundance of bacteria corresponding to A) those containing a mobile element, and B) Gram positive bacteria. A statistically significant difference (adjusted P <0.05) was detected across phenotypes in both traits. VWR group was statistically lower at week 6 compared to week 0 in both traits.

Pairs	Aerobic	Anaerobic	Contain Mobile Elements	Facultative Anaerobes	Biofilm Formation	Gram Negative	Gram Positive	Potentially Pathogenic	Oxidative Stress Tolerant
SED (wk0-6)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
VWR (wk0-6)	n.s	n.s	Yes ↓	n.s	n.s	Yes ↑	Yes ↓	n.s	n.s
MSED (wk0-6)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
MVWR (wk0-6)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
WT vs MUC2$^{-/-}$	Yes ↓	Yes ↑	Yes ↑	Yes ↓	Yes ↓	Yes ↓	Yes ↑	n.s	Yes ↑

Table 9. BugBase's phenotypic trait predictions

n.s = no significant difference, yes=a significant (adjusted P<0.05) difference was detected between the described pairs. The arrow indicates the direction of the change.

3.4 Discussion

MUC2$^{-/-}$ vastly differ than WT in their colonic cytokine, SCFA, and microbial profiles - MUC2$^{-/-}$ mice displayed clinical and histological symptoms of moderate colitis corresponding to the expected severity of this model at 11 weeks of age in our facilities (personal observations). The colonic gene expression of inflammatory cytokine TNF-α, and the mucosal defense factor RELM-β, as well as antimicrobial peptide RegIII-γ were upregulated in MUC2$^{-/-}$ animals, as observed previously (Morampudi *et al*., 2016). Notable, the anti-inflammatory cytokine IL-10 was overexpressed in MUC2$^{-/-}$ compared to WT. While in a healthy state, the expression of IL-10 may be associated with increased tolerance to inflammatory events, in MUC2$^{-/-}$ animals, this upregulation is essential in the host's efforts at suppressing the excessive inflammation resulting from continuous exposure to bacterial ligands. Indeed, MUC2$^{-/-}$ + IL-10$^{-/-}$ double knock-out mice

show highly exacerbated colitic symptoms (van der Sluis et al., 2008) compared to deletion of either genes separately. The increase in IL-10 has also been previously observed in chemical models of colitis (Marc D Cook et al., 2013b; Szalai et al., 2014). We further detected significant overexpression of CXCL9 in MUC2$^{-/-}$ animals. CXCL9 is a chemokine involved in regulating leukocyte trafficking likely in response to exposure of bacterial ligands to host cells. CXCL9 overexpression has also been reported in IBD patients (Singh, Venkataraman, Singh, & Lillard Jr., 2007). Overall, the cytokine profile of MUC2$^{-/-}$ animals reflect those expected in human IBD.

MUC2$^{-/-}$ mice born without a mucosal layer house drastically less diverse and different bacterial community than WT mice, as evident by the clear clustering of this group from WT in our PCoA plots. The dominating taxa in WT mice were generally of the Bacteroides, Clostridiales, and Lachnospiraceae, while MUC2$^{-/-}$ animals were dominated by members of the S24-7 family and *Akkermansia muciniphilia* species of the Verrucomicrobia phyla. *A.muciniphilia* is perhaps the most surprising finding in this group as this species is known -and named- for its ability to degrade mucin, and is vastly considered as a beneficial bacteria in a variety of chronic diseases including IBD (Lopez-Siles *et al*., 2018; Naito, Uchiyama, & Takagi, 2018; Reunanen *et al*., 2015). The implications of this finding are beyond the scope of the current study; however, it does warrant the reassessment of the characterization of *A.muciniphilia* as a mucin loving species to one that thrives in the absence of mucin. The bacterial phenotypic traits of MUC2$^{-/-}$ animals were predicted to be higher in abundances of Gram negative, aerobic, and biofilm forming groups compared to WT mice. Lastly, the cecal SCFA of MUC2$^{-/-}$ mice were composed of significantly less butyrate and higher propionate concentrations compared to SED animals. The increased propionate levels in these animals is likely associated with the high abundances of *A.muciniphilia*, a prominent propionate producer (Rajilić-Stojanović, Shanahan, Guarner, & de Vos, 2013; Reichardt *et al*., 2014). Overall, we found the MUC2$^{-/-}$ model of colitis to capture many components of human IBD, especially those with impaired mucosal integrity.

Wheel running in MUC2$^{-/-}$ mice does not reduce severity of chronic colitis

Contrary to our primary hypothesis, we found that 6 weeks of wheel running in MUC2$^{-/-}$ mice did not significantly affect severity of clinical symptoms, histopathological scores, colonic expression of inflammatory cytokines, or abundances of cecal SCFAs, and only influenced the abundance of a single low abundant taxon in these animals. These findings contrast others that show protective effects of VWR or FTR in chemically-induced models of colitis (Bilski *et al*., 2015; Marc D Cook *et al*., 2013b; Saxena *et al*., 2012; Szalai *et al*., 2014). The fundamental difference between those studies and ours is in the model of colitis used. Previously, VWR was initiated in healthy animals prior to disease induction with chemical toxins, whereas in our study, wheel running is imposed over an existing disease state as a therapeutic intervention. This would suggest that PA prior to disease onset primes various components of intestinal health, enhancing its tolerance to injury. The effects of PA following disease-onset on the other hand, are either abolished or are overmined by stronger disease signaling. This is supported by our findings that wheel running in WT but not MUC2$^{-/-}$ animals lead to significantly lower levels of pro-inflammatory colonic cytokines, increased anti-inflammatory IL-10, and increased levels of beneficial SCFAs.

VWR significantly attenuates pro-inflammatory, and upregulates anti-inflammatory cytokines in WT mice

VWR mice, in the presence of the mucin2 gene, showed downregulation of inflammatory cytokines TNF-α, IFN-γ, and TGF-β compared to SED animals, all of which have been implicated in IBD (Strober & Fuss, 2011). TNF-α is perhaps the most studied cytokine in relation to IBD as it plays a crucial role in innate and adaptive immunity and is directly involved in apoptotic processes in the intestines (Popa, Netea, van Riel, van der Meer, & Stalenhoef, 2007). It is found in significantly higher abundances in IBD patients (Komatsu *et al*., 2001) as well as murine colitis (Mueller, 2002), making its regulation an obvious target for disease

management. In fact, TNF-α inhibition using monoclonal antibodies is the most common target of biological therapies for moderate to severe IBD. The role of IFN-γ in colitis pathogenesis is less consistent across the literature, however its overproduction has been shown in CD (Fais *et al*., 1994; Sasaki, Hiwatashi, Yamazaki, Noguchi, & Toyota, 1992) and UC patients (Verma, Verma, & Paul, n.d.). In DSS-induced colitis models, neutralization antibodies against IFN-γ significantly reduced disease severity (Obermeier *et al*., 1999), while IFN-$\gamma^{-/-}$ mice were completely protected from disease symptoms (Ito *et al*., 2006). Anti-IFN-γ antibody treatments in human IBD are less effective however, with their efficacy dependent on baseline C-reactive protein levels (Abraham, Dulai, Vermeire, & Sandborn, 2017), highlighting the need for treatment personalization. TGF-β is pleiotropic cytokine that is ubiquitously produced by many cells and is involved in various immune functions including both anti- and pro-inflammatory actions. These include suppression of immune responses through recruitment of Tregs which in turn produce IL-10, but TGF-β can also elicit potent Th17 responses to combat extracellular bacteria (Ihara, Hirata, & Koike, 2017). TGF-β is found in higher concentrations in intestines of IBD patients (Babyatsky, Rossiter, & Podolsky, 1996; McCabe, Secrist, Botney, Egan, & Peters, 1993), due to increased exposure of microbial ligands to host epithelial cells. Inversely, the attenuated levels of this cytokine in our VWR animals then may reflect a decrease in bacterial antigen exposure to the IEC suggesting reduced levels of host-microbe interactions in the mucosa. Alternatively, reduced TGF-β could also indicate reduced Treg activity in VWR mice, however, the increase in Treg derived IL-10 in these animals does not support this notion. IL-10 is an anti-inflammatory cytokine ubiquitously secreted by Tregs and is the primary driver of immunosuppressant actions in the intestines. Polymorphism in IL-10 promoter have been linked to IBD, making IL-10 supplementation a potential target for IBD therapy, however, clinical studies of IL-10 therapy to date have not been significantly effective (Asadullah, Sterry, & Volk, 2003). The significant increase in IL-10 in VWR mice suggests higher Treg activity which is associated with reduced inflammation. This is in agreement with others who showed a

significant increase in murine intestinal IL-10 following treadmill running or swimming (Hoffman-Goetz, Spagnuolo, & Guan, 2008; Viloria et al., 2011). However, it is unclear whether this reflects a beneficial increase in anti-inflammatory events, or simply an adaptive response to changes in the microbial composition. Gram-negative bacteria preferentially stimulate IL-10 production and are associated with higher virulence due to increases in abundance of LPS bound to Gram-negative bacterial walls (Hessle, Andersson, & Wold, 2000). The higher expression of IL-10 in VWR animals then is likely correlated with increased abundance of Gram-negative bacteria observed in these mice. Further investigations are needed to determine the consequence of these changes.

