FACTORS AFFECTING THE GUT MICROBIOME

By

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Dedication

To the family and friends who've been there, through the good and the bad.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The recent boom in microbiome research, made possible by advances in sequencing technology, has revealed that the gut microbiome plays an important role in human health. A relatively stable community, gut microbes are essential in humans for the development of the immune system and processing of nutrients, such as vitamins. Changes to these communities have been observed in disease states, such as: inflammatory bowel disease, diabetes, obesity, colon cancer and even atherosclerosis. Factors such as antibiotic use and diet can alter the gut communities, and the microbiome has become a target of therapeutic interest for certain chronic inflammatory diseases.

Here, we further examine factors affecting the gut microbiome, namely exercise, food supplements and probiotics. We have used deep sequencing methods to focus on the bacterial communities across three studies. Taxonomy was assigned to sequences and compared across samples using a bioinformatics approach.

The first study examines the impact of exercise on the murine gut microbiome. Using a machine learning model, we identify moderate but measurable changes in gut bacteria as a result of moderate exercise. We identify previously associated and novel taxa associated with exercise. The second study examines the impact of fruit-derived antioxidants on the gut microbiome in a model of lung cancer. Anthocyanin-rich haskap berry extracts have a strong impact on gut bacteria and warrant further investigation as a dietary supplement to prevent lung cancer relapses. Lastly, we analyzed microbial succession in the production of a dairy-free, coconut kefir product. Kefir is made by fermenting coconut milk using traditional kefir grains containing bacteria and yeasts. We find that coconut kefir contains many of the same beneficial bacterial as traditional dairy kefir.

The results illustrate that factors like exercise and dietary supplements have an impact on gut microbes in healthy subjects. While the effects of haskap extract were also seen in a model of murine lung cancer, these factors should be further examined in disease models. As research continues to unravel how the microbiome is related to various diseases, understanding how to shape the microbial community through exercise, diet and probiotics will become increasingly important. The research here provides a first glance at many of these systems using primarily murine animal models. Further studies in humans will extend these results further to better leverage the use of the microbiome to aid in human health.

LIST OF ABBREVIATIONS USED

ANOVA	Analysis of variance
CFS	Chronic fatigue syndrome
CR	Calorie restriction
CRC	Colorectal cancer
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
FC	Forced control
FDR	False discovery rate
FE	Forced exercise
GI	Gastrointestinal
HUMAnN	HMP Unified Metabolic Analysis Network
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
CXCL8	Chemokine ligand 8
ITS2	Internal transcribed spacer 2
KC	Keratinocyte chemoattractant
NNK	Nicotine-derived nitrosamines
OTU	Operational taxonomic unit
PCA	Principal component analysis

PCoA	Principal coordinate analysis
PCR-DGGE	Polymerase chain reaction with denaturing gradient gel electrophoresis
PEAR	Paired-End reAd mergeR
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology
RF	Random forests
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acid
STAMP	Statistical Analysis of Taxonomic and Functional Profiles
T2D	Type II diabetes
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimenthylamine N-oxide
TNFα	Tumor necrosis factor alpha
VC	Voluntary control
VE	Voluntary exercise
VSEARCH	Vectorized search

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CHAPTER 1: INTRODUCTION

1.1 HUMAN GUT MICROBIOME

Although it is only recently that sequencing technologies have advanced enough to allow us a deeper look into microbial communities, the term 'microbiome' has been around for almost 40 years. In 1988, in his book *Mycoparasitism and plant disease control*, John M Whipps stated that "A convenient ecological framework in which to examine the biocontrol systems is that of the microbiome. This may be defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties". With the invention of next-generation sequencing platforms such as 454 sequencing and Illumina, the term evolved to refer to all the microbes and their functions present in a community.

Recently, there has been a large flourish of research on the human microbiome, which encompasses all of the microbes (mainly bacteria, eukaryotes and viruses) present in and on the human host. There is particular interest in the microbial communities of the gut and the role they play in human health, with some scientists referring to the gut microbiome as a "forgotten organ" (O'Hara & Shanahan, 2006). Like an organ, the microbiome contributes significant metabolic capacity to the body, as well as mechanisms that help maintain physiological homeostasis, especially in the mucosal immune system. In this thesis, the term microbiome will refer to bacteria in the gut, unless otherwise stated.

1.1.1 HUMAN MICROBIOME IN HEALTH AND DISEASE

Changes in the gut microbiome have been linked to obesity, diabetes, cardiovascular disease, inflammatory bowel disease (IBD), and colon cancer (Ettinger et al., 2014; Serino et al., 2014; Everard et al., 2013; Qin et al., 2012; Sha et al., 2012; Scanlan et al., 2008; Turnbaugh et al., 2006) and it is fast becoming a target of therapeutic interest.

Many early studies in microbiome research looked at the impact of gut bacteria on obesity. Mice raised in sterile environments that are uncolonized by microbes (germ-free mice) are resistant to diet-induced obesity (Fiebiger et al., 2016). On the other hand, the obese microbiome increases capacity in the host to harvest energy for the body and the overweight phenotype can be transferred to germ-free mice by giving them microbes from obese mice (Turnbaugh et al., 2006). Overall, several studies have shown that an increase in bacteria in the Firmicutes phylum with a lower abundance of those in the Bacteroidetes phylum are associated with obesity (Turnbaugh et al., 2006, Louis et al., 2016). It is also reported that the bacteria *Akkermansia muciniphila* inversely correlates with weight (Everard et al., 2013; Louis et al., 2016).

The microbiome is highly studied in inflammatory bowel disease, since gut bacteria influence mucosal immunity. There are two main forms of IBD; ulcerative colitis (UC), characterized by continuous inflammation of the mucosa in the distal colon and Crohn's disease; characterized by intermittent inflammation of deeper tissue layers, often patchy anywhere along the intestines (Abraham & Cho, 2009; Khor et al., 2011). UC is often treated by surgical resection of the colon, and studies often focus on Crohn's disease, which is prone to relapse despite drug therapies and even despite surgical removal of actively diseased segments. It is

hypothesized that this inflammation is driven by both genetics and microbial gut antigens. While over 200 genetic risk loci have been identified for IBD, they only explain 8% and 13% of disease heritability for UC and Crohn's (Liu et al. 2015). Dysbiosis in the gut microbiome has been detected in IBD, in the form of overall lower species diversity, and a noteworthy increase in certain *Gammaproteobacteria* and a decrease in certain taxa in the *Firmmicutes* phylum (Sha et al., 2012; Kostic et al., 2014).

As the fourth leading cause of cancer related death worldwide, causative factors of colorectal cancer (CRC) are being increasingly investigated. Short-chain fatty acids (SCFAs) are reported to help regulate the growth of intestinal stem cells, which undergo rapid turnover and are a common starting point for colon cancer (Woo & Alenghart, 2017; Oke & Martin, 2017). Dysbiotic shifts in the microbiome also have the potential to contribute to inflammation in the gut and the development of cancer (Oke & Martin, 2017).

Type II diabetes (T2D), a growing problem in the western world, is a metabolic disease mainly caused by obesity-linked insulin resistance. Two studies have shown that changes occur to gut microbiome in T2D in humans. The first, by Lersen et al. (2010) shows that it is associated with lower levels of *Firmicutes* and *Clostridia* and slightly higher levels of *Bacteroidetes* and *Proteobacteria*. The second, by Qin et al. (2012) found higher levels of opportunistic pathogens and lower levels of butyrate-producing bacteria (known to be beneficial). Overall, it is hypothesized that there is some microbial dysbiosis that occurs in T2D and leads to an increase in potentially harmful, Gram (-) bacteria (Lersen et al., 2010).

Cardiovascular disease (CVD) is extremely prevalent in western societies and often coincides with other chronic inflammatory diseases such as obesity and type II diabetes. While there is not a lot of research on the microbiome and cardiovascular disease, a couple notable

studies have surfaced in recent years. Diets high in fats and simple sugars lead to risk of metabolic syndrome, as well as promote bacteria that have an increased capacity for energy intake. Metabolic syndrome defines a number of risk factors for CVD that include obesity, hypertension, high blood lipids and insulin resistance (Lavie et al., 2014). It is proposed that adding more plant-based fibers and fresh, unprocessed foods can help return the gut microbiome to a healthier state and reduce metabolic burden (Ettinger et al., 2014).

A ground-breaking study by Wang et al. (2015), actually demonstrated the ability to drug the gut microbiome to reduce atherosclerosis. Choline is converted to Trimethylamine (TMA), a precursor for Trimethylamine N-oxide (TMAO), a metabolite associated with atherosclerosis formation, in the gut by microbes. The bacterial enzyme responsible for this conversion was identified and inhibited by a small molecule drug, successfully reducing circulation TMAO levels in mice. While studies looking at a direct microbial target are rare, they are crucial for the development of therapies for future clinical practice.

1.1.2 HOST-MICROBE INTERACTIONS

The intestinal microbiome has been shown to be an important contributor to normal host physiology, including immune development and the metabolism of energy and drugs (Kinross et al., 2011; Cani et al., 2009). Changes to the host environment, such as selective pressures brought about by antibiotic use (Ramond et al., 2015) or inflammation due to bacterial infection (Sekirov et al., 2010) can disrupt the normal community. In healthy individuals, the microbes are generally able to reestablish their functional niches thereafter (Raymond et al., 2015; Sekirov et al., 2010; Dethlefsen et al., 2008). It has also been shown that factors, such as diet and exercise

contribute to changes in an individual's gut microbiome throughout their lifetime (Allen et al., 2015; David et al., 2013).

Age and sex are two factors that also have an influence on the microbiome. Aging in mice is associated with increased *Rikenellaceae, Lachnospiraceae, Ruminococcaceae* and *Clostridiaceae* species (Langille et al., 2014) and these trends are also observed in humans (Biagi et al., 2010). It has been shown that gut microbial diversity is lower in juvenile rodents (Mika et al., 2015). Humans and mice exhibit both physiological and behavioural differences between sexes, and this extends to the microbiome. In mice, the microbiome of females and males diverges starting in puberty and continuing on into adult life (Jašarević et al., 2015). Transfer of gut microbes from one sex to the other has been associated with changes in hormone levels consistent with the donor sex. Little research has been done on sex-based differences in the human microbiome, although it is known that pregnant women undergo distinctive shifts in their gut microbiomes (Jašarević et al., 2015).

1.1.2.1 DEVELOPMENT OF IMMUNITY

While our gut microbes benefit from the nutrients we consume, we benefit from them in a number of ways. Mammals have co-evolved with their bacterial communities, and the microbiome plays a key role in early life in the development of mucosal immunity, as well as the metabolism of indigestible nutrients and production of certain essential vitamins (Sommer & Bäckhed, 2013; Round & Mazmanian, 2009). It is vital that the immune system develops tolerance to the body's own bacteria – improper development of this cooperation is believed to lead to local and systemic inflammation (Round & Mazmanian, 2009). While being non-

responsive to healthy bacteria, the mucosal immune system must protect the body from invading pathogen, and microbial composition has been shown to influence mucosal properties, lymphoid structure and development of immune cell differentiation and response (Sommer & Bäckhed).

Bacteria both produce beneficial metabolites and induce the immune system to produce important molecules. Upon encountering microbial structures, immune receptors in the mucosa signal for the production of IgA and antimicrobial peptide secretion by epithelial cells, crucial mediators in balancing the relationship with commensals and overt invasive bacterial infection. A number of bacterial species produce SCFAs from the fermentation of dietary fibers, which to date are the most important known microbial metabolite (Kinross et al., 2011). As well as facilitating the resolution of inflammation through toll-like receptors (TLRs), SCFAs (e.g. butyrate) regulate the differentiation of intestinal stem cells, reducing risk of colon cancer (Woo et al., 2017).

1.2 MODIFYING THE MICROBIOME

1.2.1 DIET

Our gut microbes are heavily influenced by diet and respond very rapidly when diet is altered. For example, David et al. (2013), demonstrated that within 2 days, consumption of animal- or plant-based diets containing substantially different macronutrients significantly shifted specific taxa in the gut. Despite daily fluctuations (David et al., 2014), not all individuals are affected the same way by specific dietary changes and long term dietary trends shape the major compositional features of a microbiome. For example, carnivorous versus herbivorous

diets increase levels of different bacteria to break down the different major macronutrients (David et al., 2013).

1.2.2 EXERCISE

Changes in the microbiome have been observed in diseases such as obesity, diabetes, cardiovascular disease, inflammatory bowel disease and colon cancer (Ettinger et al., 2014; Serino et al., 2014; Everard et al., 2013, Qin et al., 2012; Sha et al., 2012, Scanlan et al., 2008, Turbaugh et al., 2006). Exercise has been shown to have beneficial effects on these diseases states (Hagg et al., 2005; Peters et al., 2001), in part through the modulation of levels of inflammation (Pedersen et al., 2000). Furthermore, exercise has been shown to have both acute and chronic effects and it is these chronic effects that have positive outcomes on disease states (Chen et al., 2014). However, what remains unclear is whether the chronic effects of exercise on inflammation alter the intestinal microbiome.

The impact of exercise with both age and nutrition has been studied in rodents (Queipo-Ortuño et al., 2013; Zhang et al., 2013; Mika et al., 2015). Mika et al. (2015) showed in rats that age affects the impact of exercise on the microbiome. Young rats (three weeks old) were more susceptible than adult rats (ten weeks old) to changes in microbial diversity as a result of exercise. Queipo-Ortuno et al. (2013), fed 6-week old male rats either restrictively or *ad libitum,* with and without free access to an exercise wheel, and found that exercise increased gut bacterial diversity when rats had unlimited food access. This study was only performed over a six day period and limited by the use of PCR-DGGE (denaturing gradient gel electrophoresis), an insensitive method for microbial composition analysis. In contrast, a study examining the effects

of both calorie restriction and voluntary exercise on the gut microbiome, also using six-week old male rats, found that exercise alone had no significant effects on microbial composition (Zhang et al., 2013). Conclusions were, however, largely focused on the diet component of the experiment and fecal sampling did not start until 62 weeks into the study. A study by Cook et al. (2013), demonstrated that voluntary wheel running attenuated, while forced treadmill running exacerbated disease progression in a colitis mouse model. When they compared the effects of voluntary and forced exercise on gut microbial diversity in healthy mice, they found both to have distinct and significant effects on community structure (Allen et al., 2015). In humans, significant differences in the microbiome of elite athletes undergoing high volume, high intensity exercise were identified, but could not be clearly separated from diet differences (Clarke et al., 2014).

1.2.3 DRUGS

Given the metabolizing potential of microbes (Haiser & Turnbaugh, 2013), the gut microbiome can be altered by drugs and also can also alter the effect of drugs on the body through first pass metabolism. A well-known example of this phenomenon occurs with the cardiovascular drug Digoxin. Digoxin increases contractility of the heart by inhibition of Na/K-ATPase membrane pumps and is used to treat heart failure and arrhythmias (Haiser et al., 2014; Gheorghiade, 2006). It was discovered that in about 10% of patients, a bacteria *Eggerthella lenta* was reducing the drug to its inactive form, therefore decreasing the efficacy of Digoxin before absorption in these patients. A diet rich in arginine is able to suppress the bacterial genes responsible for this phenomenon and allow the effects of Digoxin to take place in these patients.

Gut bacteria can also prevent drugs from being excreted. Many drugs are metabolized and conjugated in the liver, and when they are excreted into the intestine in the bile they are not able to be reabsorbed and exit the body with other waste products. However bacterial enzymes such as ß-glucoronidases are able to deconjugate drugs and allow their reabsorption from the intestinal lumen and the bloodstream. This can be dangerous when toxic drugs are being administered, such as irinotecan, a frontline therapeutic in the treatment of colon cancer (Haiser & Turnbaugh, 2013). The bacterial enzymes allow prolonged exposure of the drug in the body and causes severe diarrhea, weight loss and anorexia. While antibiotic treatments suppress these harmful effects, they leave patients susceptible to pathogens such as *C. difficile* (Haiser & Turnbaugh, 2013).

Lastly, it has also been shown that gut bacteria affect the expression of drug metabolizing genes. Germ-free mice (mice that lack microbes), have higher levels of important drug metabolizing genes. This has the potential to cause harm by inactivating beneficial drugs or by rapidly converting prodrugs into their more toxic active forms (Bjorkholm et al., 2009).

1.2.3.1 ANTIBIOTICS

One of the biggest drivers of microbial changes in the gut are antibiotics. While very effective at reducing the mortality and morbidity caused by pathogenic bacteria, they also target beneficial organisms (Blaser & Cox, 2015). Blaser and Cox have shown that antibiotics reduce the diversity of microbes that develop in the gut when administered to young mice and also that these mice become overweight. Disruption of the microbiome by antibiotics can allow succession by pathogenic bacteria such as *C. difficile* as well as promote the development of

antibiotic resistance (Langdon et al., 2016). The use of antibiotics has been linked to the development of a number of immune-related diseases such as asthma and allergies (Langdon et al., 2016).

1.2.3.2 PROBIOTICS AND PREBIOTICS

The mechanisms by which the gut microbiome affects health have not yet been uncovered. The gut microbiome can differ extensively between individuals, and researchers have uncovered several links between certain bacterial species and important functions. Some of these key commensal organisms and their roles were discussed above, and some of them are discussed in further detail below.

Probiotics describe microbes, mainly bacteria, but also some yeast, that can be consumed for health benefits. For example, it has been shown that the consumption of *Lactobacillus sp.* can alleviate symptoms of lactose intolerance (Rosa et al., 2017). While a number of bacterial species thought to be beneficial have been sold as probiotics, not many have been objectively studied. Many fermented foods are also considered probiotics. Cheese and dairy milk have been praised for their potential for appetite control and weight loss, in part because of microbes and microbial metabolites (Montel et al., 2014).

Kefir is a fermented dairy product made using microbial 'grains', held together by a polysaccharide matrix (Nielsen et al., 2014). Containing mostly lactobacillus bacteria and yeast, the health benefits of kefir have been described since it originated in Russia over 4000 years ago (Rosa et al., 2017). Recently, as probiotics have become an area of increasing interest in health, several scientific studies have examined the health benefits of fermented dairy products. For

example, bacteria found in kefir have been shown to be protective against a number of Gram (+) pathogens, mediate asthma and inflammation and even have anti-tumour activity through increased immune response (Rosa et al., 2017; De LeBlanc et al., 2007).

Even though kefir has been touted for its probiotic health benefits the microbes within kefir grains and kefir fermented products have been studied very little. One recent study used high-throughput sequencing and metabolomics to examine the relationship between flavour production and microbes in dairy kefir (Walsh et al., 2016). They identified that earlier stages of fermentation were associated with *Lactobacillus kefiranofaciens* being the dominant bacteria and 'cheesy' and 'fruity' being the dominant flavours, and a shift to *Leuconostoc mesenteroides* in later stages was associated with a more 'buttery' flavour.

One of the described benefits of fermented dairy products such as yoghurt and kefir is the reduction of lactose intolerance (Marlin, 2003). Unfortunately, some individuals are still sensitive to lactose levels within these fermented products. A local Halifax business owner has succeeded in producing a coconut kefir product which is completely free of dairy, made by fermenting coconut milk with traditional kefir grains.

Microbes require certain nutrients to survive and thrive. Prebiotics are consumable products that will promote healthy bacteria. For example, the consumption of indigestible fibers such as inulin have been shown to promote the beneficial *Bifidobacterium* species. To date, even less research exists on prebiotics than probiotics, but they could prove to be a simple and safe method of improving the gut microbiome and overall health.