Taken together, the reduction of these pro-inflammatory cytokines and increase in anti-inflammatory IL-10 in VWR animals suggests a primed anti-inflammatory state in healthy but not diseased intestines marking them as important targets for prevention and remission maintenance therapy.

VWR significantly augments SCFAs content in WT but not MUC2$^{-/-}$ mice

SCFAs are metabolic by-products of bacterial fermentation of dietary fibers in the colon and are involved in various physiological processes of the host. Aberrant intestinal SCFAs content has been implicated in various diseases such as irritable bowel syndrome, cardiovascular disease, certain cancer types, and IBD (Floch & Hong-Curtiss, 2002; Tedelind, Westberg, Kjerrulf, & Vidal, 2007; Venter, Vorster, & Cummings, 1990). The most abundant of these, acetate, propionate, and butyrate (>95%), are markedly decreased in IBD patients (Huda-Faujan *et al*., 2010), while their exogenous delivery can reduce inflammation via inhibition of TNF-α release from neutrophils (Segain *et al*., 2000; Tedelind *et al*., 2007). Overall, increases in these SCFAs, especially butyrate, appear to positively influence IBD (Plöger *et al*., 2012). We found an overall higher abundance of total cecal SCFAs, acetate, butyrate, and propionate in response to wheel running in WT but not MUC2$^{-/-}$ animals. This is in accordance

with other reports showing higher butyrate concentrations in wheel running rats (Matsumoto *et al*., 2008) and exercised humans (Jacob M. Allen *et al*., 2018), as well our findings from Chapter 2 showing a positive association between higher butyrate levels and VO_2peak. The increase in these SCFAs may simply reflect higher energy demands of colonocytes which utilize SCFAs as their primary energy substrate. Interestingly, when we analyzed SCFAs content in relative terms, we saw a significant increase in relative abundance of butyrate, but not acetate, or propionate. This suggests a preference in VWR animals for production of butyrate and its accompanying anti-inflammatory properties. These findings further support the patterns of anti-inflammatory priming in these animal, suggesting an overall healthier intestinal environment in VWR mice. The mechanisms behind PA-induced changes in SCFAs are not known, however the microbiota is likely to play an important part. SCFAs affect microbiota dynamics as they are directly involved in chemical balance and pH regulation of the intestines (van Hoek & Merks, 2012). Bacterial composition in turn can also affect SCFAs production, establishing a bidirectional affiliation.

VWR has limited but significant effects on the intestinal bacteria composition

Comparisons of the overall bacterial community in $MUC2^{-/-}$ animals showed no differences as a function of time or wheel running. In WT animals however, a significant shift in the weighted UniFrac distances in VWR but not in SED animals was observed. The Bray-Curtis distances at week 0 and week 6 were also significantly different in VWR animals, however, SED also showed a significant shift, albeit to a lesser extent, indicating time as an influential factor in community composition when using this metric. Univariate analyses of individual taxa however, were not able to detect changes in any features in the SED animals, the observed community shift was then, presumably, not mediated through any one specific bacteria. The VWR group on the other hand had significantly reduced abundances of *Anaerotruncus colihominis* species and an unknown genus, also from the Ruminococcaceae family at week 6. Interestingly, and

perhaps counterintuitively, members of the Gram-positive Ruminococcaceae family are known butyrate producers (Vital, Howe, & Tiedje, 2014) which would advocate a lower capacity for butyrate production in VWR animals. This suggests then, that the elevated SCFAs levels observed in VWR mice is likely derived from subtle changes across multiple other taxa in which our statistical tests were unable to detect. These patterns of change do not align with previous reports of impacted taxa in response to PA. For example, Allen *et al*. (2017) showed increases in Ruminococcus genera among various others in their wheel running mice, while Lamoureux *et al*. (2017) showed a decrease in Ruminococceae family in forced-treadmill running but not VWR mice. These differences are likely a result of differences in animal vendors, facilities, DNA extraction methods and sequencing, bioinformatics analysis, as well as statistical testing methods. This topic is discussed in more detail in Chapter 5. Alpha diversity was not significantly different across time in any groups, however the rate in change of observed species in VWR animals was significantly higher than those of SED. This suggests the intestinal milieu of VWR mice tolerate colonization of a more diverse consortium of bacteria; the mechanism behind this is not known.

Overall, wheel running had limited influence on the bacterial population, and the current experiment likely lacked sufficient power to detect subtle changes of all influenced taxa. Analyzing the bacterial consortia based on their phenotypic traits however, revealed additional information regarding the effect of wheel running on the overall community. Following wheel running, WT mice had significant reduction (-14%) in total abundance of Gram-positive bacteria. This is supported by the observed decreases in members of the Gram-positive Ruminococceae in these mice, as well as the attenuated expression of RegIII-γ, an antimicrobial peptide that specifically targets the surface peptidoglycan layer of Gram-positive bacteria. The implications of this phenotypic shift in microbiota of healthy individuals is not known but may provide a clue for understanding the adaptations of the intestinal environment to the physiological stresses of PA. Furthermore, mirroring the shift in Gram-positive phenotype was the lowered relative

abundances of bacteria containing mobile elements. These refer to microevolutionary processes such as transposons i.e. segments of DNA with the ability to move locations within the genome, and bacterial plasmids which are involved in horizontal gene transfer. These events are typically associated with sharing of virulence factor across bacterial cells and increased resistance to antibiotics. The higher abundances of mobile elements in these mice is likely not indicative of antibiotic-resistance but rather associated with higher abundances of Gram-negative bacteria representing more mobile-elements. The results of these predictions should be interpreted with caution however, as these mobile elements can rapidly become population specific within an individual thus precluding inference across similar experimental groups (Brito et al., 2016).

Summary

In contrast to our hypothesis we found that 6 weeks of wheel running did not ameliorate any symptoms of colitis in $MUC2^{-/-}$ animals, nor did it influence any components of the intestinal environment in these animals. However, we found that wheel running in healthy WT animals imposed various physiological effects on the gut, including downregulation of pro-inflammatory and upregulation of anti-inflammatory cytokine gene expression, and increased concentration of total SCFAs including butyrate, acetate, and propionate. In addition, wheel running lead to a shift in bacterial community structure corresponding to both a higher overall diversity and higher abundances of Gram-negative bacteria. As these physiological changes have been associated with protection against chronic inflammatory diseases such as IBD, we conclude that PA prior to disease onset can prime the intestines, enhancing their tolerance to injury. These benefits however are lost when PA is imposed on an existing disease state. Overall, the findings here suggest that PA in humans may be an important preventative therapy against intestinal diseases such as IBD.

Chapter 4: Low volume wheel running and associated intestinal adaptations[3]

4.1 Background

With rapid improvements of high-throughput sequencing technologies and the concurrent cost reduction of these methods, the study of human microbiota in health and diseases has moved away from culture-dependent methods allowing for rapid and novel discoveries. We have reached an inflection point of human microbiome research where we transition from exploration and association to application and causation. For example, an individual's unique intestinal bacterial population has been established as an important predictor of the host's response to various pharmaceutical interventions (Bisanz, Spanogiannopoulos, Pieper, Bustion, & Turnbaugh, 2018; Petrosino, 2018). While this can lead to discovery of disease subsets and shift the paradigm towards personalized medicine, there is further potential in manipulating the host microbiome towards either healthier states as a prevention method against dysbiosis-associated diseases, or towards a more responsive state to augment drug intervention outcomes. To this end, PA has been proposed as a potential modulator of the human microbiota (Campbell & Wisniewski, 2017; Codella, Luzi, & Terruzzi, 2018). In Chapter 3, we showed that PA in healthy mice led to significant changes in the intestinal environment, including reduction of pro-inflammatory and increase in anti-inflammatory cytokines, increased production of SCFAs, as well as a limited but significant shift in the bacterial community composition. The PA-derived changes in gut microbiota have been directly linked with protection against chemically-induced models of murine colitis (Barlow, Yu, & Mathur, 2015). In their study, Allen *et al*. transplanted microbiome of voluntary-wheel running (VWR) mice into germ-free recipients and induced dextran-sodium sulphate (DSS) colitis following a 4-week colonization

[3] A portion of the data from this experiment has been published. Spielman LJ, Estaki M, Ghosh S, Gibson DL, Klegeris A. The effects of voluntary wheel running on neuroinflammatory status: Role of monocyte chemoattractant protein-1. Mol Cell Neurosci. 79:93-102

period. They showed significant protection against colitis symptoms in recipient mice only when their donor was physically active but not if they were sedentary. This study elegantly showed the benefits of VWR against murine colitis to be both mediated through the microbiome and transferable across hosts. Several other studies have also shown PA to either correlate with changes in the microbiota (Bressa *et al*., 2017; Clarke *et al*., 2014; Estaki *et al*., 2016; Petersen *et al*., 2017; Yuan *et al*., 2018) or directly alter them (Jacob M. Allen *et al*., 2018; Jacob M Allen *et al*., 2015; Evans *et al*., 2014; Mika *et al*., 2015; Petriz *et al*., 2014).