Plant-derived polyphenols, such as blackberry anthocyanins, are known to have antioxidant and anticancer properties. More recently, these compounds have also been shown to modulate lipid metabolism and obesity. A study by Eposito et al (2015) showed that not only

could blackberry anthocyanins attenuate weight gain and improve glucose metabolism in mice but also demonstrated that these benefits are dependent on the gut microbiome. Administration of antibiotics abolished the metabolic improvements otherwise seen in mice given anthocyanins, indicating that gut microbes play an important role in the metabolism of these compounds. However, the microbiome was not profiled during this study, limiting our knowledge on how anthocyanins alter the microbial community composition. Other studies have demonstrated the capacity of gut microbiome to metabolize anthocyanins and other plant phenols such as ellagic acid, quercetin and a variety of hydroxycinnamic acids (Faria et al., 2014; Rechner et al., 2002; Saha et al., 2016).

1.2.3.3 FECAL TRANSPLANTS

The microbiome is a relatively stable community, which can shift as a result of inflammation from infection or a course of antibiotics. This is commonly what happens in patients who acquire persistent *C. difficile* infections (Crow et al., 2015). Hospital patients who are immunocompromised or who are on antibiotics are susceptible to acquiring the pathogen, which can wreak havoc on the normal microbiome. In some individuals, even after treatment with antibiotics, a healthy microbial community is not able to re-establish and the infection persists, cause severe diarrhea and pain (Culligan & Sleator, 2016). Early clinical trials found that following a fecal transplant from a healthy microbiome donor, these patients had success rates ranging from 83-100% (Chapman et al., 2016). Microbes are isolated from the feces of a healthy person and administered either orally or intestinally through an enema to the patient, who has been given a round of antibiotics. While it is difficult to permanently introduce a handful of

species to your gut, it appears that a complete intestinal community introduced together has the established interactions to be successful. Fecal transplants are not yet considered standard practice, though are being considered as more common place and being tested for the treatment of other diseases, such as IBD (de Groot et al., 2017).

1.3 ANALYSING THE MICROBIOME

1.3.1 MOUSE MODELS

Even though our interest lies in studying human health, there are limitations to studies conducted on humans. It is difficult to standardize a human's daily environment and control for factors that could be influencing their microbiome such as diet, exercise, chemicals and stress. Murine models allow finer control and more flexibility in treatment protocols and easier sample collection. The anatomy and physiology of the mouse gut is similar to that of the human gut and their microbiome are both dominated by two major phyla, *Bacteroidetes* and *Firmicutes* (Nguyen et al., 2015).

One way to study the gut microbiome in mice is to treat them with antibiotics. Usually this is a broad spectrum cocktail used to knock down the largest number of microbes (Langdon et al., 2016). The advantage to this method is that it can be applied at any point during the lifetime, to a variety of different disease models. The disadvantage is mainly that antibiotics can interact with other drugs being administered, or have their own physiological effects on parameters, such as weight (Langdon et al., 2016).

Another method is to use germ-free mice. These are mice raised in a sterile environment, completely uncolonized by bacteria. This allows the experimental comparison to conventionally raised mice that have bacteria, without drugs like antibiotics and enzyme inhibitors as confounding factors. However, an important consideration when using germ-free mice is that normal physiological development has been shown to be altered in these individuals. Altered immunity and organ function are common differences in germ-free mice (Fiebiger et al., 2016).

Similar to germ-free mice, gnotobiotic mice are colonized by known species, added intentionally by researchers. The biggest asset to gnotobiotic models is the ability to "humanize" the microbiome, that is, to colonize mice with microbes from the human gut. Key studies in microbiome research have been made using this technique, including the work by Turnbaugh et al. (2006) showing that lean mice colonized with microbes from overweight humans became overweight despite having similar calories available as control mice had in their food.

1.3.2 SEQUENCING

Since the development of nucleic acid sequencing techniques in the 1960's, these technologies have seen great improvements, especially in the past two decades (Reis-Filho, 2009). The advent of next-generation sequencing technologies has allowed a plethera of new microbiome research due to the high-throughput capabilities and continually decreasing time and cost constraints (Arnold et al., 2016). Early sequencing technologies, such as Sanger sequencing, were only able to process one cultured species at a time. There are thousands of bacterial species in the gut, and it is estimated that the vast majority of them are unculturable in the lab (Eckburg et al., 2005). We have only able to get a small snapshot of the true diversity in microbial

communities (Morgan & Huttenhower, 2012). Since Sanger sequencing was taken over by pyrosequencing and then Illumina technologies, we are now able to characterize whole microbial communities, including eukaryotes and viruses as well as bacteria. Sanger sequencing involves the selection incorporation of chain-terminating nucleotides during DNA replication, followed by separation of the strands by size using gel electroporation. Pyrosequencing removed the need for electroporation using sequencing by synthesis. Nucleotides are selectively added to the reaction and upon incorporation into the growing strand, the release of pyrophosphate is detected using a luciferase-catalyzed reaction (França et al., 2002).

Illumina technologies also use sequencing by synthesis and simultaneous amplification of single-strands of DNA in separate clusters on a flow cell. The fragment is tagged with adaptor regions of DNA that bind to complementary DNA adaptors on the flow cell. The complementary DNA strand is made using the original as a template. The double stranded DNA is denatured and the original DNA strand is washed away, leaving the complementary strand now bound to the flow cell. The strand folds over, so that the DNA adaptor on the other end binds to its complementary adaptor on the flow cell. The complementary strand is then synthesized using this template, and the strands are denatured, leaving two single-strands of DNA attached to the flow cell. The process, known as "bridge-amplification" is repeated many times until a cluster of the forward and reverse DNA fragments is formed. The reverse strands are cleaved and washed away so that only the forward strand is sequenced. Sequencing then begins by attaching a primer to the single strand. Fluorescent primers are added to this primer and the complementary strand grows. Every time a correct nucleotide is added, a fluorescent signal is emitted, which is detected by the sequencer. Each nucleotide has its own signature light signal. Once the strand is synthesized, bridge-amplification occurs once again to allow the reverse strand to be sequenced

(Illumina Inc., 2014). When there is overlap between the forward and reverse reads, they are aligned after sequencing to allow better identification from reference databases.

DNA barcodes can be added to the adaptor sequences, allowing pooling of samples, called 'multiplexing'. Also, despite the number of steps involved in sequencing by synthesis it happens simultaneously in millions of clusters on the flow cells and so that the process happens very rapidly.

When analyzing which microbes are present in a community, it is easiest to sequence a singular gene common to all organisms, the 16S ribosomal RNA gene in bacteria and the internal transcribed spacer 2 (ITS2) in fungi. The 16S gene codes for part of the small ribosomal subunit in bacteria and contains both well-conserved and variable regions (de la Custa-Zuluaga & Escobar, 2016). The ITS2 region in a non-transcribed gene spacer that is excised when the small ribosomal subunit of the rRNA matures and is widely variable among species (Ji et al., 2017). Primers are designed to span one or two variable regions, which have enough genetic separation to allow grouping into species. Sequences that are 97% identical are grouped into what are known as operational taxonomic units (OTUs), and this is the threshold for species assignment.

In order to determine microbial functions present in a community, metagenomics sequencing is performed using Illumina technologies. In this case all of the DNA in a sample is sequenced, and then sequenced DNA reads are compared to a reference database in order to determine which genes are present.

1.3.3 BIOINFORMATIC APPROACHES & REFERENCE DATABASES

Reference sequence databases are an important part of sequencing analysis. They allow the annotation of relevant information to the millions of base pairs of DNA that have been obtained. Both metagenomic and 16S sequencing analyses use databases to assign information to sequencing data. In microbiome data, taxonomy is the classification assigned to each organism found in the sample, generally at the species level where possible.

With metagenomic data both taxonomy and function can be assigned to sequence reads. For example, a common method of assigning functions to metagenomic data is to use the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa & Goto, 1999) at the level of gene ortholog groups, modules, or pathways. To assign taxonomy to metagenomic data, a tool known as MetaPhlAn compares sequencing reads to a number of reference genomes using clade specific genes (Truong et al., 2015).

One of the most popular databases in this research is the Greengenes 16S OTUs database which provides a vast catalog of full length 16S sequences from previously cultured and environmentally sampled microbes. Similarly, the ITS UNITE database was used to assign fungal reads to ITS2 sequencing data (Abarenkov et al., 2010). While it is useful to assign OTUs using a reference database that has species annotation, no microbial database is by any means complete. Assigning taxonomy using only a reference database is known as “closed-reference OTU picking”. To avoid losing information from reads that do not map to an OTU in the reference database, the remaining sequences are assigned to OTUs “de novo”, meaning they are clustered in groups of 97% sequence identify and assigned a novel identification number. This is known as “open-reference OTU picking”. It is also possible to assign all OTUs completely de

novo, known as “de novo OTU picking”, which can be useful for datasets with many unknown species.

The OTU assignments are output into a biome formatted table. The information given in the table is the number of times each OTU is observed in each sample. It is important to note that the abundances of the OTUs are relative, since some of the raw sequences were filtered for quality. Singletons, OTUs that are only observed once, are also filtered out of the final table to reduce the computational burden in later analyses as well as to remove some noise from the data (McDonald et al., 2012).

There are two measures of diversity used to analyze the taxonomic data. The first is alpha diversity, which is a measure of species richness in each sample. There are a number of methods to measure alpha diversity but the simplest is to count the number of different OTUs observed in a sample. The sample with the highest number of different OTUs is considered to have high alpha diversity, or species richness. The second is beta diversity, which is a measure of the compositional diversity between samples. Even if two samples have the same number of bacterial species, if the taxa are different or the overlapping taxa are present at very different levels then the samples are dissimilar. Beta diversity is calculated in a matrix, where the levels of each OTU is compared across all samples. Taxa can also be weighted by phylogenetic distance.

1.4 OBJECTIVES

The main objectives of the studies presented in this thesis are to confirm factors that affect the gut microbiome. While it has been shown that diet plays an important role in shaping

these communities, our studies focus on the impacts of exercise, food supplements and probiotics.

The first objective was to understand the microbial communities in the mouse gut as a result of moderate exercise. The second objective, was to analyze the impact of fruit-derived antioxidants on the gut microbiome in a model of lung cancer. The third objective was to study microbial succession in the production of a dairy-free, coconut kefir product.

We hypothesized that since exercise has beneficial physiological effects on inflammation, which is related to changes in the gut microbiome, that we would see shifts in the gut microbiome as a result of the exercise regimens. The gut microbiome has been shown to play a role in the metabolism of antioxidant compounds such as anthocyanins, and so we also proposed that the haskap anthocyanins would also influence the bacterial communities. While coconut kefir is made with traditional kefir bacteria and yeasts, we were unsure what kind of ecological succession we would see in the microbial community of this dairy-free version of the fermented beverage.

CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMAL WORK

2.1.1 EXERCISE STUDY

Forty-two 6-10 week old C57BL6 mice (eleven male, thirty-one female) were obtained from Charles River Laboratory (Canada). All mice were housed individually on a 12-h light/dark cycle in the University Animal Care facility for the duration of the experiment. Mice were assigned to either a voluntary exercise group (n=10), a forced exercise group (n=11) or a non-exercise control group (n=21), and were caged singularly during the exercise period. All protocols were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the Dalhousie University Committee on Laboratory Animals.

2.1.1.1 VOLUNTARY EXERCISE PROTOCOL

Twenty female mice were used for the eight-week voluntary exercise portion of the study. Female mice were chosen as it has been reported that they perform higher levels of voluntary physical activity than male mice (Lightfoot et al., 2004) and we wanted to maximize exercise volume to maximize the microbiome effects observed. Mice were acclimatized to individual housing for one week and then were randomly assigned to a voluntary wheel running (VE) group (n = 10), or a sedentary control (VC) group (n = 10). Mice in the VE group were housed individually in cages that contained running wheels giving the mice 24 hour access to the running wheel. Each running wheel was connected to a data-logger, which counted the number of wheel revolutions per day for the 55 day exercise protocol. Using the diameter (d) of the

wheel, total number of running wheel revolutions were converted to metres travelled per day (πd).

2.1.1.2 FORCED EXERCISE PROTOCOL

Eleven mice (five female, six male) were randomly assigned to a forced treadmill running (FE) group. Both male and female mice were chosen to compare sex differences in the effects of exercise on the microbiome. While Allen et al. (2015), reported microbiome differences due to voluntary exercise in male mice, our observations on voluntary exercise in female mice did not reveal any microbiome shifts. Mice were acclimatized to individual housing for one week, and then FE mice acclimatized to the treadmill for five days prior to starting the training protocol. Following acclimation to the treadmill, a forced running protocol was administered for six weeks. Five days a week, FE mice were run for 40 minutes, starting at a speed of 15 m/min for weeks one and two, and increasing by 2.5 m/min every two weeks, so that by weeks five and six mice were running at a speed of 20 m/min. Benito et al. (2011), have previously defined 60 minutes of treadmill running at 36 m/min as vigorous exercise for rats, and we therefore defined our forced treadmill running protocol (40 minutes at 15-20 m/min) as moderate exercise in mice. Control (FC) mice were placed in a clean, empty cage for a comparable amount of time to mimic handling stress. Mice were trained on the LE8700 Single Lane Treadmill equipped with a rest platform and an electronic control unit (Panlab Harvard Apparatus). When mice stopped running they were gently nudged off the rest platform and back onto the treadmill belt.

2.1.1.3 DIET AND BODY MASS COMPOSITION

All exercising and control animals had ad libitum food (Prolab RMH 3000, LabDiet, Brentwood, MO) and water. Food was weighed and distributed to each mouse's cage every seven days, and uneaten food from the previous seven days was weighed to determine how much food had been consumed.

To assess body composition mice were weighed every two weeks. Dual-energy X-ray absorptiometry (DEXA) was also used to assess body mass composition of the mice. In brief, mice were anesthetized with isofluorane and placed in a prostrate position. They were scanned using a Lunar PIXImus2 (GE Medical Systems) DEXA machine. Whole-body scans, minus the head, were taken and bone marrow density (g/cm^2), bone marrow content (g), body area (cm^2), lean mass (g) and fat mass (g) were determined. DEXA scans were taken at week-6 of both exercise protocols.

2.1.1.4 FECAL AND MUCOSAL SAMPLE COLLECTION

Starting on day zero of the experimental timeline, fecal samples were collected on a bi-weekly basis by placing each mouse in a separate clean cage, waiting until they passed fecal pellets, and then transferring these to autoclaved microfuge tubes using sterile forceps. Fecal samples were stored at -80ºC until they were prepared for analysis.

At the end time point of both experimental protocols mice were sacrificed by cervical dislocation while under isofluorane anesthesia. The terminal half of the colon was removed from the animals and any fecal contents were flushed out with cold PBS using a rat feeding tube. An

incision was then made longitudinally along the colon and mucosal contents were scraped off using a glass coverslip and deposited into a microfuge tube. All fecal and mucosal samples were frozen immediately using liquid nitrogen and stored at -80°C.

2.1.2 HASKAP ANTHOCYANIN STUDY

Fifty 4-week old female A/J mice were obtained from Charles River Laboratory (Canada). A/J mice are a strain that is genetically susceptible to developing tumours when exposed to carcinogens. Tobacco smoke, as well as its isolated carcinogens, have been administered to A/J mice, which subsequently develop lung tumours (Ge et al., 2015; Witschi, 2004). All mice were housed individually on a 12-h light/dark cycle in the Carleton Animal Care facility for the duration of the experiment. There was a one week adaptation period to the single housing before the experimental treatments were started.

2.1.2.1 HASKAP STUDY TIMELINE

Mice were assigned to one of six treatment groups for the 25-week duration of the study. Regular mouse chow was abundantly available to all treatment groups throughout the experimental period. Two were control groups: (A) Fed regular mouse diet for the duration of the study, injected with saline as a sham and (B) Fed haskap berry extract (0.2 g/mouse) daily for the duration of the study, with saline injected as a sham. Four were experimental groups, all injected with a single dose (100mg/1kg body weight) of nicotine-derived nitrosamine (NNK) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone after three weeks of the study: (C) Fed normal

mouse food for the duration of the study (D) Fed haskap berry extract for three weeks and fed normal mouse diet for the following 22 weeks, (E) Fed haskap berry extract for the duration of the study and (F) Fed normal mouse food for three weeks and fed haskap berry extract for the following 22 weeks. Tobacco smoke contains thousands of chemicals and its unrefined administration to mice dilutes the tumour producing effect of some of its more potent carcinogens. Toxicology of many of these compounds has been assessed and NNK has been found to be a potent activator of tumorigenesis in the mouse lung (Witschi, 2004). Increased levels of NNK metabolites in the urine have also been associated with increased risk of lung cancer in humans (Peterson, 2016).

Three weeks into the study, mice were injected intraperitoneally with either saline or 100mg/kg body weight NNK (2mg in 0.1ml saline), as described in the study timeline. Haskap berry extracts were administered by mixing with powdered regular chow, in the form of a jelly cube. During the adaptation period prior to the experimental time period, all mice were given daily placebo jelly cubes to prepare them for the dietary supplementation. Ethical endpoints were administered to mice who showed serious signs of acute toxicity in response to the NNK, including severe difficulty in breathing, immobility and abnormal vocalization.

2.1.2.2 FECAL SAMPLE COLLECTION

At the experimental endpoint, fecal samples were collected from each mouse's cage and stored in 15 ml sterile Falcon tubes at -20ºC until they were required for analysis.

2.2 COCONUT KEFIR STUDY

2.2.1 COCONUT KEFIR PRODUCTION

Coconut kefir is made using a method very similar to traditional dairy kefir. First, the kefir grains, which are kept in dairy milk at 4°C, are removed from the fridge and left to rest until they come to room temperature (about 1 hour). Then, the grains incubate for another hour in coconut milk, to prime them for overnight fermentation. Finally, grains are transferred to fresh coconut milk and left to ferment overnight. The product is bottled and sealed and stored at 4°C.

2.2.2 KEFIR SAMPLES

A schematic of the sampling timeline outlining collection at four different stages is illustrated in Figure 18. Grains were sampled: 1) after incubating in dairy milk, 2) after incubating in coconut milk and 3) after the overnight fermentation. Milk samples were also taken at these time points, with the addition of a sample of fresh coconut milk. Samples were taken in triplicate at all time points for initial 16S sequencing. Eleven samples were then taken of the final product for metagenomic sequencing. All samples were stored at -80°C until analysis.

2.3 DNA ISOLATION, LIBRARY PREPARATION AND SEQUENCING

DNA was isolated from fecal and mucosal samples using the PowerFecal™ DNA Isolation Kit (MO BIO Laboratories, Catalog #: 12830-50), and from kefir milk and grain

samples using the PowerFood™ DNA Isolation Kit (MO BIO Laboratories, Catalog #: 21000-50). In brief, a tube containing garnet or glass beads and lysis buffer, samples are heated and then homogenized by bead-beating (Disruptor-Genie). After centrifugation, non-DNA organic and inorganic cell contents are precipitated from the supernatant. A high-concentration salt solution is then added to the supernatant to allow DNA to selectively bind the silica membrane of a spin-filter column. After being bound to the column and washed, purified DNA is eluted in low-salt conditions.

Variable regions V6-V8 of bacterial 16S ribosomal RNA genes were amplified from all purified DNA using the PCR conditions and primers from Comeau et al. (2011), modified for use on the Illumina MiSeq. The V4-V5 region was also amplified for the exercise study samples using primers and conditions from Walters et al. (2015). Finally, in the kefir study the internal transcribed spacer region 2 (ITS2) was also amplified from samples using the primers and conditions from Walters et al. (2015). The forward and reverse primers used Nextera Illumina index tags and sequencing adapters fused to the 16S and ITS2-specific sequences. Each sample was amplified with a different combination of index tags to allow for sample identification after multiplex sequencing. Following amplification, paired-end 300+300 bp V3 sequencing was performed for all samples on the Illumina MiSeq.