In rodent studies using voluntary PA such as that in Chapter 3, the volume of wheel running drastically differs across studies, ranging from ~ 2.5-10 km/day. Mice in our previous experiment yielded significant and beneficial physiological changes in their gut environment. Unexpectedly however, these animals ran considerably less than expected based on previous reports. The healthy WT cohort ran an average of ~1.3 km/day, almost half the distance ran by the next lowest report amongst these studies (Lamoureux *et al*., 2017), which are themselves considered below average in these strains (J. Goh & Ladiges, 2015). VWR models are well known for their individual variability, however the minimum daily running distances typically exceeds 5 km, far above the distances we observed. This raises an important question as whether the microbial changes associated with PA are volume-dependent or are rooted in physiological adaptations independent of work load. For example, does access to a free-wheel alone initiate adaptation responses associated with increased work and energy expenditure, or does the volume of work itself invoke such changes? To better understand the role of PA load in relation to changes in the intestinal microbiota and health, we chose to manipulate total running in our VWR models. In this study we chose to focus on a model of low-VWR as the effects of higher volumes have already been reported, and, increasing movement loads in VWR models is not possible. We hypothesized that low levels of wheel running are insufficient to instigate microbial or immunological adaptations in the gut.

4.2 Methods

Animals - All procedures involving the care and handling of the mice were approved by the UBC Committee on Animal Care, under the guidelines of the Canadian Council on the Use of Laboratory Animals. Female C57BL/5 mice were purchased from Charles River (Vancouver, Canada) and kept under specific pathogen-free conditions until ~ 6 weeks of age. All animals were housed in a temperature-controlled room (22 $\pm$ 2°C) on a 12h light/dark cycle with access to tap water and irradiated food *ad libitum.* Wheels used in this experiment were same as those reported in Chapter 3, however to produce a model of low physical activity, the wheel's axels were tightened to limit the ease of their movement. This method allows the mice to participate in voluntary running however at a significantly reduced rate. Using a random number generator, animals were then assigned to individual cages with either free access to a voluntary wheel (VWR) or without wheel access (SED) for ~ 6 weeks, n=8/group. Animals were acclimated to the wheels 3 days prior to start of the wheel running measurements. Body weights and food and water intake were measured weekly for the duration of the experiment. Food weight measurements consisted of a week's remaining pellets on the cage lid, plus any stashed away pellets in the cage bottoms.

Tissue Collection - Mice were anaesthetized with isoflurane and blood was collected via intracardial puncture followed by immediate termination by cervical dislocation. Whole blood was kept on ice for ~30 minutes then centrifuged (1800 g) for 10 minutes at 4°C to separate serum which was aliquoted and kept in -80°C until further analysis. Colon and ileum tissues were collected as follows: starting from distal end, three ~1.5 cm sections from each tissue were collected with the most distal section being fixed in formalin for histological staining, the middle section was stored in RNAlater (Thermo Fisher Scientific) for RNA extraction, and the most proximal section was snap-frozen in liquid nitrogen and stored -80°C for DNA extraction.

Histology - Colon cross-sections were fixed in formalin for 24 hrs, washed with phosphate buffered saline (PBS, pH 7.4), transferred to 70% ethanol and sent for paraffin-embedded sectioning and Hematoxylin and eosin (H&E) staining at Wax-it Histology Services (Vancouver, Canada). Tissue slides were coded throughout the microscopy analyses and investigators blinded to the groupings. H&E stained sections were viewed under 200x magnification on an Olympus IX81 and the full image stitched together using Metamorph® software. Stitched images were imported into ImageJ (Schneider *et al*., 2012) (v. 1.51r) for further analyses. To compare the overall structural size and health of colon sections between groups and we compared total relative size of the muscularis externa, total number of goblet cells in a section, and average crypt lengths.

RT-qPCR - The mRNA gene expression for tumor-necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), resistin-like molecule beta (Relm-β), regenerating islet-derived protein 3 (RegIII-γ), transforming growth factor beta (TGF-β), chemokine C-X-C motif ligand 9 (Cxcl9), and claudin 10 (Cldn10) were measured in colon tissues. Total RNA was purified from tissues using Qiagen RNEasy kits (Qiagen) according to the manufacturer's instructions with an additional initial bead beating step (3x30 seconds, 30 Hz) on a Retsch MixerMill MM 400 homogenizer. Next, cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) in 10 µl reactions. The RNA and cDNA products' purity and quantity were assessed by a NanoDrop spectrophotometer (Thermo Scientific). The cDNA products were normalized to ~ 40 ng/µl with DNAse free sterile water prior to qPCR reactions. A total of 10 µl RT-qPCR reactions consisted of: 0.2 µl of each forward and reverse primers (10mM), 5 µl of Sso Fast Eva Green Supermix (Bio-Rad), 3.6 µl DNAse free water, and 1 µl of cDNA template. Reactions were run in triplicates using the Bio-Rad CFX96 Touch thermocycler and analyzed using Bio-Rad CFX Maestro software 1.1 (v4.1). The median quantitation cycle (Cq) value from each sample was used to calculate the $2^{-\Delta\Delta Ct}$ based on the reference gene TATA box binding protein (Tbp). A list of all the

primer sets, their melting temperature, efficiencies, and detailed thermocycler protocol used in this study are described in Appendix B.

DNA extraction and 16S rRNA amplicon preparation - DNA was extracted from colon and ileum tissues using QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions following 3 x 30 s of homogenization using metal beads on a Retsch MixerMill MM 400 homogenizer. Amplicon libraries were prepared according to the Illumina16S Metagenomic Sequencing Library Preparation manual. In brief, the V3-V4 hypervariable region of the 16S bacterial rRNA gene was amplified using recommended degenerate primer sets 341F: CCTACGGGNGGCWGCAG, and 805R GACTACHVGGGTATCTAATCC, which create an amplicon of ~460 bp. Amplicons were purified using AMPure XP beads and adapters and dual-index barcodes (Nextera XT) were attached to the amplicons to facilitate multiplex sequencing. Following another clean-up step, libraries were quality controlled on an Experion automated electrophoresis system (Bio-Rad), and sent to The Applied Genomic Core (TAGC) facility at the University of Alberta (Edmonton, Canada) where they were normalized using fluorometric method (Qubit, Thermo Fisher Scientific) and sequenced using the Illumina MiSeq platform with a V3 reagent kits allowing for 2 x 300 bp cycles.

Bioinformatics - All bioinformatics processes were performed within the QIIME2 platform (Caporaso *et al*., 2010) using the various build-in wrappers described below. All used software packages, versions, and parameters are available under the 'provenance' tabs of the QIIME2 artifacts will be made available online. This file can be viewed locally on a browser by drag and dropping the file onto https://view.qiime2.org/. Poor quality scores of sequences on the 3' ends precluded sufficient overlap for appropriate merging of paired-end sequences. We therefore proceeded our analyses with the forward reads only, trimmed to an equal length of 200 bp. Sequencing were denoised using Deblur (Amir *et al*., 2017) with default settings. The output of this process is a feature table of amplicon sequence variants (ASV) that is a higher resolution analogue of traditional OTU tables. A Naïve Bayes classifier that was trained on the specific

region targeted by our primer sets using the most recent available version of the Greengenes (13_8) was used to assign taxonomy at the genus level. For microbial analyses encompassing phylogenetic information, MAFFT-aligned (Katoh & Standley, 2013) sequences were used to produce a phylogeny tree using FastTree2 (Price, Dehal, & Arkin, 2010) with default settings. To predict the functional repertoire and phenotype of the microbiome, we used BugBase (Ward *et al*., 2017) which utilizes PICRUSt's (Langille *et al*., 2013) extended ancestral-state reconstruction algorithm for metagenome composition prediction. As these tools require features to be classified against Greengenes taxonomy, we used QIIME2's VSEARCH (Rognes *et al*., 2016) plugin to pick closed-reference OTUs from our denoised feature table at 97% similarity threshold against the 97% Greengenes OTUS database.

Statistical Analysis - All statistical analyses were performed using R version 3.5.1 unless stated otherwise. All scripts available upon request.

Body weights and food/water intake - To account for natural differences in starting body weights, total weight gained relative to starting body weights was calculated at each week. Body weights, food and water intake comparisons across the 6 weeks were assessed using repeated measures using linear mixed-effects regressions (LMER) using the *lme4* package with individual animals assigned as the random effect. Significance was set at $P<0.05$.