Metagenomic DNA libraries were also prepared from the final coconut kefir milk samples using the Illumina Nextera XT kit. Briefly, samples are "tagmented" (enzymatically "sheared" and tagged with adaptors), PCR amplified while adding barcodes, purified using columns or beads, normalized using Illumina beads or manually, then pooled for loading onto the NextSeq.

2.4 SEQUENCING ANNOTATION

2.4.1 16S RIBOSOMAL RNA GENE ANNOTATION

Analysis of 16S sequencing data was done on a Linux virtual machine, using the Microbiome Helper workflow, specific to 16S analysis, obtained from GitHub (https://github.com/mlangill/microbiome_helper/wiki/16S-standard-operating-procedure). Paired-end reads were stitched together using PEAR (Zhang et al., 2014) and then low quality reads that are less than 400 base pairs long and have less than 90% of their bases at a quality score of 30 or more and were filtered. Chimeras were removed using VSEARCH (https://github.com/torognes/vsearch). OTUs were generated within QIIME (Caporaso et al., 2010) through the open-reference OTU picking protocol (Rideout et al, 2014) at 97% identity against the GreenGenes database v13_5 (McDonald et al., 2012). Open-reference picking assigns OTUs by first mapping sequence reads first to a reference genome database using SortMeRNA (Kopylova et al., 2012). Any sequences that fail to align with known sequences are aligned *de novo*, meaning that they are clustered with each other based on similarity using SumaClust (https://git.metabarcoding.org/obitools/sumaclust/wikis/home). OTUs with low counts (based on a dynamic cutoff of 0.1% of the total number of sequences per sample) were removed, which has been previously shown to ensure that the number of OTUs is accurately represented (Comeau et al., 2017). The graphical software package STAMP (Parks et al., 2014) was also used to determine whether levels of individual taxa were significantly altered using unpaired t-test with Benjamini-Hochberg FDR and a p-value cutoff of 0.05

Functions of genes and biochemical pathways can also be inferred using PICRUSt software (Langille et al., 2013). Taxonomy identified using QIIME (Kuczynski et al., 2011) is searched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) and known functions are retrieved and assigned. Comparison of functional pathways can then be visualized in STAMP (Parks et al., 2014).

2.4.2 INTERNAL TRANSCRIBED SPACER ANNOTATION

Analysis of ITS2 sequencing data was done on a Linux virtual machine, using the Microbiome Helper workflow, specific to ITS2 analysis, obtained from GitHub (https://github.com/mlangill/microbiome_helper/wiki/ ITS2-Fungi-Standard-Operating-Procedure). The taxonomic analysis was similar to that of the 16S data, except that the ITS UNITE (Abarenkov et al., 2010) database was used instead of GreenGenes. No functional analysis was done on the fungal data.

2.4.3 METAGENOMIC ANNOTATION

Analysis of metagenomic sequencing data was done on a Linux virtual machine, using the Microbiome Helper workflow, specific to metagenomic analysis, obtained from GitHub (https://github.com/mlangill/microbiome_helper/wiki/Metagenomic-standard-operating-procedure). Paired-end reads were stitched together using PEAR (Zhang et al., 2014) and Bowtie2 (Langmead & Salzberg, 2012) was run to screen out contaminant sequences, in this case reads that map to PhiX viral and human genomes. Then low quality reads that are less than 400

base pairs long and have less than 90% of their bases at a quality score of 30 or more and were filtered using Trimmomatic (Bolger et al., 2014). Taxonomy of sequences was identified using MetaPhlAn2 (Truong et al., 2015) and functional assignment was given using HUMAnN (Abubucker et al., 2012). Taxonomic and functional information was examined using STAMP (Parks et al., 2014).

2.5 STATISTICAL METHODS

For all analyses, the p-value cutoff is 0.05, and standard error is reported. Once 16S and ITS2 data has been generated, sample composition can be visualized in one of two ways. The simplest method is to summarize the taxa in stacked barplots using the designated QIIME script. Barplots allows visualization of the proportions of microbial species in a sample or pooled samples at any taxonomic level (phyla down to species). To test for differences in individual taxa between samples, statistical evaluation can be performed in STAMP. To compare two samples, a Welch's two-sided t-test can be performed with Benjamin-Hochberg false discovery rate (FDR) correction in STAMP. To compare individual taxa across multiple experimental groups, and ANOVA test can also be performed in STAMP, with Benjamin-Hochberg FDR.

2.5.1 ALPHA DIVERSITY (SPECIES RICHNESS)

Species richness of each sample was calculated by counting the number of observed OTUs. Bar plots were used to illustrate the observed OTUs in each treatment group, comparisons of the alpha diversity in different groups were done using two sample *t*-tests.

2.5.2 BETA DIVERSITY (COMMUNITY STRUCTURE)

In order to look at the overall variation between samples, UniFrac principal coordinate axis plots were generated using information from the OTU table to illustrate microbial diversity between treatment and control samples in each study (Lozupone & Knight, 2005). Linear equations were fitted to the data, so that each equation explained the most amount of variation possible (principal components). The three largest principal components (PCs) were then assigned to the X-, Y- and Z-axes of a three-dimensional plot. Each sample is assigned a value based on its PC and plotted, with relative proximity to other samples in three-dimensional space correlating to sample similarity. The distance between each sample can be weighted to take into account phylogenetic relationships between the microbes. This is known as weighted UniFrac. Unweighted UniFrac only takes into account the counts of each different species of microbe. These PCs can then be visualized in a program called Emperor. Samples are plotted in 3D space along the top three PC axes, which explain the most amount of variation in the data.

In order to test for statistical significance of the distance matrix, QIIME uses a script that performs an Adonis test (Caporaso et al., 2010). Adonis is a non-parametric multivariate analysis of variance which in this case compares the abundance of each bacteria in a sample to its abundance in other samples. It tests the null hypothesis that the bacterial composition of the samples is the same in control and exercise groups. The R^2 value is the effect size and indicates the percent variation that can be explained by the tested variable (Anderson, 2001).

2.5.3 MACHINE LEARNING

In the exercise study, in order to examine subtle changes in community structure, we employed a form of machine learning, known as supervised learning, where features (in this case OTUs present in the samples) are used to predict the class (experimental condition) to which a sample belongs. Fecal OTU data from the two exercise cohorts was input into the python software Scikit-Learn (Pedregosa et al., 2011) to build separate random forests (RF) models. Using a leave-one-out cross-validation method, the models were trained to classify samples from both cohorts as either exercise or control, based on their OTU profile. A parameter search from 1 to 30 trees was tested to determine the highest accuracy. Accuracy was reported as the mean of 100 iterations of modeling and testing. Importance of features as output by Random Forests were averaged across the iterations and were used to determine the taxa with the highest contribution to classification model.

The random forests model was run with the OTU data 100 times, for both the voluntary and forced cohorts. The average weights for each OTU were computed and the top 30 OTUs for each cohort were selected. The model was run again 100 times using only the selected OTUs and the average classification accuracy for samples at each time point was calculated using both true and randomized sample labels.

2.6 INFLAMMATORY CYTOKINE ANALYSIS

At the end time point of both experimental protocols mice were sacrificed by cervical dislocation while under isofluorane anesthesia. The chest cavity was then rapidly opened, the

aorta was cut and blood was collected from the chest cavity and placed in a 2 ml microfuge tube. The blood sample was allowed to sit at room temperature for 15 minutes and then spun at 10,000g for 15 minutes at 4°C. The serum was then removed and stored at -80°C until required for analysis.

Inflammatory marker serum concentrations (IL-1β, KC, IL-10, TNFα) were measured in blood samples collected two days post-exercise-endpoint using a custom mouse multiplex assay (Biorad, Catalog #: 12002231). The assay was prepared according to manufacturer's instructions and read using a MagPix multiplex reader (Biorad). Initial serum samples were diluted by a factor of four. A one-way ANOVA was used to determine if any differences existed between groups for the cytokines.

CHAPTER 3: MODERATE EXERCISE HAS LIMITED BUT DISTINGUISHABLE EFFECTS ON THE MOUSE MICROBIOME

3.1 EXERCISE AND THE MICROBIOME

In this study, we investigate the direct effects of exercise on the gut microbiome using both voluntary and moderate forced exercise models in 6-10 week old C57BL6 mice, while controlling for diet and measuring changes in food intake, body mass composition, and host immunological expression. In the voluntary cohort, one of the exercise mice had to be euthanized after coming in contact with potentially contaminated facility floors, leaving 10 control mice and 9 exercise mice for analysis. In the forced cohort, one of the exercise mice did not regain consciousness after being anaesthetized for a routine blood draw, leaving 11 control mice (6 female, 5 male) and 10 exercise mice (5 female, 5 male) for analysis.

3.2 VOLUNTARY BUT NOT FORCED EXERCISE RESULTS IN HIGHER FOOD INTAKE AND LEAN MASS IN MICE

In the voluntary wheel running cohort, each voluntary exercise (VE) mouse had access to a running wheel at all times. The cumulative distance (metres) travelled by each VE mouse was totaled for the eight weeks. Mean total distance for the group was calculated at 138,565 m, with the mice running an average of 2.5 ± 0.7 km a day. In the forced treadmill running group, each exercise mouse ran for 40 minutes, five days a week. The cumulative distance (metres) travelled by each forced exercise (FE) mouse was 21 km. The distance run per day was 600 m in weeks one and two, 700 m in weeks three and four and 800 m in weeks five and six. Food intake was

measured per mouse on a weekly basis for the duration of the experiments (seven weeks for the voluntary cohort, starting at week 1, and six weeks for the forced cohort, starting at week 0). A schematic of the experimental timeline is shown in **Figure 1**. Cumulative food intake was averaged for each week (**Figure 2**). Two-sample *t*-tests for equal means were done to compare mean cumulative food intake between exercise and control mice at each time point. Mice in the voluntary control (VC) group ate significantly less food overall than their voluntary exercise (VE) counterparts, starting at week two ($p < 0.05$). Mice in the forced exercise (FE) group had comparable food intake to the forced controls (FC) at each time point ($p > 0.5$).

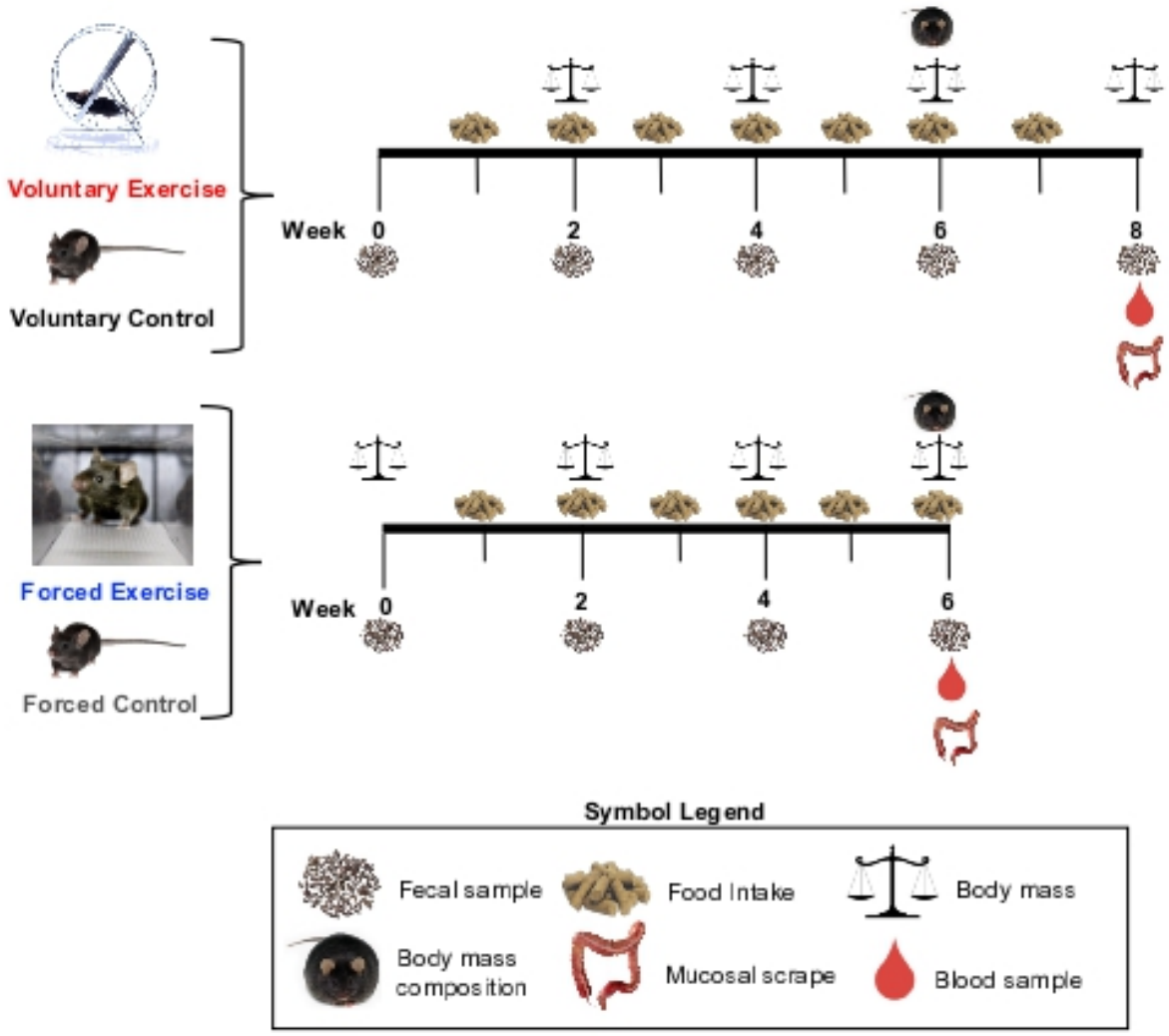

Figure 1 Experimental timeline for voluntary and forced exercise cohorts. Voluntary wheel running took place over 8 weeks, while forced treadmill running was performed over 6 weeks. Fecal and weight samples were taken every two weeks, while blood and mucosal samples were taken at the experimental endpoint. DEXA scans for body mass composition were performed at Week 6 of both studies. Number of mice in each cohort: Voluntary exercise: n = 9; Voluntary control: n = 10; Forced exercise: n = 10; Forced control: n = 11.

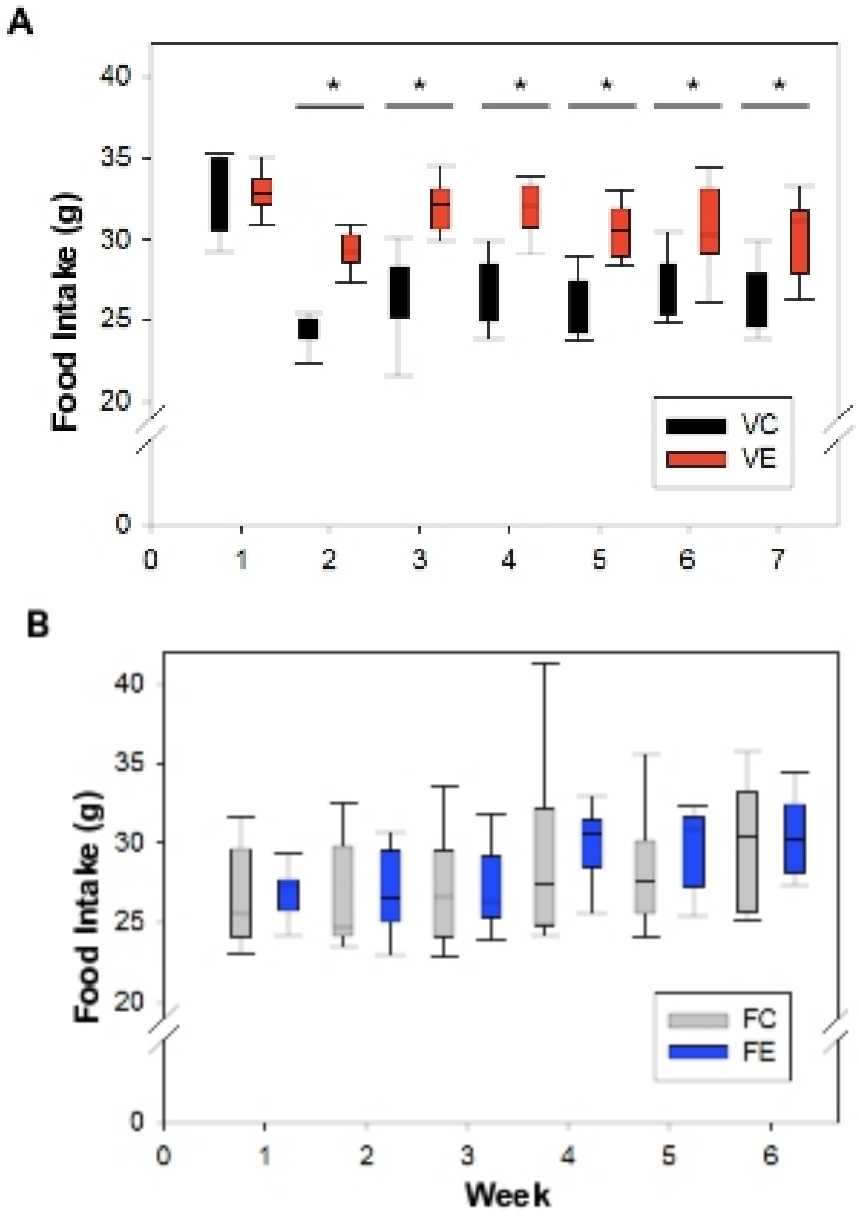

Figure 2 Voluntary but not forced exercise alters food consumption in mice. Box plots depict average weekly food intake (grams) of exercise and control mice for A) voluntary exercise and B) for the forced exercise cohorts. Comparisons were conducted using two sample t-tests with a significance cut off of $p < 0.05$. Significant differences at time points are indicated with an asterisk. Number of mice in each cohort: VE: n = 9; VC: n = 10; FE: n = 10; FC: n = 11.

Body mass measurements for all mice, in grams, were taken every two weeks (starting at week two for the voluntary cohort and week zero for the forced cohort) of the experimental timeline (**Figure 3A-B**). Two-sample *t*-tests for equal means were done to compare mean body weight between exercise and control mice at each time point. Body mass was not statistically different between control and exercised mice in either cohort at any time point ($p > 0.5$). DEXA scans showed that lean body mass (calculated as a percentage of total body mass; **Figure 3C-D**) was significantly different between voluntary exercise mice and controls (VE = 83.6 $\pm$ 1.2%, VC = 80.8 ± 0.5%, $p= 0.046$), but did not differ between forced exercisers and controls ($p > 0.1$). When the forced exercise cohort was divided into male and female, there was no significant difference between exercisers and controls.

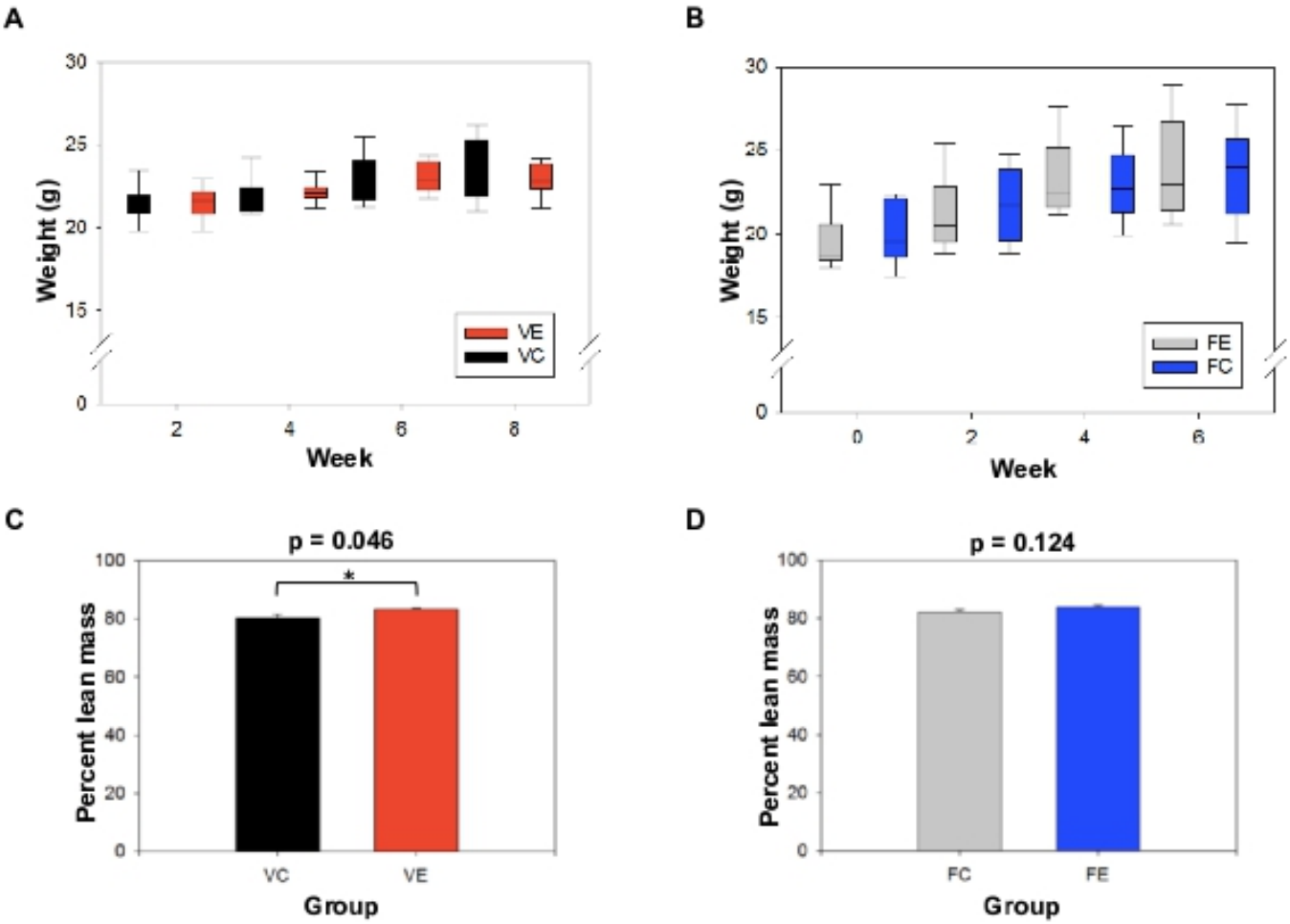

Figure 3 Voluntary but not forced exercise promotes lean body mass in mice. Box plots depict average biweekly body mass measurements of exercise and control mice for (A) voluntary exercise and (B) forced exercise cohorts. Bar graphs (2C-D) illustrate percent lean body mass for voluntary ($p = 0.046$) and forced ($p = 0.124$) cohorts, respectively. Comparisons were conducted using two sample t-tests with a significance cut off of $p < 0.05$. Significant differences at time points are indicated with an asterisk. Number of mice in each cohort: VE: n = 9; VC: n = 10; FE: n = 10; FC: n = 11.

3.3 VOLUNTARY AND FORCED EXERCISE HAVE NO MEASURABLE EFFECT ON BACTERIAL DIVERSITY IN THE MOUSE MICROBIOME

Post-filtering, the average sequence coverage for fecal and mucosal samples, respectively, was 23558 and 28865 seqs/sample. For comparison of microbial communities across experimental groups, fecal and mucosal samples were normalized to a depth of 8585 and 7081 reads/sample, respectively. Four fecal samples and two mucosal samples were excluded due to low coverage.

Fecal samples were taken every two weeks in both cohorts, while mucosal samples were taken at the experimental endpoint. There was no significant difference in alpha diversity (species richness) between exercise and control mice at week 8 in the voluntary ($p = 0.180$) or at week 6 in the forced ($p = 0.227$) cohorts (**Figure 4A-B**). Species richness for mucosal samples was calculated in the same fashion (**Figure 4C-D**) and was also not found to be different in either cohort (voluntary: $p = 0.337$, mucosal: $p = 0.289$). Taxonomic data from fecal samples was also used to generate weighted UniFrac beta-diversity principal coordinate axis plots for each time point using weighted (**Figure 5**) and unweighted UniFrac beta-diversity measurements (**Figure 6)**. An Adonis test did not reveal a significant difference in community structure between the fecal samples at each time point for the voluntary (W0: $R^2 = 0.0476$, $p = 0.493$; W2: $R^2 = 0.0751$, $p = 0.257$; W4: $R^2 = 0.0746$, $p = 0.203$; W6: $R^2 = 0.0226$, $p = 0.933$; W8: $R^2 = 0.0836$, $p = 0.146$) or forced cohorts (W0: $R^2 = 0.0370$, $p = 0.775$; W2: $R^2 = 0.0399$, $p = 0.634$; W4: $R^2 = 0.0576$, $p = 0.386$; W6: $R^2 = 0.0496$, $p = 0.414$) when using weighted UniFrac. Weighted (**Appendix – Figure 26**) and unweighted (**Appendix – Figure 27**) UniFrac beta-diversity plots were also generated using the mucosal data collected in the final week of each

study. An Adonis test did not reveal a significant difference in community structure between the mucosal samples at each time point for the voluntary (weighted: W8: $R^2 = 0.0941$, $p = 0.16$; unweighted: W8: $R^2 = 0.0746$, $p = 0.081$) or forced cohorts (weighted: W6: $R^2 = 0.0392$, $p = 0.756$; unweighted: W6: $R^2 = 0.0535$, $p = 0.682$). Altering the beta-diversity measurement to unweighted UniFrac or Bray-Curtis (at different taxonomic levels) did not result in statistical significance (data not shown). Statistical comparisons of relative abundances of individual taxa did not reveal any significant differences at any taxonomic level after multiple test correction.

A comparison of sex differences in the forced cohort was also performed using an Adonis test. At the final week of the experiment, no differences in microbial beta-diversity from the fecal samples was found between the male and female mice in either the control (W6: $R^2 = 0.1126$, $p = 0.453$) or exercise group (W6: $R^2 = 0.1246$, $p = 0.185$). This data is plotted in **Appendix - Figure 28**.

A comparison of the fecal microbiomes all mice from both cohorts at the beginning of each experiment was performed using an Adonis test. Mice microbiomes were significantly different between cohorts in both weighted (W0: $R^2 = 0.7858$, $p = 0.001$) and unweighted (W0: $R^2 = 3868$, $p = 0.001$) UniFrac beta-diversity plots as illustrated in **Appendix – Figure 29.**

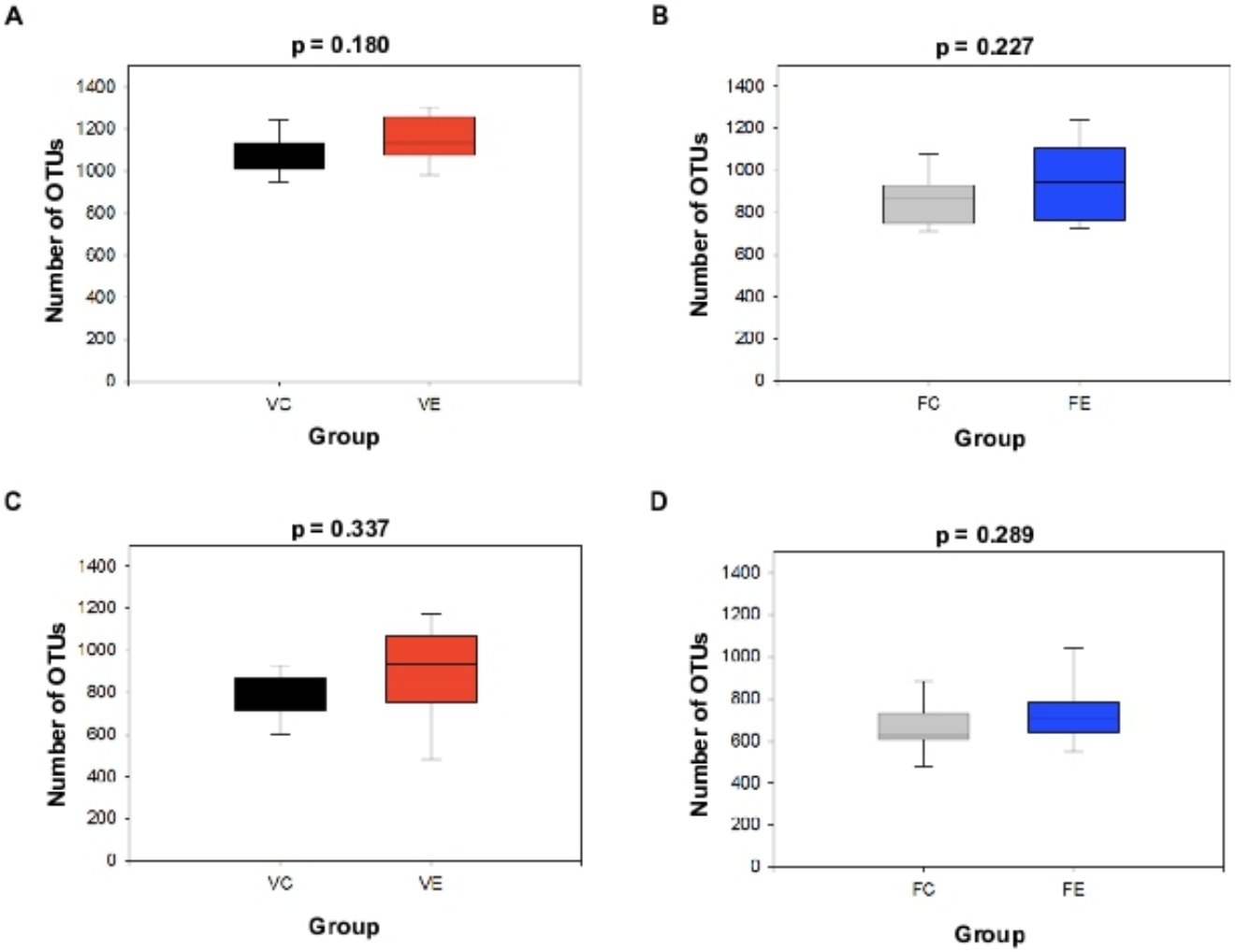

Figure 4 Voluntary and forced exercise do not affect species richness in the mouse gut. The number of OTUs from each final week (week 8 for VE and week 6 for FE) fecal and mucosal sample were counted and counts per sample were averaged for each experimental group. Box plots illustrate average species richness of control and exercise groups for both voluntary fecal (3A, $p = 0.180$) and mucosal (3C, $p = 0.337$) samples and forced fecal (3B, $p = 0.227$) and mucosal (3D, $p = 0.289$) samples. Comparisons were done using students t-tests. Number of mice in each cohort: VE: $n = 9$; VC: $n = 10$; FE: $n = 10$; FC: $n = 11$.

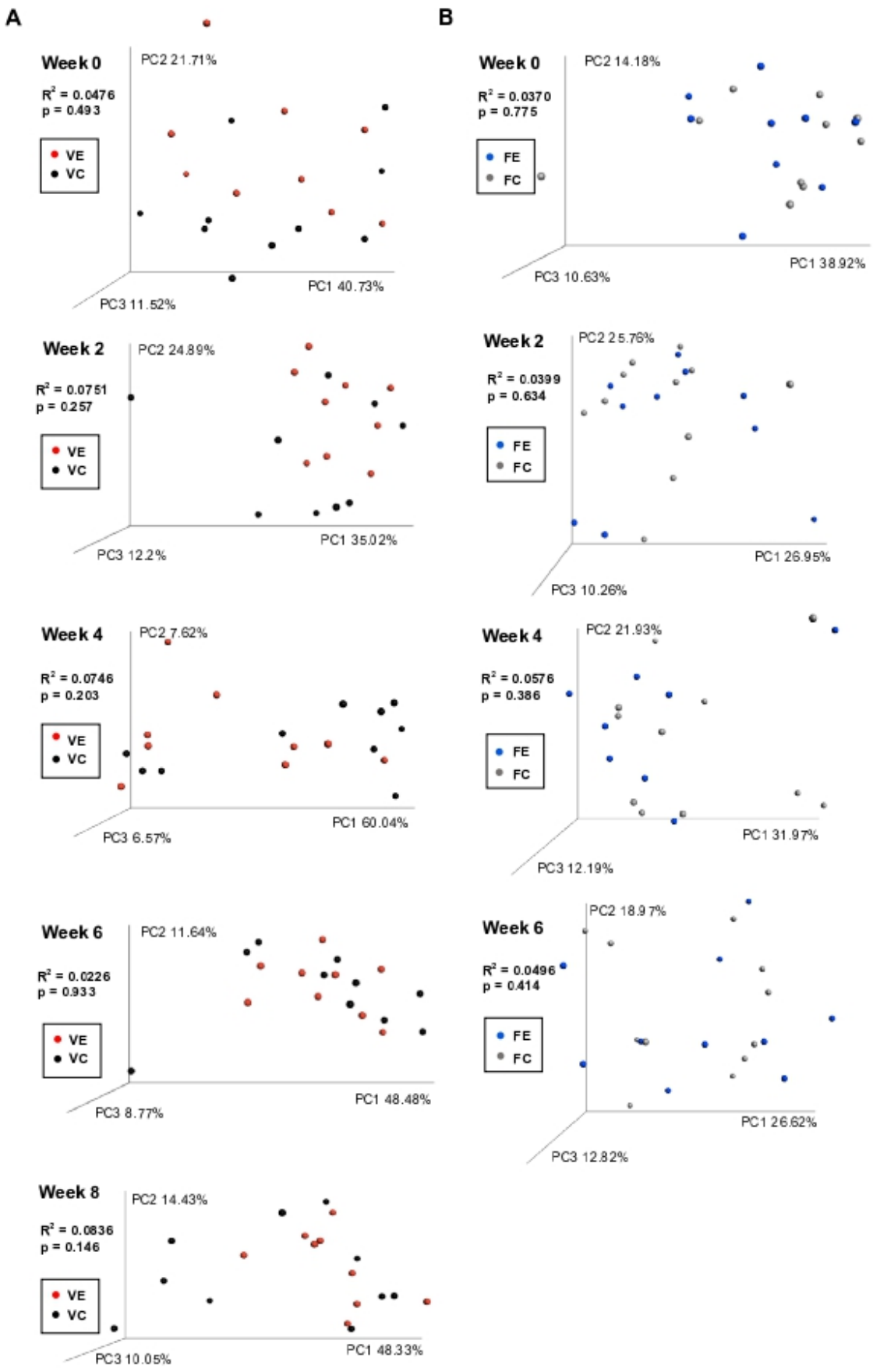
A
Week 0
PC2 21.71%
R² = 0.0476
p = 0.493
VE
VC
PC1 40.73%
PC3 11.52%
Week 2
PC2 24.89%
R² = 0.0751
p = 0.257
VE
VC
PC1 35.02%
PC3 12.2%
Week 4
PC2 7.62%
R² = 0.0746
p = 0.203
VE
VC
PC1 60.04%
PC3 6.57%
Week 6
PC2 11.64%
R² = 0.0226
p = 0.933
VE
VC
PC1 48.48%
PC3 8.77%
Week 8
PC2 14.43%
R² = 0.0836
p = 0.146
VE
VC
PC1 48.33%
PC3 10.05%
B
Week 0
PC2 14.18%
R² = 0.0370
p = 0.775
FE
FC
PC1 38.92%
PC3 10.63%
Week 2
PC2 25.76%
R² = 0.0399
p = 0.634
FE
FC
PC1 26.95%
PC3 10.26%
Week 4
PC2 21.93%
R² = 0.0576
p = 0.386
FE
FC
PC1 31.97%
PC3 12.19%
Week 6
PC2 18.97%
R² = 0.0496
p = 0.414
FE
FC
PC1 26.62%
PC3 12.82%

Figure 5 Weighted gut microbial diversity of voluntary and forced exercise mice. Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of exercise and control mice from (A) voluntary (W0: $R^2 = 0.0476$, $p = 0.493$; W2: $R^2 = 0.0751$, $p = 0.257$; W4: $R^2 = 0.0746$, $p = 0.203$; W6: $R^2 = 0.0226$, $p = 933$; W8: $R^2 = 0.0836$, $p = 0.146$) and (B) forced (W0: $R^2 = 0.0370$, $p = 0.775$; W2: $R^2 = 0.0399$, $p = 0.634$; W4: $R^2 = 0.0576$, $p = 0.386$; W6: $R^2 = 0.0496$, $p = 0.414$) exercise cohorts. Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: VE: $n = 9$; VC: $n = 10$; FE: $n = 10$; FC: $n = 11$.