Morphological parameters - Morphological parameters were compared across groups using a Welch's t-test or a Wilcoxon rank sum test if the data did not meet assumptions of normality.

Colon mRNA gene expression analysis - Given that we showed an attenuation of VWR on the colonic cytokines IL-10, TNF-α, TGF-β, IFN-γ, and RegIII-γ, from our previous study we analyzed these *a priori* cytokines separately without power adjustments for multiple testing to maximize discovery power. We used a 1-way analysis of variance (ANOVA) or a non-parametric Kruskal-Wallis test when data did not meet assumptions of the parametric tests. The remaining colonic mRNA genes CXCL9, CLDN10, and RELM-β were analyzed together using a multivariable generalized linear model (GLM) test carried out in the *mvabund* package (Wang et

al., 2012). This fits separate GLMs to each cytokine while accounting for the inter-correlation amongst them and adjusting for multiple testing. The negative binomial distribution assumption was selected for the model and the mean-variance plot was used to visually assess the model fit.

Microbial Analysis - Community structural patterns of colon and ileum bacteria across samples (β diversity) were explored in QIIME2 by calculating the Bray-Curtis and weighted-UniFrac distances across samples and plotting them onto a PCoA space using Emperor interactive graphic tool (Vázquez-Baeza *et al*., 2017). We first plotted both colon and ileum samples onto the same PCoA space, to look for obvious community structural patterns. Colon and ileum samples, as expected showed clear separation. However, 6 ileum samples (4 from VWR, 2 from SED) were dropped due to insufficient sequence coverages (<1300). As a result, we separated the colon samples and all proceeding analyses were carried on these samples alone. New PCoA plots were constructed using rarefied (4,577) distance matrices and group differences were tested using a PERMANOVA (Anderson, 2001) test. The overall within sample diversity (α diversity) was calculated based on the species richness, Shannon (H') index, and Faith's phylogenetic diversity (PD) indexes, which capture the overall richness, evenness, and phylogenetic diversity of the communities, respectively. Group differences were tested as before.

Differential abundance testing between individual genera was performed using the Analysis of Composition (ANCOM) wrapped in q2-plugin (Mandal *et al*., 2015). This approach utilizes centered log-ratio transformation to account for the compositional nature of these types of datasets. To reduce noise in the test, low abundant taxa were filtered based on the requirement that each taxon must be observed at least 50 times and be present in at least 1/3 of all samples. BugBase was used to determine high-level phenotypes of bacterial communities based on the following default traits: Gram negative vs. Gram positive, biofilm forming, mobile element containing, oxidative stress tolerance, pathogenic potential, and oxygen utilizing. Group

differences in relative abundances of these elements were tested in each group using a Kruskal-Wallis test.

4.3 Results

Wheel running, body weights, and food/water intake - VWR mice ran a mean (SD) total of 5.36 (4.6) km across the 6 weeks corresponding to ~130 (110) meters per day, showing that we successfully limited the movement of these mice to about 1/10th the distance produced in the previous experiment. The average daily food and water intake were not statistically different between groups (Figure 23B). The relative total weight gained throughout the experiment however, was significantly higher in VWR animals ($P<0.05$, F-value=4.8). By the end of the experiment, SED animals had gained on average (± SE) 18.24 ± 1.6 % of their starting body weight, while VWR mice gained 24.09 ± 1.7 % (Figure 23A).

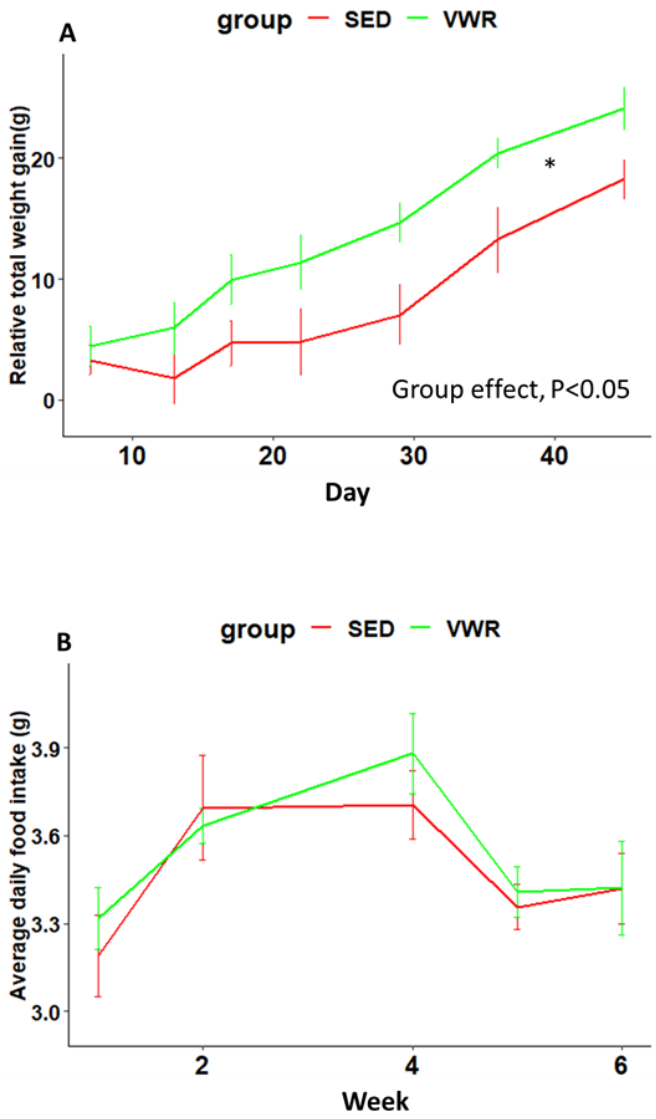

Figure 23. Weight gain and food intake

Effects of VWR on A) relative body weight gain and B) average daily food intake. Weight gain is calculated as the total weekly weight gained, as percentage of each animal's own starting body weight. There was a significant group ($P<0.05$, F-value=4.8), time ($P<0.001$, F-value=291.2), and group x time interaction ($P<0.05$, F=5.4) effect. VWR mice gained significantly more weight throughout the experiment. Average daily food-intake is calculated based on weekly food intake measurements divided by number of days in that period. Values are shown as means ± SE

Colon morphology - Morphological assessments of colons across groups showed no significant differences between total number of goblet cells counted, total muscularis externa area, or average crypt lengths (Fig 24).

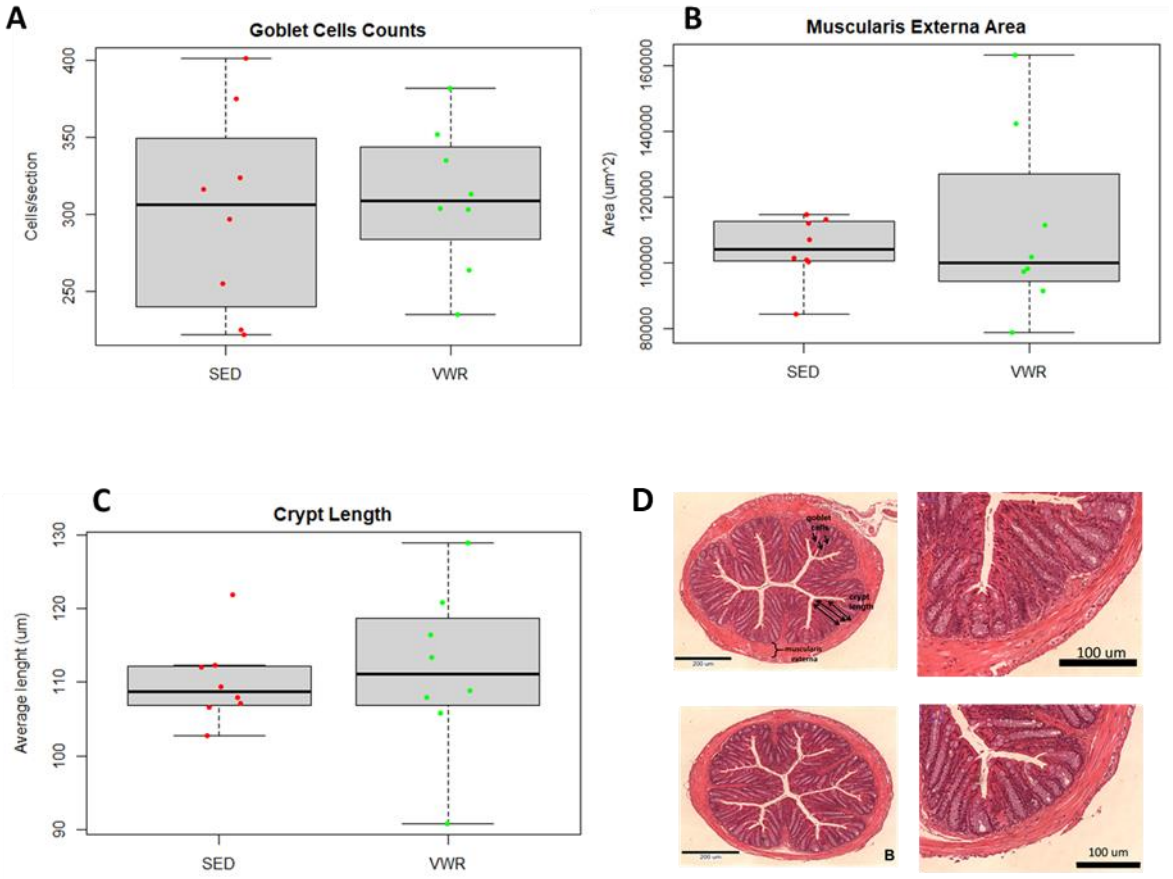

Figure 24. Morphological assessments of colons

Measurements of morphological parameters in the colon were measured using microscopy. A) total goblet cell counts in whole section, B) total area of muscularis externa area, C) average length all crypt lengths, D) Representative H&E sections of SED (top) and VWR (bottom) mice. The *bottom* and *top of boxes* are the first and third quartiles, the middle band inside the boxes is the median, the *whiskers* contain the upper and lower 1.5 interquartile range (IQR). No differences were observed between any groups in any of the measured parameters.