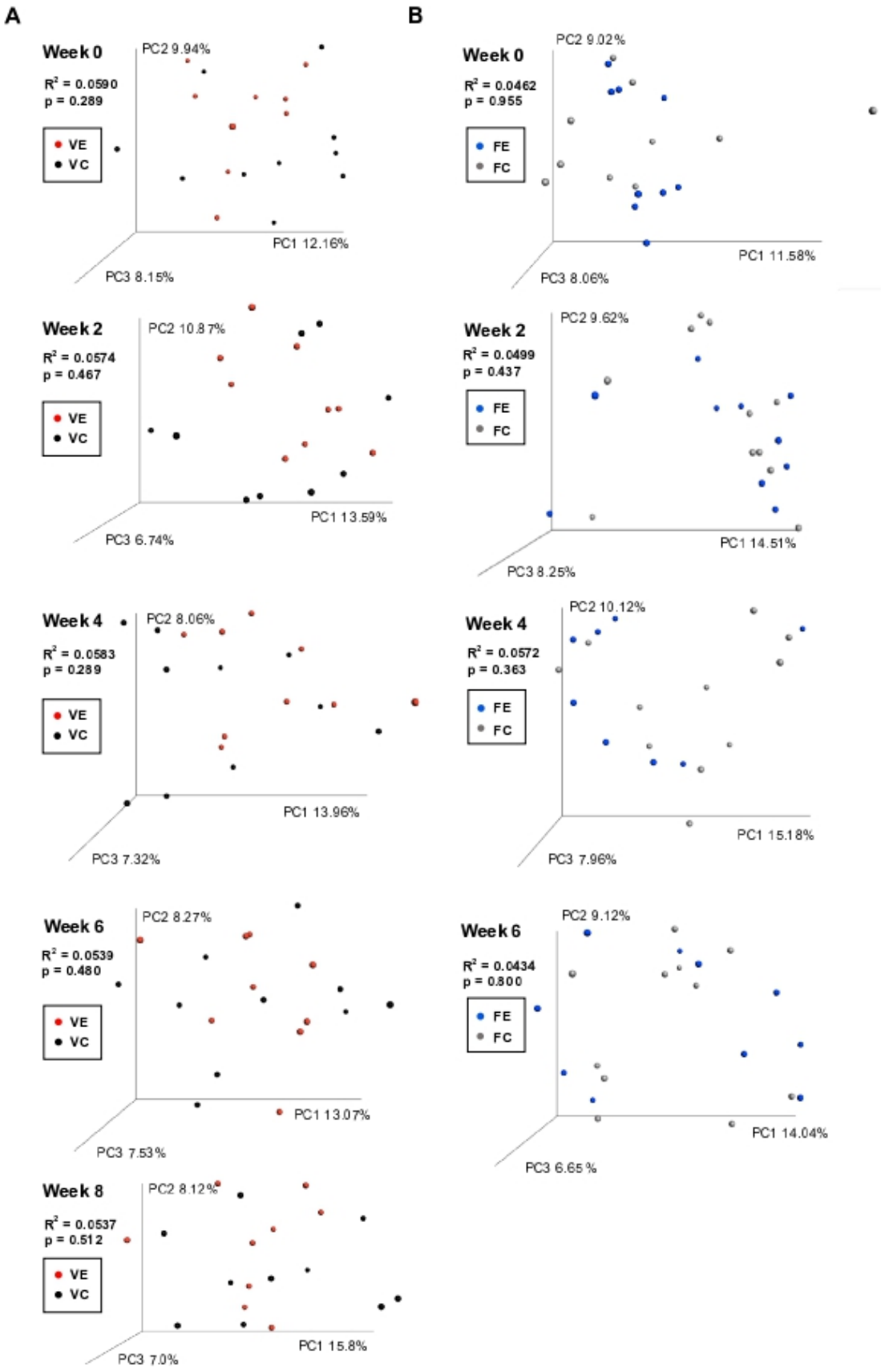
A
Week 0
R² = 0.0590
p = 0.289
VE
VC
PC2 9.94%
PC1 12.16%
PC3 8.15%
Week 2
R² = 0.0574
p = 0.467
VE
VC
PC2 10.87%
PC1 13.59%
PC3 6.74%
Week 4
R² = 0.0583
p = 0.289
VE
VC
PC2 8.06%
PC1 13.96%
PC3 7.32%
Week 6
R² = 0.0539
p = 0.480
VE
VC
PC2 8.27%
PC1 13.07%
PC3 7.53%
Week 8
R² = 0.0537
p = 0.512
VE
VC
PC2 8.12%
PC1 15.8%
PC3 7.0%
B
Week 0
R² = 0.0462
p = 0.955
FE
FC
PC2 9.02%
PC1 11.58%
PC3 8.06%
Week 2
R² = 0.0499
p = 0.437
FE
FC
PC2 9.62%
PC1 14.51%
PC3 8.25%
Week 4
R² = 0.0572
p = 0.363
FE
FC
PC2 10.12%
PC1 15.18%
PC3 7.96%
Week 6
R² = 0.0434
p = 0.800
FE
FC
PC2 9.12%
PC1 14.04%
PC3 6.65%

Figure 6 Unweighted gut microbial diversity of voluntary and forced exercise mice.

Unweighted UniFrac principal coordinate axis plots compare gut microbial diversity of exercise and control mice from (A) voluntary (W0: $R^2 = 0.0590$, $p = 0.289$; W2: $R^2 = 0.0574$, $p = 0.467$; W4: $R^2 = 0.0583$, $p = 0.289$; W6: $R^2 = 0.0539$, $p = 480$; W8: $R^2 = 0.0537$, $p = 0.512$) and (B) forced (W0: $R^2 = 0.0462$, $p = 0.955$; W2: $R^2 = 0.0499$, $p = 0.437$; W4: $R^2 = 0.0572$, $p = 0.363$; W6: $R^2 = 0.0434$, $p = 0.800$) exercise cohorts. Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: VE: $n = 9$; VC: $n = 10$; FE: $n = 10$; FC: $n = 11$.

3.4 MODERATE EXERCISE DOES NOT ALTER EXPRESSION OF INFLAMMATORY MARKERS

The results showed that serum levels of IL-1β, IL-6, TNFα did not differ significantly between the voluntary and forced exercise groups nor did the concentrations differ between either exercise group and the controls. The only marker found to have significantly different expression was KC, a neutrophil marker, homologous to CXCL-8 in humans (**Table 1**). KC was increased by almost 45% in voluntary exercisers alone ($p << 0.001$).

Table 1 Inflammatory cytokine profiles of control and exercise mice

Group	IL-1β (pg/ml)	IL-6 (pg/ml)	TNFα (pg/ml)	KC (pg/ml)
Controls	212.79 ± 16.32	3.76 ± 0.40	155.68 ± 23.78	26.77 ± 1.45
Voluntary Exercisers	227.10 ± 13.32	3.87 ± 0.45	173.02 ± 30.63	38.52 ± 1.52[a]
Forced Exercisers	238.90 ± 11.07	4.96 ± 0.95	229.47 ± 60.37	27.85 ±1.93

[a] Significantly different as compared to control group

3.5 MACHINE LEARNING IDENTIFIES SHIFTS IN THE MOUSE GUT MICROBIOME IN RESPONSE TO EXERCISE

Using Scikit-Learn [25], a machine learning classification method known as Random Forests, was trained and tested using a leave one out approach on the OTU tables. In comparison to statistical comparisons of single taxa, machine learning can identifying shifts in community structure that involves multiple taxa. Machine learning was able to distinguish between the microbiomes of VE and VC mice with 97% accuracy at week eight, and between FE and FC mice with 86% accuracy at week six (**Figure 7**). Compared to a randomized model (where sample labels are randomized), the exercise microbiome could be accurately classified after six weeks of forced exercise and eight weeks of voluntary exercise (**Figure 7**).

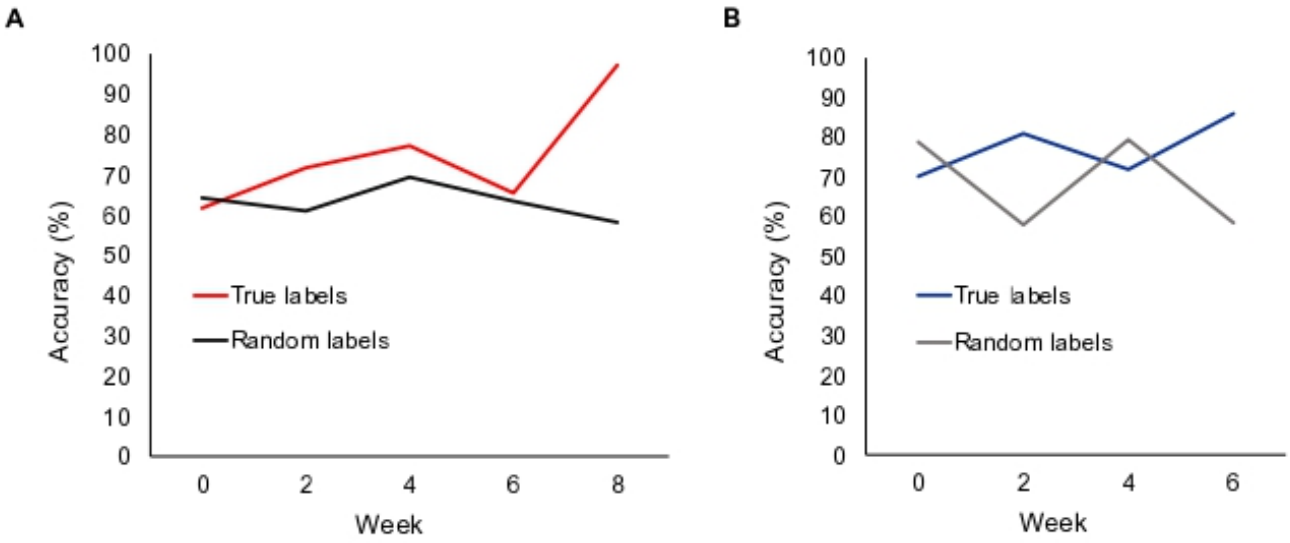

Figure 7 Accuracy of random forests model in classifying exercise versus control samples. Sample OTU tables from exercise and control fecal samples for (A) the voluntary and (B) the forced exercise experiments was used to train a random forests classifier. Accuracy of the model using true category labels is plotted over time for both voluntary (97% at week 8) and forced (86% at week 6) cohorts. Accuracy using randomized category labels is also plotted over time for the voluntary (58% at week 8) and forced (58% at week 6) cohorts.

The most important features/OTUs for classification as determined by the Random Forests model were inspected (**Table 2, 3**). We observed that in the voluntary exercise cohort, out of the top 30 taxa, 23 belong to the phylum *Bacteroidetes*, four to *Firmicutes*, two to *Proteobacteria* and one to *Actinobacteria*. Of the 23 taxa in the *Bacteroides* phylum, 18 are part of the *S24-7* family, four are *Bacteroidaceae* and one is *Rikenellaceae*. The four *Firmicutes* fall under the order *Clostridiales* (**Table 2**). In the forced exercise cohort, 24 out of the top 30 taxa were from the *Firmicutes* phylum, while six were *Bacteroidetes*. Out of the 24 taxa in the *Firmicutes* phylum, 19 are in the order *Clostridiales*, four are in *Lactobacillales* and one did not have an assigned order. Five out of the six taxa in the *Bacteroidetes* phylum have the *Bacteroides* genus, and the other is a *Parabacteroides* (**Table 3**).

Table 2 Top 30 OTUs important in classifying control versus voluntary exercise samples

OTU ID	Phylum	Order	Family	Genus	Weight
213896	Firmicutes	Clostridiales			0.13141
332364	Firmicutes	Lactobacillales	Lactobacillaceae	Lactobacillus	0.09947
259111	Firmicutes	Lactobacillales	Lactobacillaceae	Lactobacillus	0.0786
184484	Firmicutes	Clostridiales	Lactobacillaceae		0.06421
259372	Firmicutes	Lactobacillales	Lactobacillaceae	Lactobacillus	0.05582
186871	Firmicutes	Clostridiales			0.05352
New. ReferenceOTU231	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.04629
2740953	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.04055
4417335	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.03515
New.ReferenceOTU3155	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.03174
347496	Firmicutes	Clostridiales			0.02939
274380	Firmicutes	Clostridiales			0.02882
New.ReferenceOTU7685	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.02678
344527	Firmicutes	Clostridiales	Ruminococcaceae	Ruminococcus	0.02443
230268	Firmicutes	Clostridiales	Ruminococcaceae	Ruminococcus	0.02365
New.Cleanup.ReferenceOTU10206	Firmicutes	Lactobacillales	Lactobacillaceae	Lactobacillus	0.02333
316428	Firmicutes	Clostridiales	Ruminococcaceae	Oscillospira	0.02314
192111	Bacteroidetes	Bacteroidales	Porphyromonadaceae	Parabacteroides	0.02216
New.CleanUp.ReferenceOTU6362	Firmicutes	Clostridiales	Mogibacteriaceae		0.02062
534498	Firmicutes				0.02026
New.ReferenceOTU1251	Firmicutes	Clostridiales			0.01929
New.CleanUp.ReferenceOTU2315	Firmicutes	Clostridiales			0.01335
4402077	Firmicutes	Clostridiales			0.01333
New.ReferenceOTU15378	Firmicutes	Clostridiales			0.01333
210073	Firmicutes	Clostridiales			0.01191
New.ReferenceOTU10920	Firmicutes	Clostridiales	Ruminococcaceae	Ruminococcus	0.0116
258969	Firmicutes	Clostridiales	Lachnospiraceae		0.01128
New.CleanUp.ReferenceOTU28	Firmicutes	Clostridiales			0.01076
258283	Firmicutes	Clostridiales	Lachnospiraceae		0.0097
New.ReferenceOTU15157	Firmicutes	Clostridiales	Ruminococcaceae	Oscillospira	0.00602

Green indicates taxa that increased with exercise and red indicates taxa that decreased with exercise.

Table 3 Top 30 OTUs important in classifying control versus forced exercise samples

OTU ID	Phylum	Order	Family	Genus	Weight
New.CleanUp.ReferenceOTU352	Bacteroidetes	Bacteroidales	S24-7		0.09377
348680	Proteobacteria	Enterobacteriales	Enterobacteriaceae		0.07741
568410	Bacteriodetes	Bacteroidales	S24-7		0.0549
New.ReferenceOTU52	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.05477
New.ReferenceOTU71	Bacteriodetes	Bacteroidales	S24-7		0.05009
New.ReferenceOTU193	Bacteriodetes	Bacteroidales	S24-7		0.04908
258910	Bacteroidetes	Bacteroidales	S24-7		0.04517
266203	Firmicutes	Clostridiales			0.04439
New.ReferenceOTU393	Bacteroidetes	Bacteroidales	S24-7		0.0421
New.ReferenceOTU19449	Bacteroidetes	Bacteroidales	S24-7		0.0399
New.ReferenceOTU10	Bacteriodetes	Bacteroidales	S24-7		0.03804
New.ReferenceOTU83	Bacteriodetes	Bacteroidales	S24-7		0.03677
348821	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.03671
264325	Bacteriodetes	Bacteroidales	Rikenellaceae		0.03401
336676	Bacteriodetes	Bacteroidales	S24-7		0.03109
New.ReferenceOTU542	Bacteriodetes	Bacteroidales	S24-7		0.03093
177435	Bacteriodetes	Bacteroidales	S24-7		0.02969
162639	Bacteroidetes	Bacteroidales	S24-7		0.02783
New.ReferenceOTU1066	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	0.02718
307416	Firmicutes	Clostridiales			0.02566
311482	Bacteriodetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.02298
214159	Bacteriodetes	Bacteroidales	S24-7		0.02174
New.ReferenceOTU31060	Bacteriodetes	Bacteroidales	S24-7		0.01995
549837	Proteobacteria	Pseudomonadales			0.0151
215214	Bacteriodetes	Bacteroidales	S24-7		0.01309
New.ReferenceOTU505	Bacteriodetes	Bacteroidales	Bacteroidaceae	Bacteroides	0.0128
180555	Firmicutes	Clostridiales	Lachnospiraceae	Coprococcus	0.01198
349175	Bacteriodetes	Bacteroidales	S24-7		0.00691
New.CleanUp.ReferenceOTU14118	Firmicutes	Clostridiales			0.00402
New.ReferenceOTU25958	Bacteriodetes	Bacteroidales	S24-7		0.00197

Green indicates taxa that increased with exercise and red indicates taxa that decreased with exercise.

3.6 SUMMARY

Contrary to previous studies (Cook et al., 2013; Allen et al., 2015), our initial observations of bacterial diversity indicate limited alterations to the microbiome in response to moderate exercise. Inflammatory profiles were also not found to be altered from this exercise. It is important to note that cytokines in the peripheral blood as well as muscle tissue could reveal changes that were not observed using blood collected from the chest cavity upon sacrifice. However, a supervised Random Forest trained model was able to classify mice as sedentary or exercising based on their microbiome with 97% accuracy for voluntary exercise modality and 86% for forced exercise modality. Our results illustrate that exercise has a moderate but measurable effect on gut microbial communities in mice. Compared to other known environmental drivers such as diet, moderate exercise may play a more limited role in shaping the gut microbiome. These methods can be used to provide important insight into other factors affecting the microbiome and our health. Our results are from healthy, young, non-obese mice, and more study is needed to understand the dynamics and interplay between exercise and these other important factors on the human microbiome.

CHAPTER 4: EFFECTS OF HASKAP POLYPHENOLS ON THE MOUSE MICROBIOME IN EXPERIMENTAL MODELS OF LUNG CANCER

4.1 ANTHOCYANINS

Haskap berries, originating in Japan and Russia, are extremely rich in polyphenols, including anthocyanins. A study being conducted at Dalhousie University to examine the antitumour potential of haskap berry extracts in in a mouse lung cancer model. We took this opportunity to examine impact that haskap berry extracts affect the gut microbiome in this model.

4.2 NNK BUT NOT HASKAP REDUCES SPECIES RICHNESS IN MICE

As defined in the methods, mice were assigned to one of the following groups: (A) Fed regular mouse diet for the duration of the study, injected with saline as a sham (B) Fed haskap berry extract daily for the duration of the study, with saline injected as a sham. (C) Fed normal mouse food for the duration of the study and injected with NNK after three weeks of the study (D) Fed haskap berry extract for three weeks, injected with NNK and fed normal mouse diet for the following 22 weeks, (E) Fed haskap berry extract for the duration of the study and injected with NNK after three weeks (F) Fed normal mouse food for three weeks, injected with NNK and fed haskap berry extract for the following 22 weeks. To simplify the analyses, groups D, E and F, which all had exposure to both NNK and haskap extract were combined. Although there were originally 50 mice in the study, it was not possible to obtain fecal samples from all mice due to

ethical endpoints and time constraints. An outline of the experimental timeline is shown in **Figure 8.**

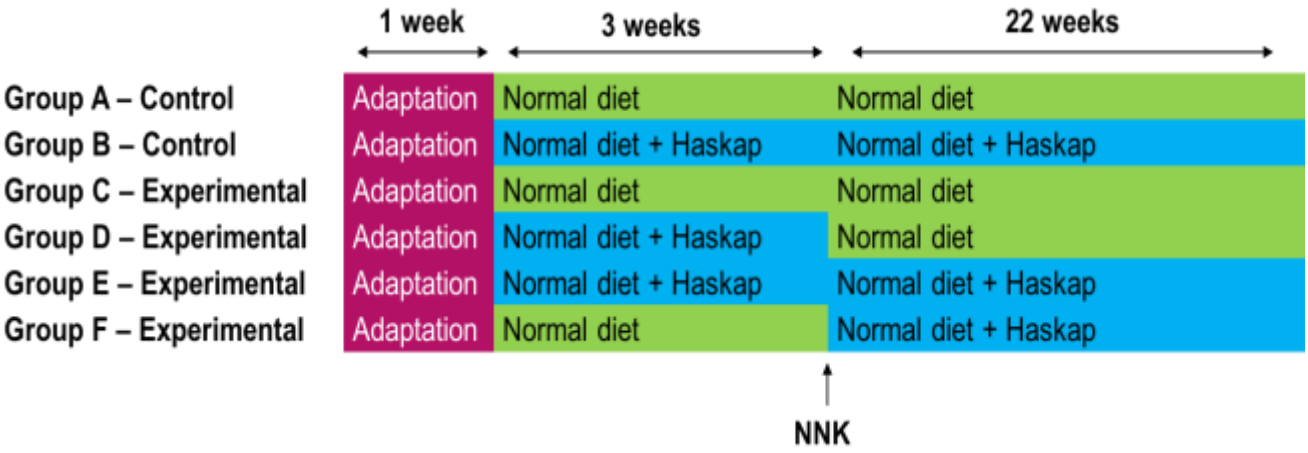

Figure 8 Experimental timeline for haskap administration in a mouse model of lung carcinoma. Regular mouse diet was available to all the treatment groups throughout the experimental period. Apart from their regular chow, haskap berry extracts were fed (0.2 g/ mouse) by mixing it with powdered regular chow for the treatment groups as presented in Figure 1 (Normal diet + haskap). A placebo, their normal diet was fed accordingly (normal diet). Saline was injected to groups A and B as a sham, and NNK was injected (single dose of 100 mg/ 1kg body weight ip.) to the mice in groups C, D, E, and F.

After taxonomic assignment of sequences, observed OTUs were counted for each group and compared via t-test. Species richness in the microbiome of the control group fed a normal diet was compared to that of the control group administered haskap extract. A two-sample *t*-test revealed that there was no difference in the number of observed OTUs between the two control groups ($p = 0.507$), as shown in **Figure 9.** Species richness in the microbiome of the experimental NNK group fed a normal diet was compared to that of the NNK group administered haskap extract. A two-sample *t*-test revealed that there was no difference in the number of observed OTUs between the two experimental groups ($p = 0.953$), as shown in **Figure 9.** Species richness in the microbiome of the control mice was compared to that of the mice that received NNK. A two-sample *t*-test revealed more OTUs in the control group, compared to the mice receiving NNK ($p = 0.018$), as shown in **Figure 9.**

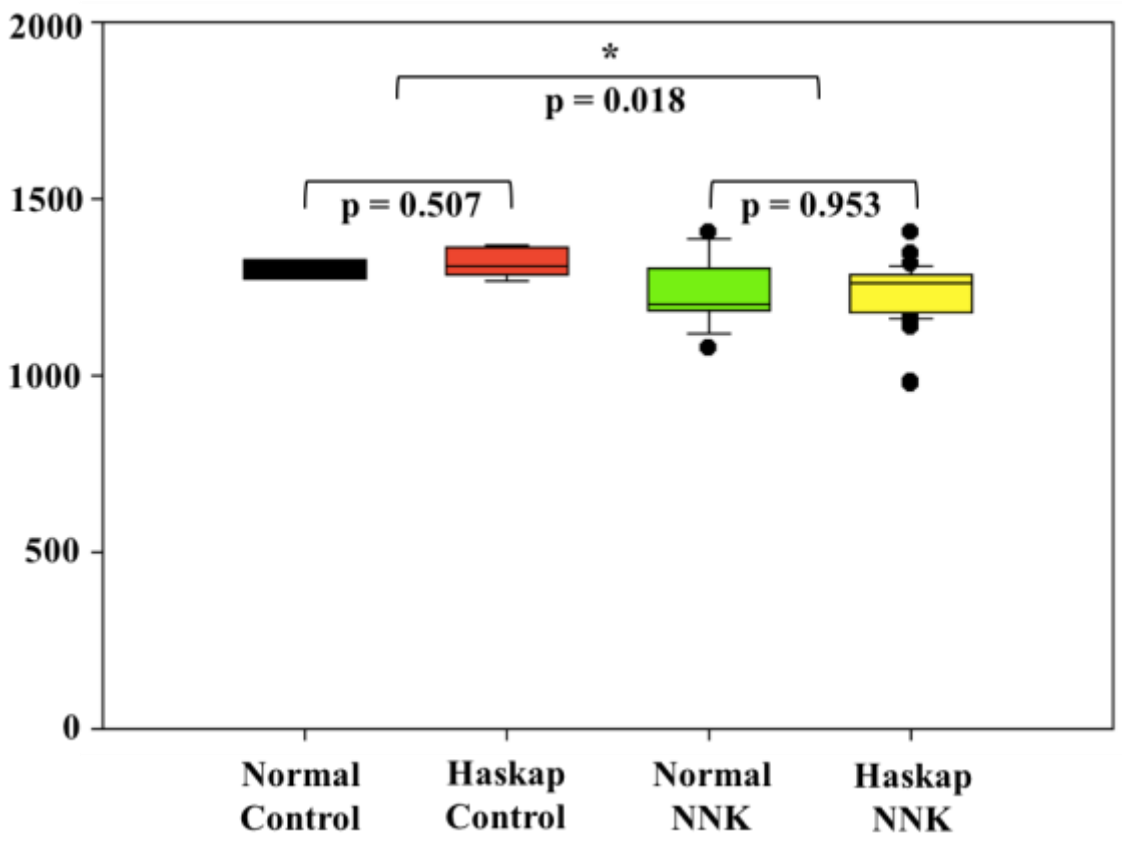

Figure 9 NNK carcinogen but not haskap anthocyanins reduce species richness in the mouse gut. The number of OTUs from each sample were counted and averaged for each experimental group. Box plots illustrate average species richness of control and NNK groups for both normal and haskap diet. Comparisons were done using paired t-tests. There was no difference in species richness between haskap and normal diet for either controls ($p = 0.507$) or NNK ($p = 0.953$). When average species richness of all control samples was compared to that of all the NNK samples, it was found that NNK samples has lower species richness ($p = 0.018$).

4.3 HASKAP AND NNK ALTER MICROBIAL DIVERSITY IN MICE

Taxonomic data from fecal samples was used to generate weighted UniFrac beta-diversity principal coordinate axis plots for all samples using weighted UniFrac beta-diversity measurements (**Figure 10**). An Adonis test revealed a significant difference in community structure between the fecal samples when comparing all groups using weighted UniFrac (R^2 = 0.1478, p = 0.005). However, statistical comparisons of relative abundances of individual taxa did not reveal any significant differences at any taxonomic level after multiple test correction.

When the weighted UniFrac beta-diversity of fecal samples from control mice were compared with an Adonis test, it was found that mice who consumed haskap extract had significantly (R^2 = 2505, p = 0.046) different community structure (**Figure 11**). When fecal samples from the NNK treated mice were compared, it was found that mice who consumed haskap extract also had significantly (R^2 = 0.1170, p = 0.003) different community structure (**Figure 12**).

When the weighted UniFrac beta-diversity of fecal samples from mice that consumed regular mouse diet were compared with an Adonis test, it was found that mice who had received NNK had significantly (R^2 = 0.1921, p = 0.024) different community structure from those who had not (**Figure 13**). It was also found that using multiple group comparison with Benjamin-Hochberg FDR multiple test correction in STAMP, that *Bacteroides acidifaciens* was significantly different across all experimental groups (p = 0.040). Both NNK groups had higher levels of *B. acidifaciens* compared to control groups (**Figure 14**). Mice who received both NNK and normal diet had higher levels than those who received both NNK and haskap.

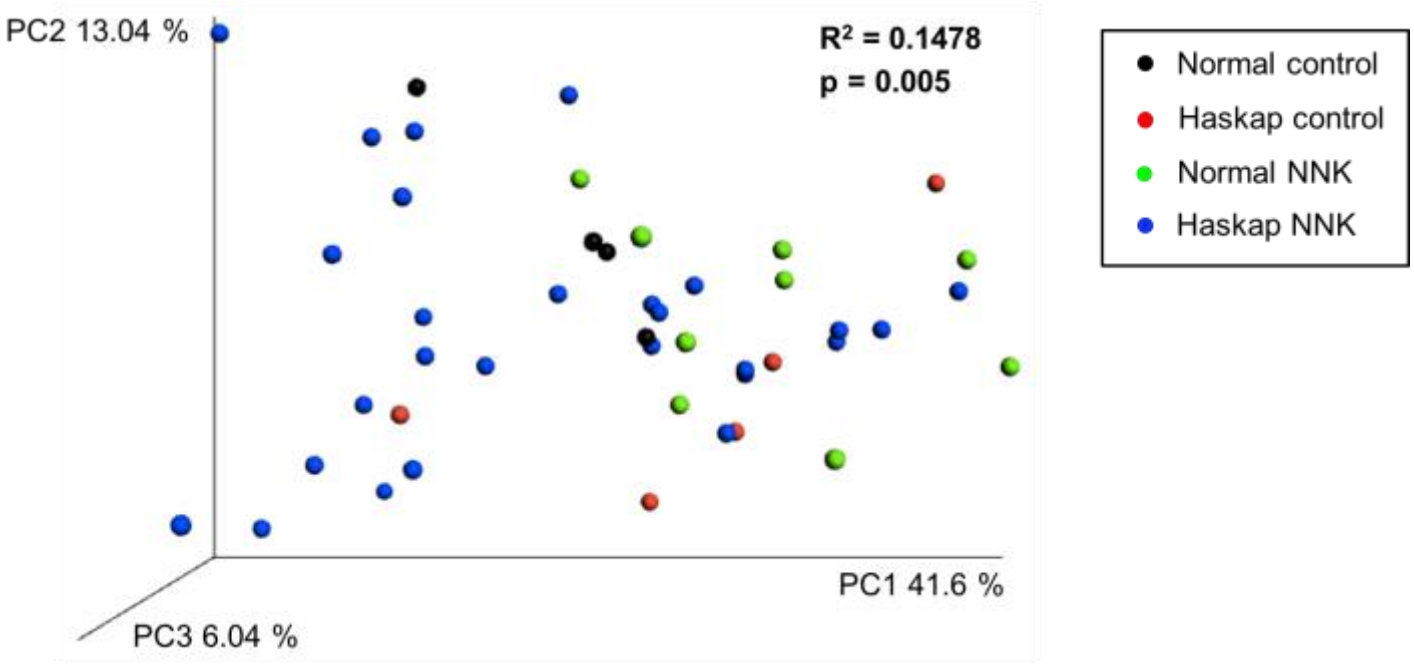

Figure 10 Comparison of microbial diversity in mice treated with or without NNK and receiving either normal or haskap diet. Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of all experimental cohorts. An Adonis test revealed a significant correlation between treatment group and microbial diversity ($R^2 = 0.1478$, $p = 0.005$).

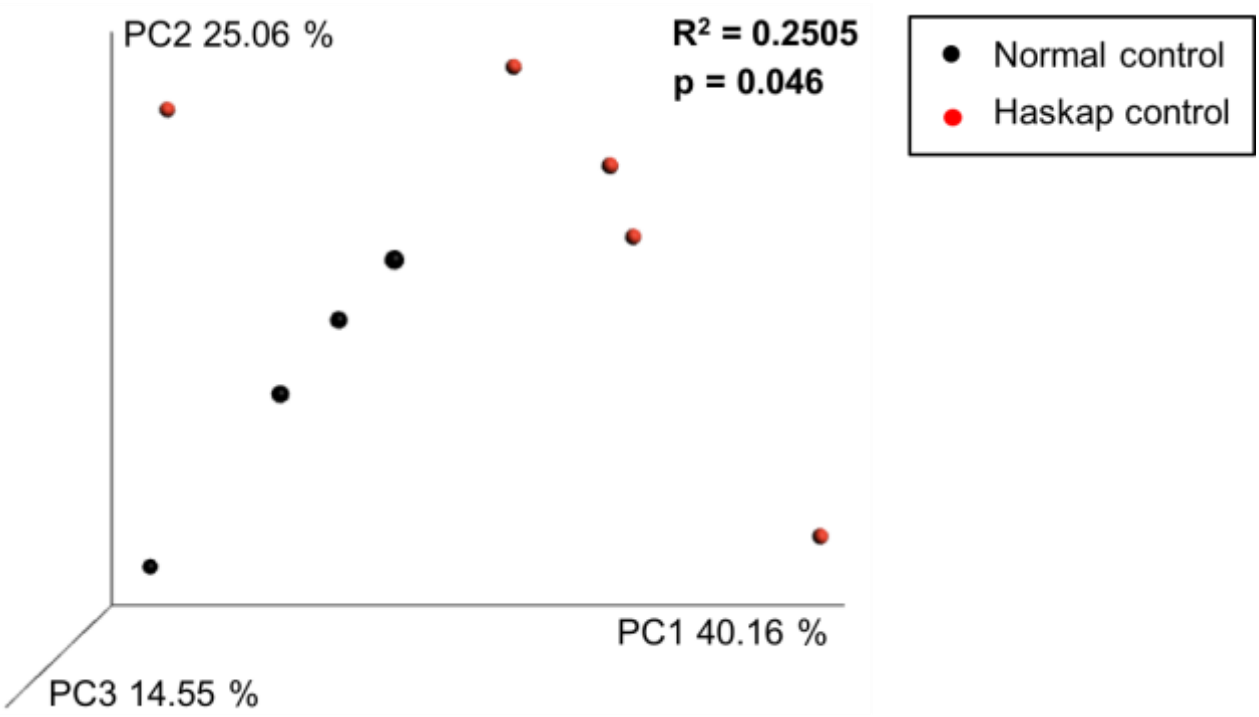

Figure 11 Haskap diet alters gut microbial diversity in healthy mice. Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of control mice receiving normal and haskap diets. An Adonis test revealed a significant correlation between diet type and microbial diversity ($R^2 = 0.2505$, $p = 0.046$).

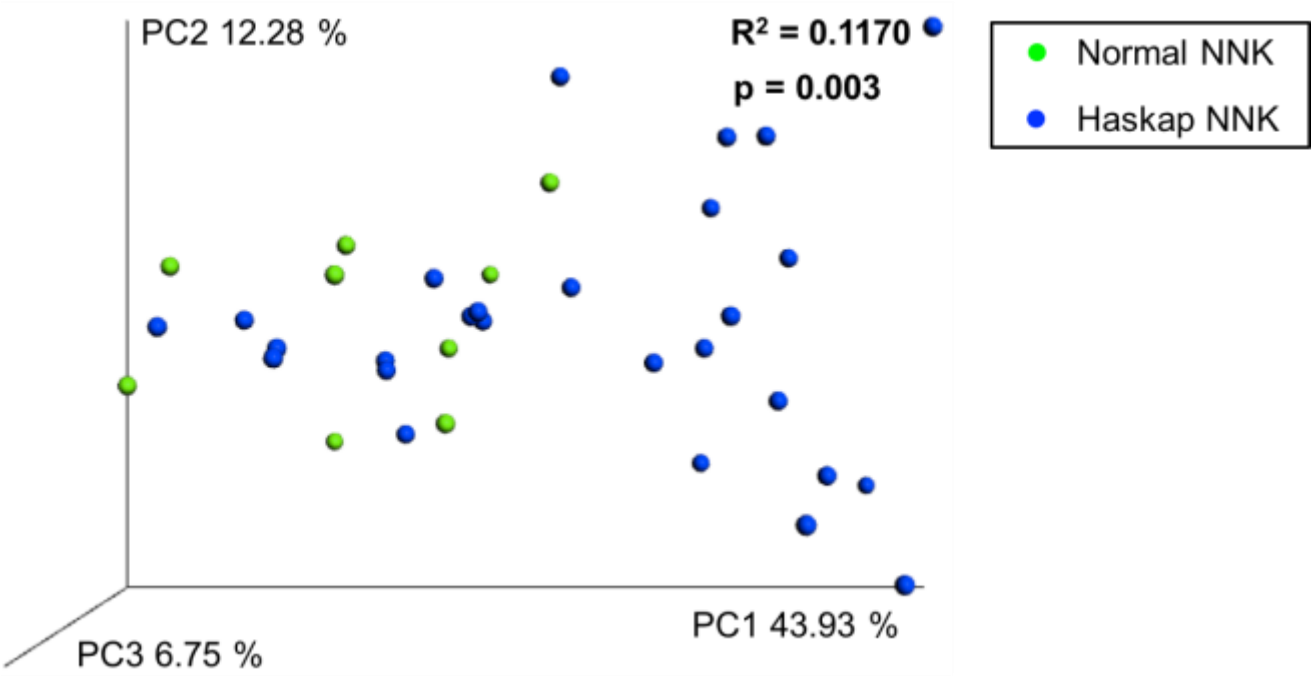

Figure 12 Haskap alters gut microbial diversity in a mouse model of lung carcinoma.

Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of NNK treated mice receiving normal and haskap diets An Adonis test revealed a significant correlation between diet type and microbial diversity ($R^2 = 0.1170$, $p = 0.003$).

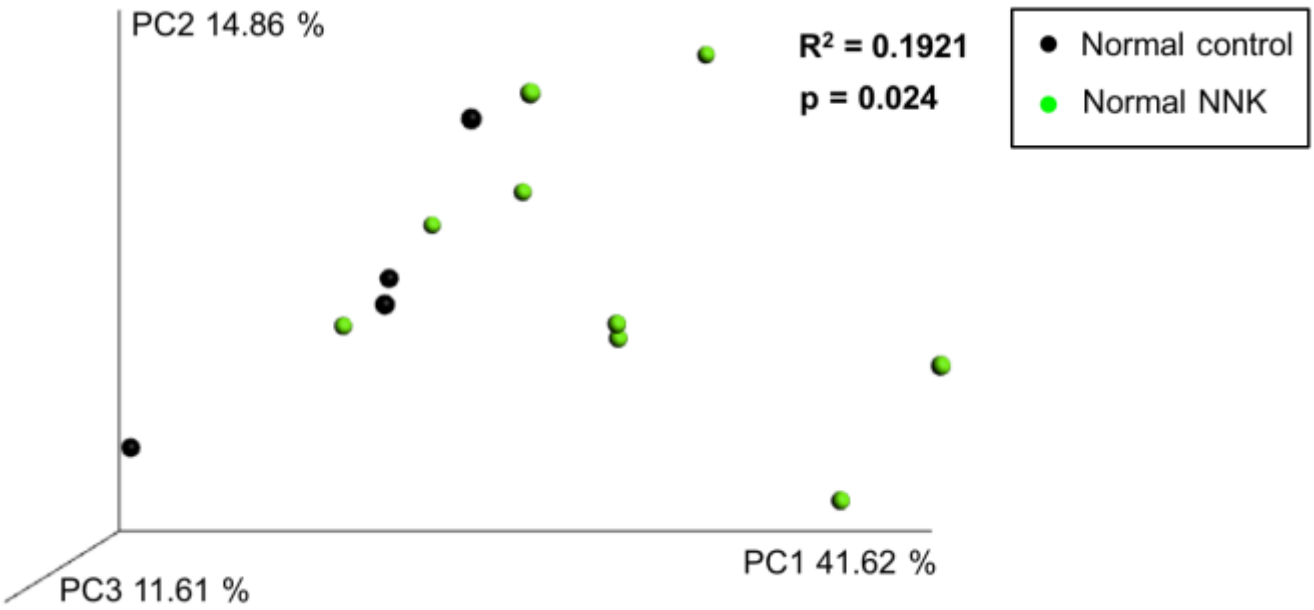

Figure 13 NNK alter microbial diversity in the mouse gut. Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of mice receiving normal diets treated with or without NNK. An Adonis test revealed a significant correlation between treatment type and microbial diversity ($R^2 = 0.1921$, $p = 0.024$).