mRNA gene expression analysis - The result of our multi-GLM tests revealed no significant group differences between colonic mRNA genes expression of CXCL9, CLDN10, and RELM-β (data not shown). Univariate analysis of our *a priori* cytokines are showed in Figure 23. We detected significantly lower relative expression of TNF-α (P<0.05, F-value=7.62), and TGF-β (P<0.05, F-value=5.77) in VWR animals compared to SED (Figure 23A-B). CXCL9, CLDN10, IFN-γ, IL-10, and RegIII-γ were not different between groups.

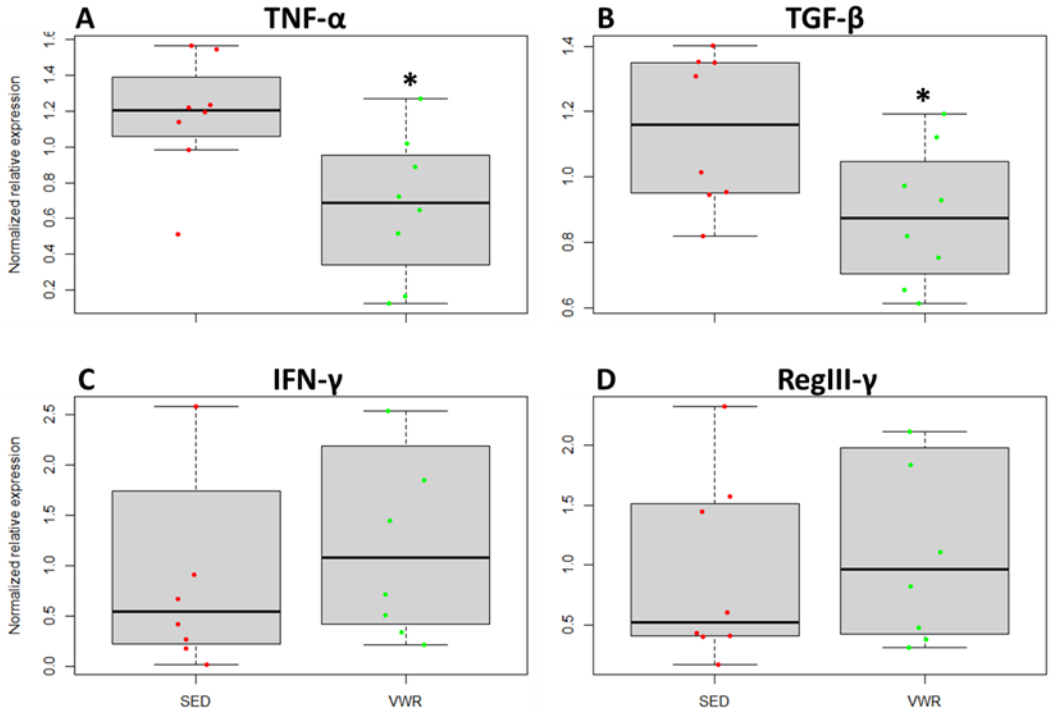

Figure 25. mRNA expression of colonic genes

The relative mRNA gene expression of selected pro- and-anti-inflammatory mediators in colon. * denotes significant difference (P<0.05) between groups.

Microbial analysis - The PCoA plots of the Bray-Curtis and weighted-UniFrac distances showed no clear clustering between VWR and SED groups (Figure 26), and no group differences were detected by the PERMANOVA test. Differential abundance testing of each genera between groups using ANCOM revealed no differences in any taxa between groups. Similarly, the phenotypic traits of the microbial communities, as predicted by BugBase were not significantly different between groups. The overall within sample diversities (α diversity) species richness, and Faith's PD were similar between groups, however Shannon's diversity was significantly ($P<0.05$, $F=5.8$) higher in VWR groups. VWR group also showed marginally higher observed OTUs (Figure 27) though this was not statistically significant ($F: 4.01$, $P<0.06$).

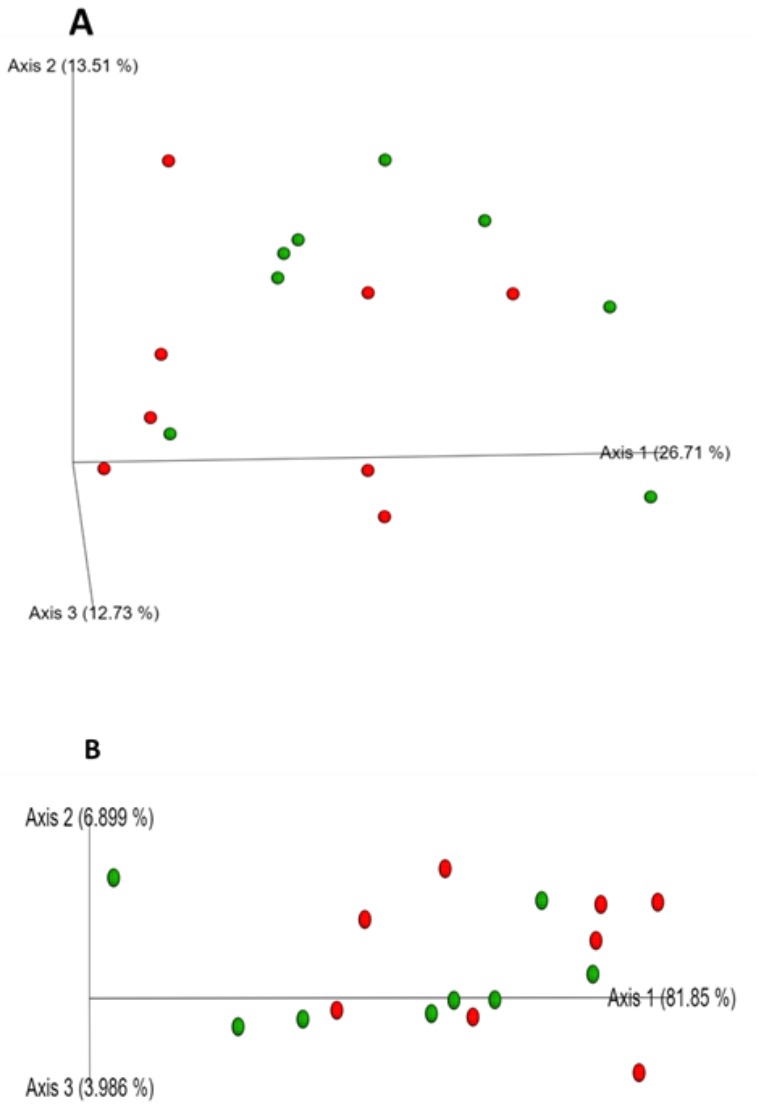

Figure 26. PCoA plots of colonic bacterial communities

Ordination plots of A) Bray-Curtis and B) weighted-UniFrac distances of bacterial communities showing no clear clustering between groups, as confirmed by PERMANOVA test. Green=VWR, Red=SED.