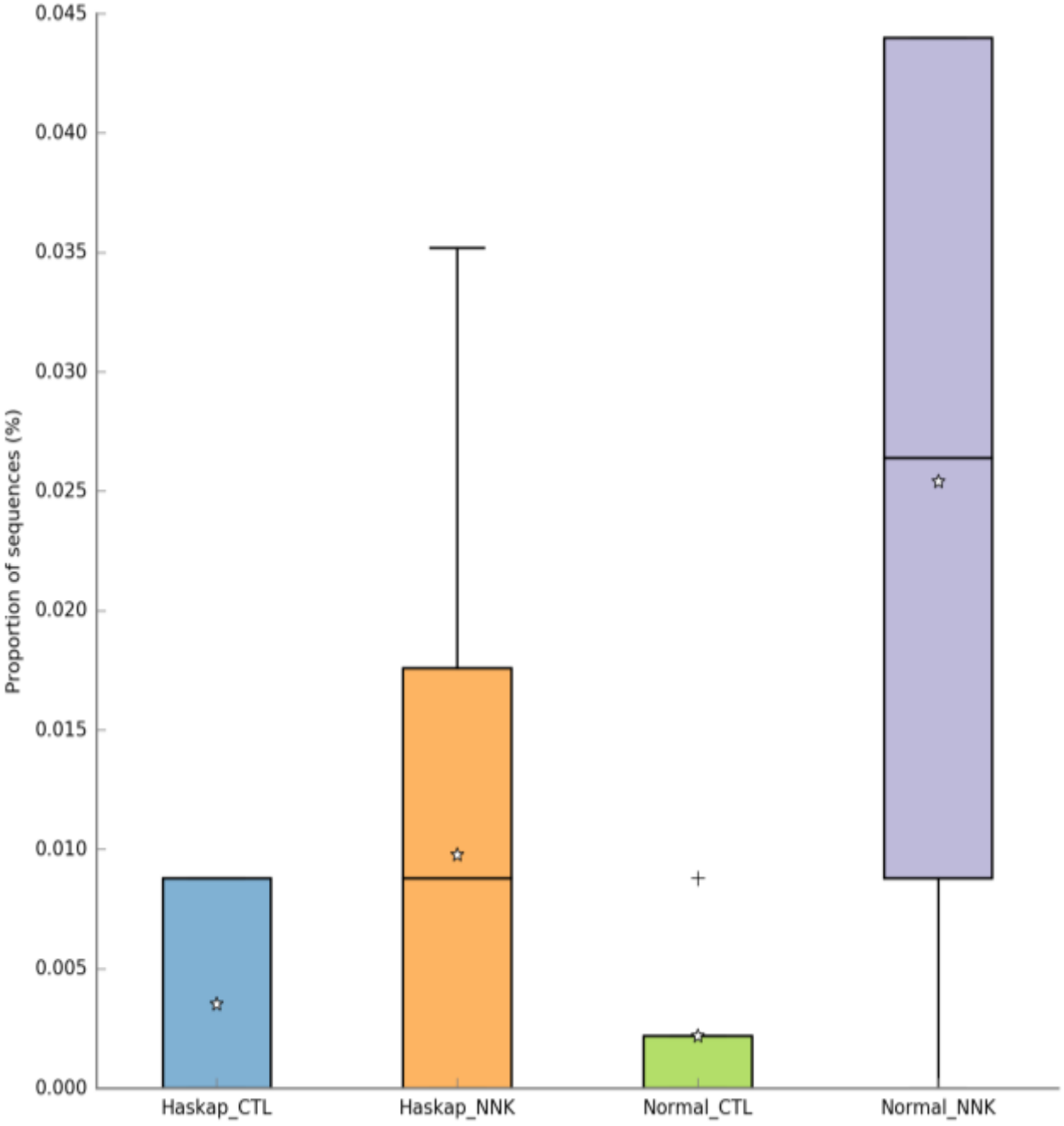

Figure 14 NNK increases levels of B. acidifaciens in the gut of mice receiving haskap or normal diet. Multiple group comparison using Benjamin-Hochberg FDR reveals that a significant difference in the levels of *Bacteroides acidifaciens* across treatment groups (p = 0.040).

When fecal samples from the haskap treated mice were compared, it was found that mice who had received NNK did not have significantly ($R^2 = 0.0505$, $p = 0.159$) different community structure from those who did not (**Figure 15**). A two-group t-test comparison with Benjamin Hochberg FDR correction in STAMP revealed one significant difference in individual taxa; an unidentified *Bilophila* species was slightly higher in NNK-treated haskap mice than control haskap mice ($p = 0.048$, **Figure 16**).

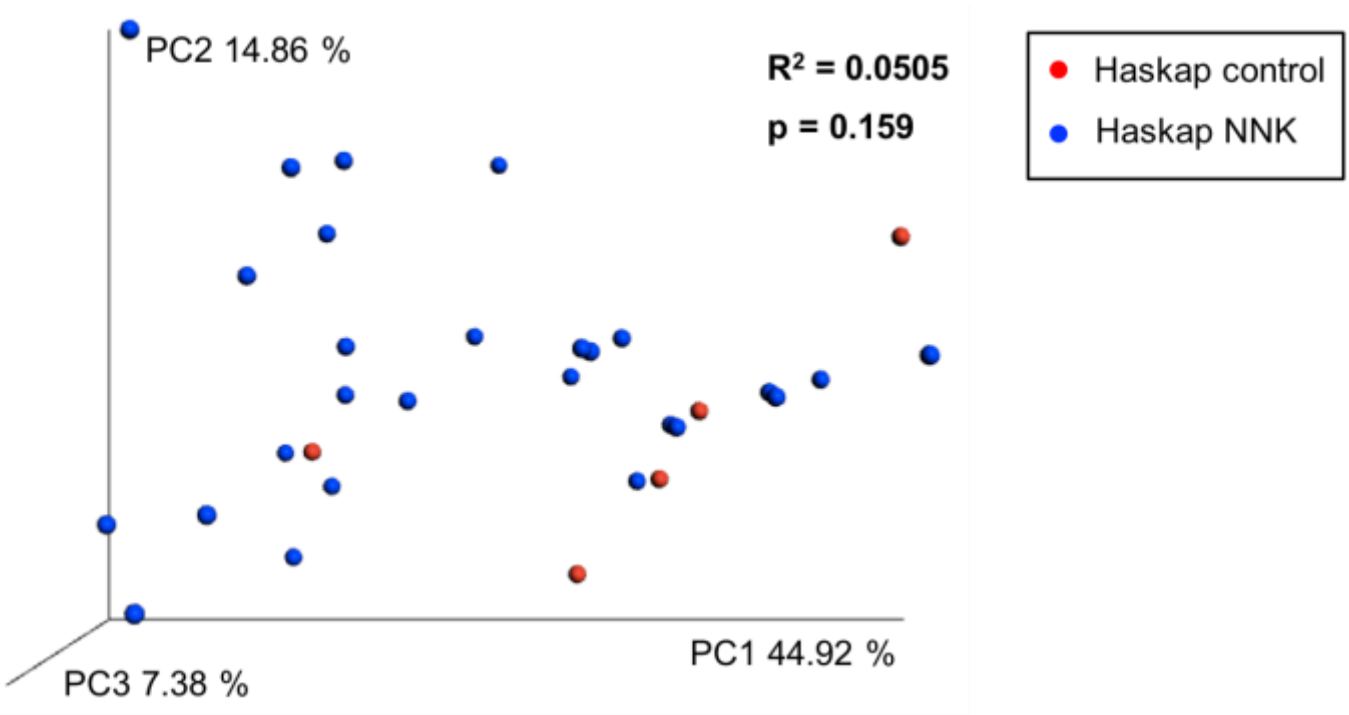

Figure 15 Microbial diversity is not altered by NNK when haskap diet is administered. Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of mice receiving haskap diets treated with or without NNK. An Adonis test revealed no difference in overall microbial diversity ($R^2 = 0.0505$, $p = 0.159$).

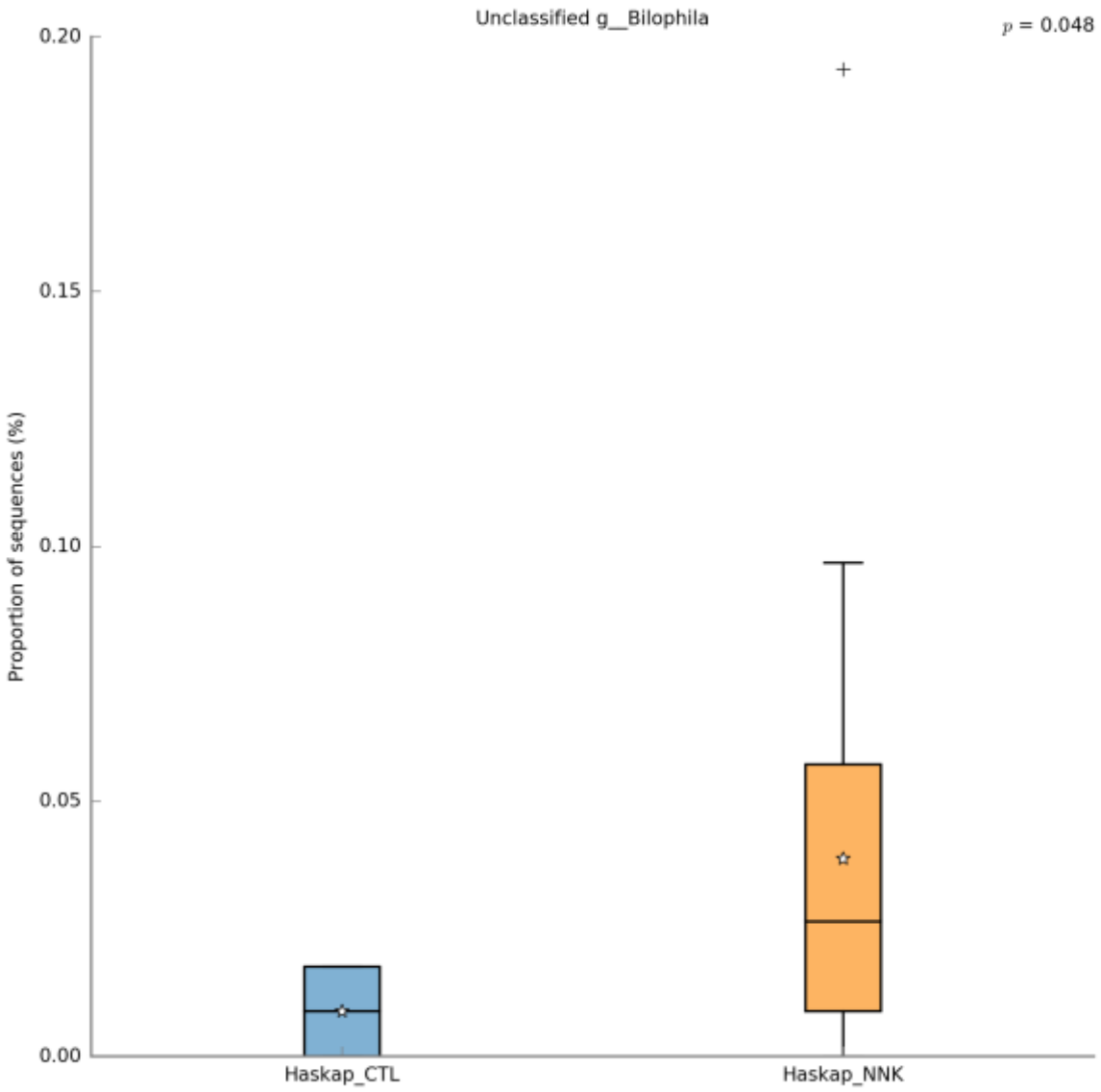

Figure 16 NNK increases levels of Bilophila in the gut of mice receiving haskap diet. Two group comparison using Benjamin-Hochberg FDR reveals that a significant difference in the levels of *Bilophila* between mice administered haskap with and without NNK (p = 0.048).

4.5 SUMMARY

Our results show that in both healthy mice and mice with lung carcinoma, a diet supplemented with anthocyanin-rich haskap extracts alters the bacterial communities in the gut microbiome. While levels of only a few taxa are significantly altered, beta diversity analysis indicates that the overall relationships between community members are altered, as well as certain functions. The NNK carcinogen itself alters the bacterial community of the gut, and reduces species diversity. It is important to note that food consumption was not measured in this experiment. It has been shown that exposure to NNK causes weight loss in mice, which can in turn indirectly alter the microbiome.

CHAPTER 5: KEFIR MICROBIAL COMPOSITION

5.1 KEFIR

In our study, we examine the microbial succession over the production of a coconut kefir product.

5.2 SUCCESSION OF MICROBES IN COCONUT KEFIR PRODUCTION

Milk and grain samples were taken from coconut kefir at the following points in production: 1) after grains rested in dairy milk, 2) after grains rested in coconut milk 3) after the final fermentation. A sample of fresh coconut milk was also taken. An outline of coconut kefir production timeline and sampling is shown in **Figure 17**. Both the 16S and ITS2 region were sequenced to analyze both bacterial and fungal content.

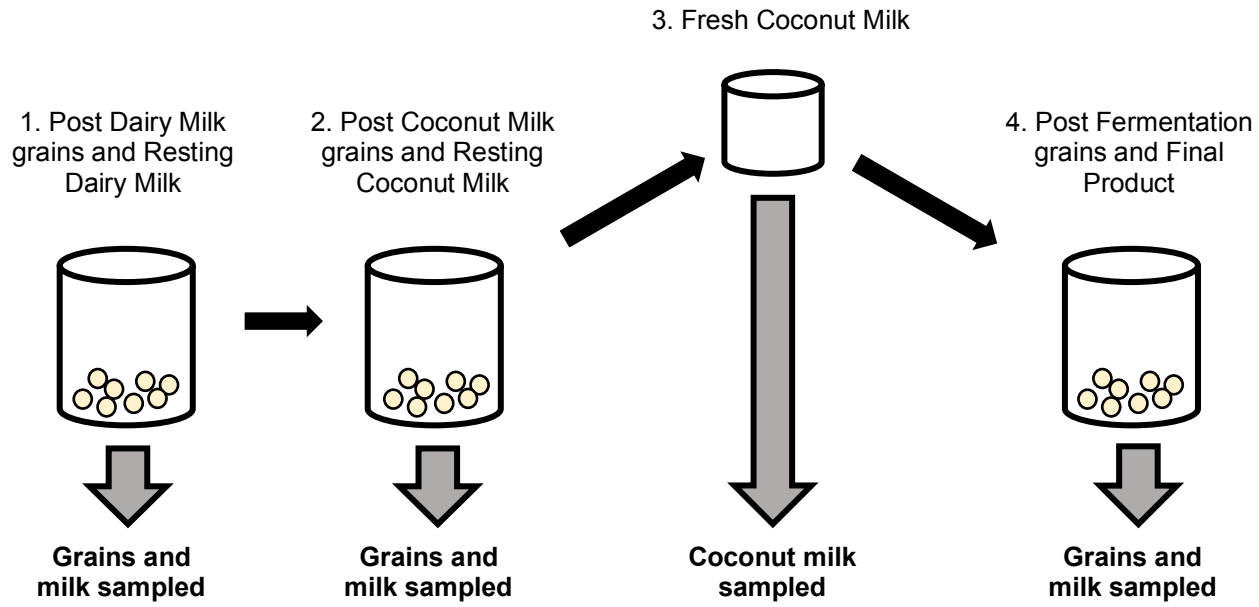

Figure 17 Outline of coconut kefir production timeline and sampling. Grain and milk samples were taken after the grains: (1) rested in dairy milk (2) rested in coconut milk and (4) fermented in coconut milk. Milk samples were taken from the fresh coconut milk prior to the fermentation (3).

5.2.1 KEFIR GRAINS ARE COMPRISED OF A LOW COMPLEXITY, STABLE MICROBIAL COMMUNITY

Species richness was determined by counting the number of bacterial and fungal OTUs in each grain sample. The grain OTU counts are depicted in bar plots (**Figure 18**). The number of observed bacterial OTUs in Post Dairy Milk grains, Post Coconut Milk grains and Post Fermentation grains ranged between 56-62 (**Figure 18A)**. The number of fungal OTUs in each was lower, ranging from 2-6 (**Figure 18B)**. Stacked bar plots (**Figure 19A**) show that the main bacterial genera present in the grains across samples are *Lactobacillus* (87.1%), *Lactococcus* (8.7%), *Leuconostoc* (1.2%) and other species in the Lactobacillaceae family (2.9%). *Saccharomyces* was the only of fungal genus found in the grains (**Figure 20A**).

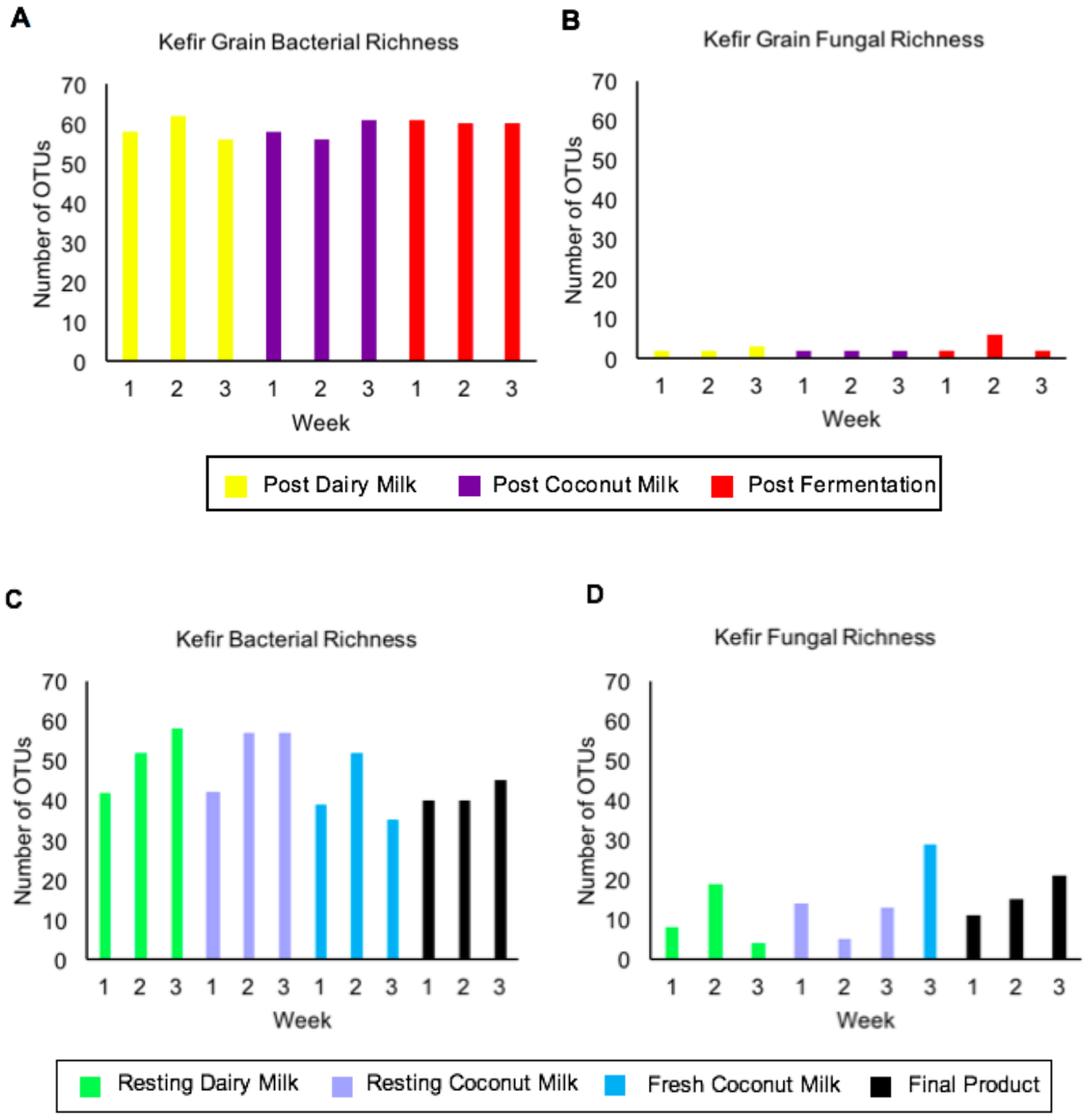

Figure 18. Bacterial and fungal species richness of kefir grain samples. (A) Kefir grain samples have between (A) 56 and 62 bacterial OTUs and (B) 2 and 6 fungal OTUs. (C) Kefir milk samples have between 39 and 58 bacterial OTUs and (D) 4 and 29 fungal OTUs.