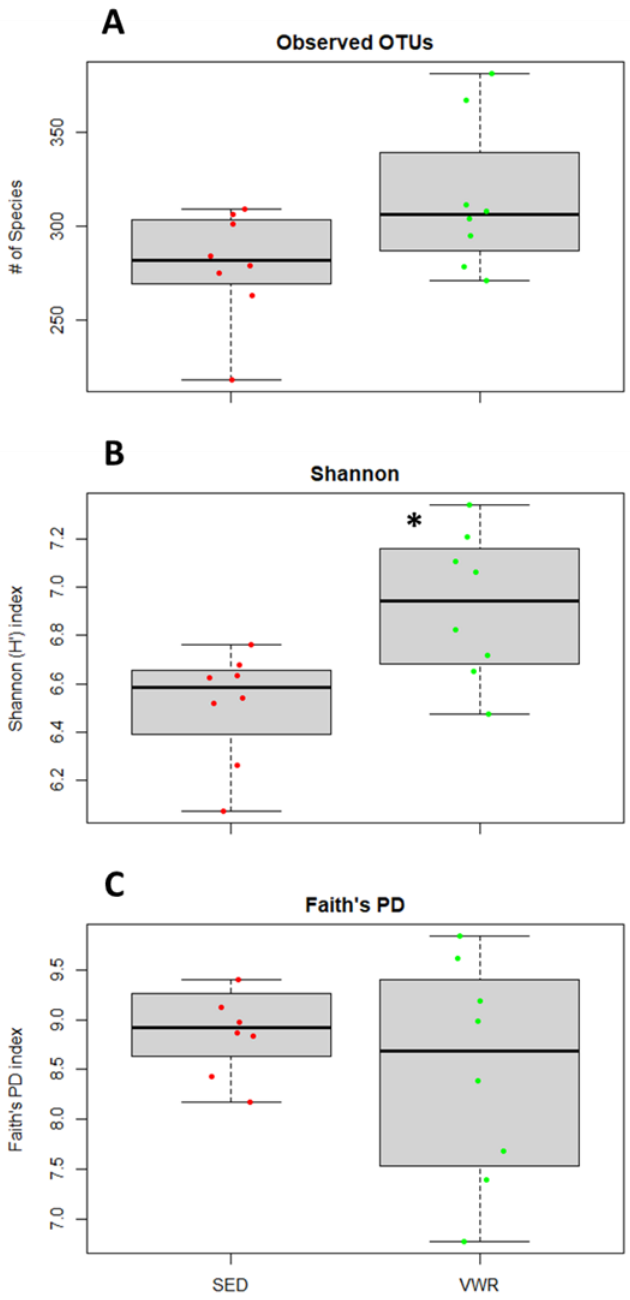

Figure 27. Alpha diversity measures of colonic bacterial communities

Comparison of within sample diversity A) observed OTUs, B) Shannon's (H'), and C) Faith's PD. * denotes a statistically significant difference between groups.

4.4 Discussion

The role of PA as a modulator of the intestinal microbiota has recently been established. We previously showed that healthy VWR mice have a significantly different intestinal environment than their SED counterparts with regards to expression of inflammatory cytokines, productions of SCFAs, and the overall bacterial community diversity and traits. These changes were true despite the observation that our animals ran significantly less than previous studies (Jacob M Allen *et al*., 2015; Evans *et al*., 2014; Lamoureux *et al*., 2017) showing PA-associated changes in mice microbiota. In this study we asked the question, can low volumes of PA also produce significant changes in the gut environment? By tightening the axels on our wheels, we were able to produce a low-volume model of VWR which corresponded to about $1/10^{th}$ of typical distances we saw in Chapter 3, which recruited beneficial changes in the gut. In agreement with our hypothesis, low VWR did not significantly alter the overall composition of the colonic bacteria or the phenotypic trait profiles. The overall diversity evenness (Shannon H') of VWR mice was significantly higher than SED, and a marginal increase was also observed in number of observed OTUs in this group. These findings follow a similar pattern to those in the previous chapter, with VWR mice housing a higher number of unique taxa, suggesting that even low VWR can lead to increased overall diversity. The mechanism behind this observation is not clear, however, lower mRNA expression of TNF-α and TGF-β in the colon of low VWR mice may provide a clue. As with normal running VWR mice in Chapter 3, a gut environment with lower expression of these cytokines may either reflect an enhanced tolerance to commensal bacterial ligands, allowing for increased bacterial colonization, or an enhanced mucosal barrier, preventing the interaction of bacterial antigens with the host cells. In contrast to findings from Chapter 3, low VWR did not induce production of the immunosuppressive IL-10 or downregulation of the inflammatory IFN-γ cytokines. This is perhaps not surprising considering the absences of changes in the overall microbial community composition in VWR mice as both

IL-10 and IFN-γ modulation are dependent on interactions with microbial antigens. In Chapter 3, we suggested the increased expression of IL-10 in VWR mice may be correlated to the increased abundance of Gram negative bacteria as these bacteria favor recruitments of IL-10. The findings here then may further support this observation as a lack of microbial changes was associated with absence of IL-10 modulation, however, the possibility that these responses rely on higher volumes of PA cannot be eliminated. As the increase in TNF-α and TGF-β has been implicated in patients with active intestinal inflammation (Babyatsky *et al*., 1996; Komatsu *et al*., 2001; McCabe *et al*., 1993), our findings here suggest that even low volumes of PA may offer some protection against intestinal diseases such as IBD. Interestingly, low VWR mice gained weight at a significantly higher rate than SED animals, despite no differences in their food and water intake habits. Given the increased energy expenditure required from wheel running, the intuitive hypothesis would be that VWR mice would either have higher food intake or gain similar or less weight than their SED counterparts. Indeed, in the experiments by Lamoureux *et al*. (2017) mice gained similar weight across 8 weeks of wheel running while consuming significantly more food. Allen *et al*. (2015) also reported VWR mice with average daily running distances of ~5.8 km had similar weight changes as SED animals across 6 weeks; food intake was not reported. One plausible explanation for this finding would be an increase in energy harvesting efficiency of the intestinal bacteria. For example, an increase in the Firmicutes to Bacteroidetes ratio has been, albeit with some controversy, associated with increased weight-gain (Barlow *et al*., 2015; Koliada *et al*., 2017). We examined the Firmicutes:Bacteroidetes across our animals and found no differences between groups. In addition to this, the lack of microbial compositional changes across any taxa in this experiment suggests the increased weight-gain is independent of the microbiome. Alternatively, low PA may be initiating energy storage events such as lipogenesis through hormonal regulation in VWR animals in anticipation of future expenditure. Further investigations are necessary to determine the mechanism behind

this observation. This study has some key limitations which are discussed in detail in the following Chapter.

Summary

In agreement with our hypothesis, we found that low levels of PA are not sufficient to induce detectable changes in the overall composition of the gut microbiota. However, even at low volumes, PA is associated with reduced intestinal expression of the inflammatory cytokines TNF-α and TGF-β, which in turn may reflect an enhanced tolerance to bacterial colonization in the gut. This may support our findings that VWR mice harbored a higher overall diversity of bacteria than SED animals. Overall, the findings here suggest that PA even at very low volumes may be beneficial in intestinal health and may be effective in protecting against future injury such as those associated with IBD or used as an adjunct therapy in IBD management. This is particularly relevant in human IBD as patients with active disease are limited in their ability to perform regular levels of PA due to disease complications.

Chapter 5: Conclusion

5.1 Summary and general discussion

The primary focus of this thesis was to explore the link between PA and intestinal health. More specifically, we were interested in the effects of PA on the microbiome and its potential benefits against intestinal injury such as those found in IBD. In reviewing the literature prior to this work, I found only one relevant study; Matusomoto *et al*. in 2008 had shown an increase of cecal butyrate concentrations, an important SCFA with anti-inflammatory properties, in VWR rats. Given that this topic was otherwise virtually unexplored we first set out to simply establish a link between PA and the microbiome. In Chapter 2, using high-throughput sequencing methods we characterized the fecal bacteriome of healthy humans with quantified CRF levels representing a wide spectrum of lifestyles ranging from sedentary to elite athletes. We found that CRF was associated with increased overall bacterial diversity regardless of age, sex, BMI, or dietary intake. The composition of bacterial communities did not follow any obvious trends, however, the predicted functions of fit individuals appeared to favor SCFAs production. Indeed, when we analyzed the abundances of fecal SCFAs, and in agreement with Matusomoto *et al (2008)*, we noticed a positive association between CRF and butyrate. Taken together, we cautiously, but optimistically interpreted the findings that endurance exercise was associated with improved intestinal health. Following this, we set to establish causality using a rodent model and further determine whether PA-associated changes were in fact beneficial to the host.

In Chapter 3, we showed that 6 weeks of wheel running in healthy mice recruited various physiological changes in their intestine, including downregulation of pro-inflammatory gene expression, increased total concentration of SCFAs, propionate, butyrate, as well as limited but significant changes to the overall bacterial communities. The VWR mice showed an increase in species richness following wheel running and reduced abundance of two members of the Gram-positive Ruminococcaceae family. Interestingly however, equal volume of wheel running in

MUC2$^{-/-}$ animals (representing a life-long model of murine colitis), did not elicit any protection. Our finding here contrasted with other reports that showed PA protected against chemically-induced models of colitis (Bilski *et al*., 2015; Marc D Cook *et al*., 2013b; Saxena *et al*., 2012; Szalai *et al*., 2014). We speculated that these differences likely derive from the possibility that in the earlier reports using chemically-induced colitis, PA primes a healthy intestine, better equipping it to tolerate the chemical injury, while in MUC2$^{-/-}$ animals, PA is imposed over an existing disease state. We concluded then that the benefits of PA are dependent on the underlying health of the intestine, or at least require the presence of a complete mucosal layer. With regards to human health, findings from this chapter suggest that a physically active lifestyle encompassing an aerobic training component can enhance the intestinal milieu. This warrants further investigation in the role of PA as 1) a preventative measure for human IBD and 2) as an adjunct therapy for remission maintenance. It should be emphasized however that regular exercise is recommended to IBD patients even during active disease to combat secondary complications of IBD, as described in Chapter 1.

In the final research chapter, we looked at the effects of low wheel running on the intestinal microbiota and health. This question was developed in response to an unexpected observation in Chapter 3 where we noticed that the volume of wheel running in our animals was significantly less than those previously reported in the literature. While wheel running is inherently variable across facilities, strains, and even littermates (J. Goh & Ladiges, 2015), our findings however more likely reflected the type of wheel used in our experiment. For example, the type of material, diameter size, footing, and the angle of the wheels can all significantly affect running distances (Reebs, St-Onge, & Reebs, 2005; Walker & Mason, 2018). In consulting with veterinarians, commercial vendors, pet-stores, and other experienced users, I hypothesized that our animals ran considerably less due to the smaller size of our wheels. I contacted the various groups involved in the mentioned studies and obtained detailed information regarding their rodent wheels. While every group had used a different wheel, all

reported diameter sizes larger than ours. Therefore, I concluded that the lower running behavior was not the result of possible confounding factors such as equipment malfunctions or undetected environmental stressors. Interestingly, even with considerably lower volume of running, our mice had experienced significant physiological adaptations. We therefore set out to test whether the benefits of PA were volume-dependent or were rooted in the physiological or psychological benefits of having access to any degree of additional movements. In Chapter 4 we tested the effects of low-VWR on mice intestinal health, by tightening wheel axels to limit their movement. To my knowledge, this method has not been previously reported. In agreement with our hypothesis we showed that low volumes of wheel running did not induce changes in the intestinal microbiota; however, similar to the normal VWR mice, the gene expression of the pro-inflammatory TNF-α and TGF-β were significantly reduced. These findings suggest that the effects of PA on the intestines are multifaceted and likely mediated through various physiological pathways. For examples, while a minimal PA threshold appears to be needed to recruit microbial changes, even very low physical movements can alter intestinal immunity, independent of the microbiome.

The combined findings from the works of this thesis suggests that PA can modulate the intestinal environment under normal conditions in a volume-sensitive manner, and that this 'priming' may be protective against inflammation-associated intestinal diseases. There are however other implications in a broader sense. For example, manipulating the microbiota through regular physical activity may have the additional benefit of enhancing the host's response to various pharmaceutical drugs that are bio-transformed through the gut. From a veterinarian and animal husbandry perspective, these findings add to the growing body of literature suggesting that sedentary animals represent a model of compromised health rather than a healthy one (Booth & Lees, 2006). The current dogma views inactive animals as healthy 'controls' in an experiment, however, from an evolutionary perspective, animals are fitted to be continuously active. The findings here support the notion that sedentary mice are associated

with a state of heightened inflammation. This has substantial biological implications with regards to animal-based research and ethical implications in the food industry where animals are often raised in captivity.

5.2 Limitations

As described, the findings from this work have significant implications across a variety of fields, however, as with any experiment, these findings are bound by certain limitations. In this section I will highlight the most important limitations as they pertain to proper interpretation of results and future work.

Of the three primary studies described here, results from Chapter 2 are the most sensitive to its limitations. Perhaps the most important of these is the integration of numerous environmental variables into complex models with a relatively small sample size. Given the observational nature of this study, combined with the complexity of nutritional information, we were subject to the “curse of multidimensionality”, and were required to limit our analyses to a subset of the variables measured. We used expert opinion, review of the literature, and a set of *a priori* factors to initially remove various dietary components from our nutritional data; further reduction was then made using objective modelling until an acceptable number of variables remained. Given our biased preference over certain dietary components, and removal of other potentially meaningful variables, many important relationships may have been overlooked. This is primarily a problem of low sample size and may be resolved with more thorough sampling efforts. Another critical consideration is the accuracy of dietary recall surveys. In our study we used a supervised 24-hrs dietary recall survey which are prone to various biases such as underreporting and memory loss (Shim, Oh, & Kim, 2014). While significant improvements have been made to resolve these issues, for example the use of food tracking mobile applications, email reminders, food journals, etc., the implementation of these methods are laborious, expensive, and may impede recruitment efforts.

The limitations of the work described in Chapter 3 are technical in nature. First and foremost, the presence of a batch effect as described previously precluded meaningful comparison of the bacterial community data across groups. As we were limited by the number of available running wheels, inevitably one cohort of animals had to be ran separately. In our experiment, VWR mice were purchased at a different date than the other three groups and amplicon preparation and 16S sequencing of these samples were performed separately. This undoubtedly resulted in the well-known phenomenon of 'batch effect' associated with Omics data (W. W. Bin Goh *et al*., 2017), which results in detection of significantly different microbial populations in samples that should otherwise be similar. This was evident in our initial PCoA plots showing a clear clustering effect between the VWR and SED animals. While recent efforts have been made to correct for these batch-effects (Davis, Proctor, Holmes, Relman, & Callahan, 2017; Gibbons, Duvallet, & Alm, 2018), these post-hoc correction methods depend on inter-run samples or gnotobiotic mock communities, both absent in our design. To circumvent this issue and eliminate erroneous interpretation of the microbial data, I chose to only examine fecal bacterial communities of each group across time, rather than across other groups. Future experiments should consider the problem of batch effects and follow appropriate designs to minimize them. This batch-effect can be extended beyond the microbial data into other measures such as SCFAs and cytokines. Given that the microbiota is tightly integrated with regulation of both SCFAs production and cytokine expression, we cannot eliminate the possibility that the reported VWR differences were linked to the pre-existing difference in this group's bacteriome. We were fortunate, however, to be provided insight into this problem: in the VWR cohort, one animal unexpectedly did not run on the wheel throughout the experiment and its SCFAs and cytokine abundances closely resembled those of SED animals, and not VWR animals. Though based on only one observation this suggests that wheel running indeed was the instigator of those changes.

In Chapter 4, we were limited by our access to only colon and ileum tissues, meaning that baseline comparison of fecal bacterial data, and cecal SCFA were not possible. The absence of pre-treatment samples significantly reduces the sensitivity of our analyses as subtle individual community changes across time are not detectable. Rather, only major separations of the two groups' communities with a high effect size would be detectable. A general consideration for all future microbial experiments would be to include longitudinal information of the community to increase sensitivity and discovery power.

One of the most difficult challenges facing HTS data, in particular 16S surveys, are the inconsistencies in changes to abundance of taxa across studies measuring the same interventions. For example, across all studies reporting on taxonomic changes associated with PA, I found almost no overlap between reported outcomes when examining the change in individual taxa/OTUs. While variability in experimental design itself, as well as factors involved in the previously described 'batch-effect' contribute to these discrepancies, there is additional subjectivity introduced at data processing and analyses steps. For example, older studies relied on clustering of sequences into OTUs with arbitrarily selected similarity thresholds. These methods often produced highly inflated OTU counts and unreliable reconstruction of known communities (Edgar, 2017). Newer denoising methods such as DADA2 (Callahan *et al*., 2016) and Deblur (Amir *et al*., 2017) used in Chapter 3 and 4, respectively, allow for inference of exact sequence variants using error models to resolve sequence ambiguity down to the level of single-nucleotide differences. Comparison of these approaches to the same data-set can yield significantly different outcomes when comparing individual taxon (personal experience). There are numerous other steps throughout the bioinformatics processes that require human input, each with potential to considerably alter the outcome. In a typical 16S survey analyses such as those presented in this thesis, there are at least 8 crucial steps that introduce user bias: choice of quality control parameters, denoising vs. OTU picking methods, sequence alignment, phylogenetic tree building approach, normalization for unequal sampling depth, reference

databases, taxonomic assignment, filtering of low abundant features, and choice of statistical testing/modelling. Given that additional options exist at each step, a single data set can yield significantly different results based on these subjective choices. These outcomes are particularly sensitive when utilizing high-precision methods such as differential abundance testing, therefore comparison of individual taxa across studies should be interpreted with caution. For metanalysis of these data, a thorough re-analysis of the data using identical bioinformatics processes is strongly recommended.

5.3 Future work

The field of 'exercise microbiology' is in its infancy, with countless questions waiting to be answered. In this section I will conclude this work by offering recommendations for future continuation of this work.

First and foremost, it is important to highlight the bidirectional nature of the PA-microbiota relationship. In this work we focused on the effects of PA on the bacterial community, however the reverse relationship also likely exists, i.e. can the microbiota alter physical performance and behavior. An interesting approach to this question could use fecal transplantation of VWR or SED mice into germ-free mice and measure the recipients' propensity to wheel running. Alternatively, previously identified bacteria associated with higher fitness levels could be introduced as cultured probiotics to examine PA behavior and performance. In Chapter 3, I proposed that the benefits of PA are dependent on the underlying health of the intestinal environment and/or the presence of a complete mucosal layer. Given that MUC2$^{-/-}$ mice are born with an underlying disease state, I would recommend future works to develop a new colitis model through inhibition of the MUC2 gene with monoclonal antibodies, allowing for temporal control over disease onset. This would further allow for induction of active disease-remission cycles, an important characteristic of human IBD. In Chapter 4 we provided evidence that the benefits of PA on the intestinal environment might be independent of the microbiota.

Confirming this relationship could occur through carrying wheel running experiments in germ-free mice, followed by colitis induction. Protection against intestinal damage would confirm a protective effect of VWR on host intestinal cells. For a more mechanistic insight, isolated epithelial cells of VWR mice could be challenged with endotoxins *in vitro* and compared with SED mice. Finally, the field has primarily focused on the effects of aerobic exercise on intestinal microbiota, while resistance training has been entirely omitted. With considerable differences in physiological adaptations of resistance training, it would be interesting to examine all previous parameters with this model of exercise instead. One important limitation in studying intestinal mucosal microbiome through fecal samples derives from the well-documented distinction between these sample types (Zoetendal et al., 2002). Unlike mucosal samples, fecal samples are easy to collect and allow for repeated surveying of microbial communities through time. However, the mucosal-associated microbes are likely to be more biologically informative as they more closely reflect the established colonies that directly interact with the host. Future studies should consider the potential differences in PA-derived changes between these two distinct microbial communities and survey both. Finally, as with all *in vivo* models of human diseases, a major limitation exists in the translation of knowledge from preclinical to clinical studies. The use of animal models in studying diseases is essential in understanding the underlying mechanisms that allow scientists to develop appropriate strategies for prevention, diagnosis, and treatments. Animal models have numerous advantageous over human studies which make them invaluable for research. For example, animal models allow for a highly controlled environment and genetically consistent subjects that are often absent in human studies. Exclusive to animal studies, genetic manipulation of desired genes allow scientist to discover genetic components of diseases and better identify at-risk populations. In addition, the rapid turnover rate of rodents allows scientists to detect age-related aspects of disease pathology within a short timeframe. Though the complexities of human and mouse physiology are relatively similar, the differences between them are still considerable and should be acknowledged whenever inferring from

preclinical findings to clinical settings. To confirm the relevance of results from this work, future studies should be carried in humans. As the findings from Chapter 3 suggest, PA is likely more beneficial in modulating intestinal health prior to disease onset. To confirm this hypothesis, a large cohort of healthy humans with sedentary or active lifestyles that are screened as high-risk for IBD should be monitored longitudinally for several years to compare incidences and severity of IBD. Alternatively, exercise prescription in populations with existing IBD may be a more direct comparison to the study reported here.

5.3 Concluding remarks

In conclusion, results from this thesis provide promising insights into the use of PA as a means to combat IBD. Combining these and previous findings from others, it is clear that PA can positively influence the intestinal environment, shifting it towards a state of heightened tolerance towards injury. While the data from our chronic colitis model did not show protection resulting from wheel running, these should be interpreted with caution as the $MUC2^{-/-}$ animals do not reflect the severity of common IBD phenotype. For example, IBD patients with defective mucosal integrity, show intermittent patches of healthy and unhealthy tissue which suggests that the observed effects of PA in healthy tissues may still be imposed on those healthy regions. In this sense, PA even following disease onset may be beneficial in humans by inhibiting the advancement of disease into healthy regions. From a personal perspective, having lived with UC for almost a decade, the benefits of PA reach beyond primary disease management. The improvements in quality of life due to various components such as stress reduction, improved body image, increased appetite, and involvement in social interactions all contribute to a holistic approach to IBD management. My final recommendations based on scientific evidence presented here, in the literature, as well as personal anecdotal experiences is for implementation of PA, to the extent that it does not cause further distress, in all IBD patients as management therapy.

I would like to conclude this ‘last lesson’ of graduate school by paying tribute to the very first lesson I was taught in it. “*You are never truly ready for an experiment, until it’s over*”.

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- Under “References” tab, choose “Update Table”.

Appendices

Appendix A

Spontaneous Colitis (MUC2 $^{-/-}$) Monitoring Sheet **Protocol:**

PI & Primary contact: Dr. Gibson 250-899-0858;
Emergency contacts: Sandeep Gill: 250-718-4287, Mehrbod Estaki: 778-214-2337, Candice Quin: 250-921-4711, Natasha Haskey: 306-290-7350, Jacqueline Barnett: 250-503-7100

Cage #: **Animal#:**

Date / Name																
From a Distance																
Normal																
Piloerection (++)																
Movement reduced (++)																
Hunched (+++)																
Inactive (+++)																
Shaking (++++) (monitor AM and PM)																
Rectal bleeding																
None																
Rectal swelling (+)																
Visible blood in stool (++)																
Large amount of blood in stool and/or cage (+++)																
Blood in stool and anus (++++)																
Rectal prolapse (++++) (euthanize)																
Stool consistency																
Normal																
Soft stool (+)																
Diarrhea (++)																
Weight																
No weight loss																
Loss of up to 5% (+)																
Loss of 5 - 10% (++)																
Loss of 10 - 19% (+++)																
Loss of more than 20% (++++)																
Hydration signs																
Normal																
Slight sunken eyes (+)																
Dehydrated/sunken eyes (+++)																
Skin tent (>2)(++++) (monitor AM and PM)																
TOTAL SCORE																

Comments:

Scoring and Action
Choose the most severe score present (one score per category)
Weight Scores, Rectal bleeding, Stool consistency: (+) assign a score of 1, (++) score of 2, (+++) score of 3, (++++) score of 4 and assign corresponding score to at a distance evaluation
Score 0-7: Action: monitor as per protocol
Score 8-10: Consider euthanasia; Action: increase monitoring to a minimum of once daily
Score 11 or more or >20% body weight loss for 2 consecutive days; Action: immediate euthanasia
Rectal prolapse; Immediate euthanasia
Endpoint: Rectal prolapse or a combined score of 11 represents gross colitis and therefore mice will be euthanized immediately.

MUC2$^{-/-}$ animal monitoring sheet

Appendix B

Primer Name	Primer Sequence	Tm (°C)	Length (bp)	Efficiency
18S-F	CGGCTACCACATCCAAGGAA	48.9	20	1.13
18S-R	GCTGGAATTACCGCGGCT	45.2	18	
IFNγ-F	TCAAGTGGCATAGATGTGGAAGAA	54.5	24	1.14
IFNγ-R	TGGCTCTGCAGGATTTTCATG	50.5	21	
TNFα-F	CATCTTCTCAAAATTCGAGTGACAA	55.4	25	1.19
TNFα-R	TGGGAGTAGAACAAGGTACAACCC	54.3	24	
TGF-β-F	GACCGCAACAACGCCATCTA	48.9	20	1.05
TGF-β-R	AGCCCTGTATTCCGTCTCCTT	50.5	21	
IL-10-F	AGGGCCCTTTGCTATGGTGT	48.9	20	0.97
IL-10-R	TGGCCACAGTTTTCAGGGAT	48.9	20	
CLDN10-F	GGCGTTGGATGGTTACATCC	48.9	20	1.27
CLDN10-R	AATCCCGGCCAAGCAAGCGA	48.9	20	
CXCL9-F	AGTGTGGAGTTCGAGGAAC	47.1	19	1.01
CXCL9-R	GAAATCATTGCTACACTGAAGAAC	54.4	24	
Tbp-F	ACCGTGAATCTTGGCTGTAAAC	52.0	22	0.89
Tbp-R	GCAGCAAATCGCTTGGGATTA	50.5	21	
RELM-β-F	ATGGGTGTCACTGGATGTGCTT	51.9	22	0.92
RELM-β-R	AGCACTGGCAGTGGCAAGTA	48.9	20	
RegIII-γ-F	CCCGTATAACCATCACCATCAT	51.9	22	0.94
RegIII-γ-R	GGCATCTTTCTTGGCAACTTC	50.5	21	

RT-qPCR Protocol:

1: 95.0°C for 0:30
2: 95.0°C for 0:05
3: 58.0°C for 0:05
Plate Read
4: GOTO 2, 39 more times
5: 95.0°C for 0:10
6: Melt Curve 65.0°C to 95.0°C: Increment 0.5°C 0:05
Plate Read

List of RT-qPCR primers used for the studies outlined in this thesis. Tm refers to the primer melting temperature. Length (bp) refers to primer length in base pairs. Primer names ending with '-F' refer to forward primers, and primer names ending in '-R' refer to reverse primers.