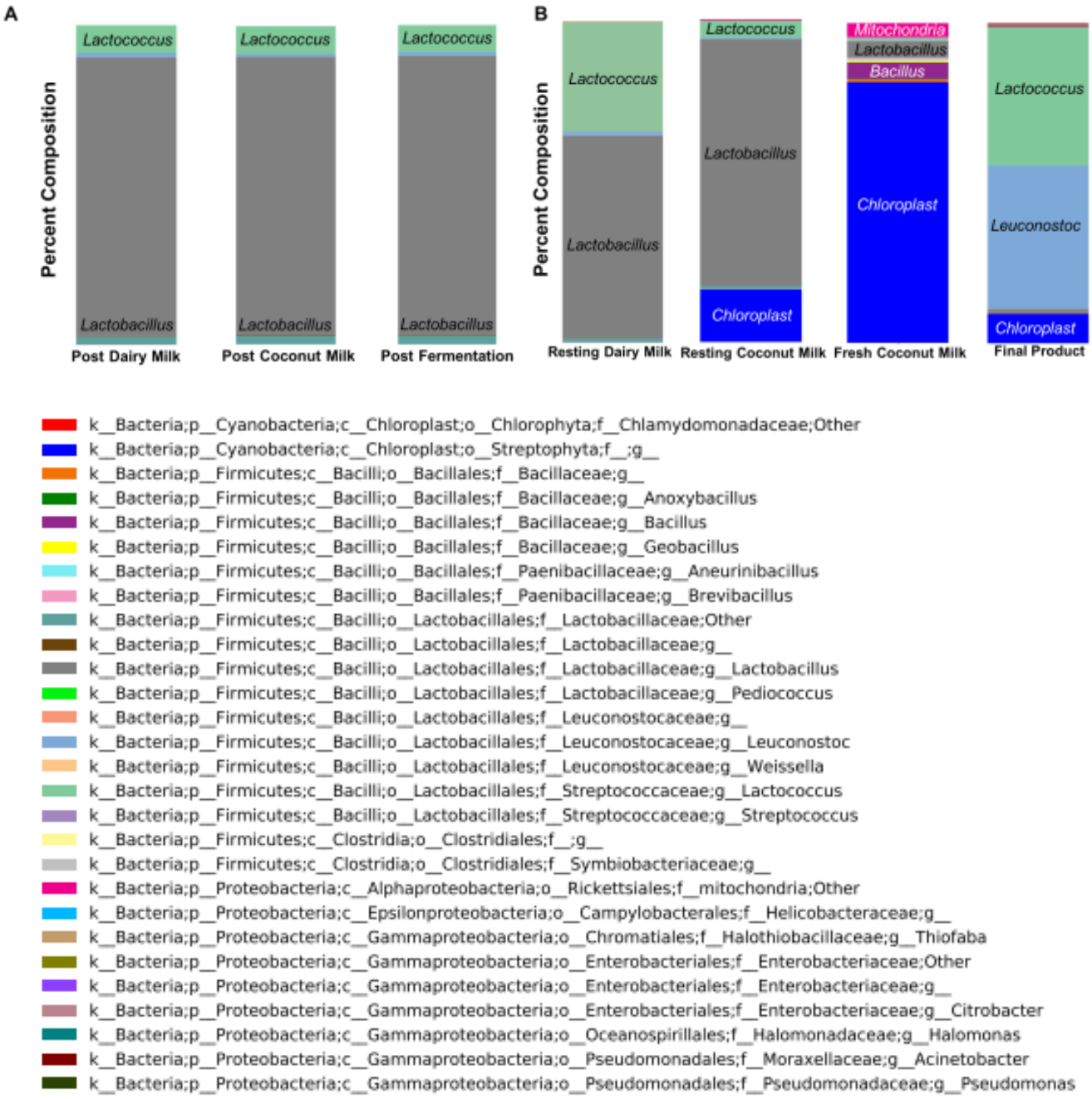

Figure 19. Bacterial taxonomic composition of kefir grain and milk samples. Colours in bar plots correspond to Taxonomy Legend. (A) Bar plots of bacteria identified in kefir grains at different sample time points. (B) Bar plots of bacteria identified in kefir milk samples at different time points.

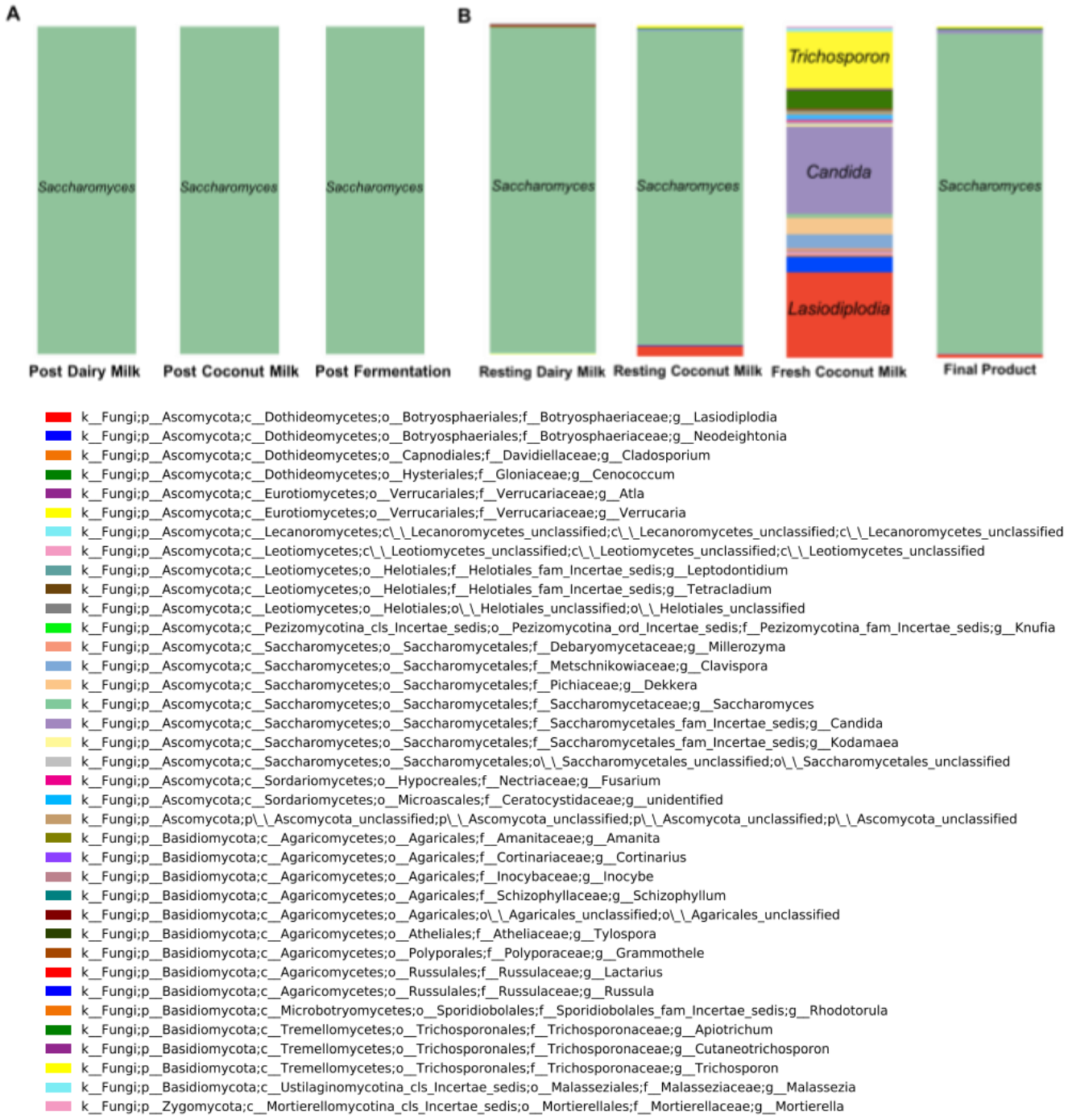

Figure 20. Fungal taxonomic composition of kefir grain and milk samples. Colours in bar plots correspond to Taxonomy Legend. (A) Bar plots of fungi identified in kefir grains at different sample time points. (B) Bar plots of fungi identified in kefir milk samples at different time points.

Comparison of bacterial diversity of the grains was examined using Principal Coordinate Analysis (PCoA) plots and tested for significance using an Adonis test. The kefir grains did not show any consistent changes in their bacterial diversity across production steps ($R^2 = 0.0445$, $p = 0.923$) (**Figure 21A**) but did show some variation from the three different batches ($R^2 = 0.8587$, $p = 0.001$) (**Figure 21B**).

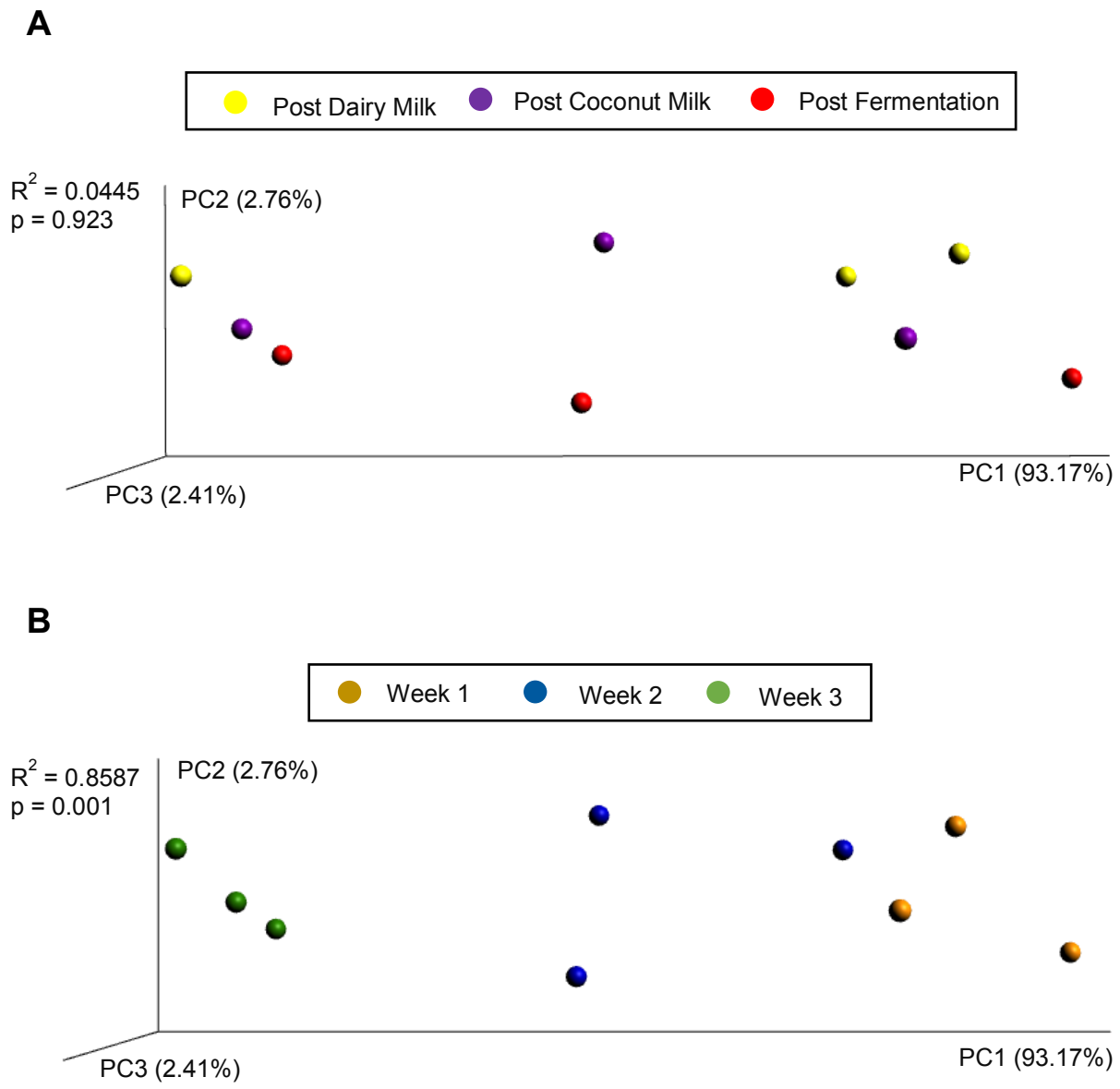

Figure 21 Kefir grain bacterial diversity by sample type and week. (A) Correlation between sample time point and bacterial diversity. (B) Correlation between batch week and bacterial diversity.

5.2.2 MICROBIAL SUCCESSION IN COCONUT KEFIR MILK OVER THE COURSE OF PRODUCTION

Species richness was determined by counting the number of bacterial and fungal OTUs in each liquid sample. The counts are depicted in bar plots (**Figure 18C-D**). The number of observed bacterial OTUs in resting dairy milk, resting coconut milk, fresh coconut milk and the final coconut kefir product range between 35-58 OTUs. The number of fungal OTUs ranging from 4-29, with the highest richness found in the fresh coconut milk. Stacked bar plots (**Figure 19B**) show that the main bacterial genera present in the Resting Dairy Milk samples are *Lactobacillus* (57.4%), *Lactococcus* (24.0%), *Leuconostoc* (1.5%) and other Lactobacillaceae species (1.2%). The Resting Coconut Milk contained mainly Lactobacillus (76.5%), Lactococcus (5.1%), with Streptophyta chloroplast DNA also being identified (16.3%), likely from the coconut plant itself (**Figure 19B**). The Fresh Coconut Milk contained mostly Lactobacillus (5.1%) and Bacillus (5.1%), with Streptophyta chloroplast (81.5%) and Proteobacteria mitochondria (4.3% - again likely from the coconut plant) also being identified (**Figure 19B**). The Final Product contained mainly Leuconostoc (44.8%) and Lactococcus (43.1%), as well as Streptophyta chloroplast (9.3%) (**Figure 19B**). Saccharomyces was the main fungal taxonomy present in the Resting Dairy Milk (98.0%), Resting Coconut Milk (95.1%) and Final Product (96.7%). The fresh coconut milk contained mainly fungi of the Botryosphaeriaceae (30.5%) and Trichosporonaceae (23.4%) families, as well other species in the Saccharomycetales (1.2%) order (**Figure 20B)**.

Comparison of bacterial diversity of the milk samples using PCoA and Adonis revealed that there are distinct microbial communities at each production stage ($R^2 = 0.8536$, $p = 0.001$) (**Figure 22A**), but not between batches ($R^2 = 0.2309$, $p = 0.102$) (**Figure 22B**).

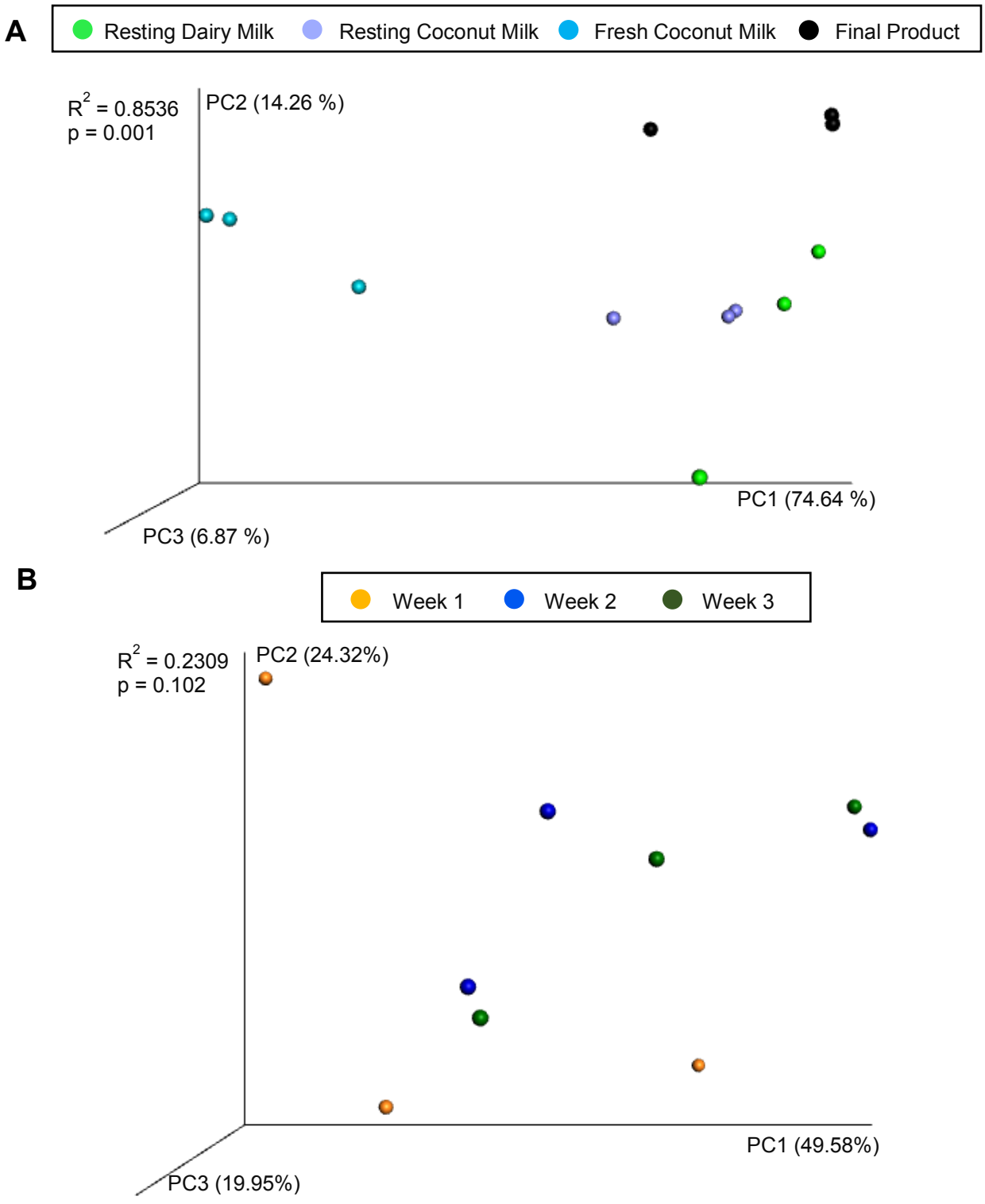

Figure 22 Kefir milk bacterial diversity by sample type and week. Correlation between bacterial diversity and (A) production steps (B) batch week.

Analysis of metagenomics sequencing on the Final Product using MetaPlAn allowed us to identify some previously categorized strains of bacteria and yeast (**Table 4**). We were able to identify the top 10 most abundant bacteria in the Final Product samples at the species level, shown in **Figure 23**. The average of the top 10 most abundance bacteria in the Final Products shown in **Figure 24.** The metagenomic taxonomy is consistent with the 16S classifications, with *Leuconostoc* (%) and *Lactobacillus* (%) species being the most abundant genera in the final coconut kefir product.

Table 4 Average microbial proportions found in coconut kefir as determined by metagenomic taxonomy

Kingdom	Phylum	Family	Genus	Species	Strain	Proportion
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc lactis	unclassified	48.0029
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	unclassified	unclassified	21.42568636
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus casei paracasei	unclassified	11.17808364
Bacteria	Firmicutes	Streptococcaceae	Lactococcus	Lactococcus lactis	unclassified	7.184455455
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus fermentum	unclassified	4.361814545
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc citreum	unclassified	3.576504545
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus plantarum	unclassified	3.350859091
Bacteria	Firmicutes	Lactobacillaceae	Pediococcus	unclassified	unclassified	0.376388182
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus kefiranofaciens	GCF_000214785	0.116597273
Bacteria	Proteobacteria	Gallionellaceae	unclassified	unclassified	unclassified	0.094120909
Bacteria	Firmicutes	Leuconostocaceae	Weissella	unclassified	unclassified	0.061115455
Bacteria	Firmicutes	Streptococcaceae	Streptococcus	Streptococcus infantarius	unclassified	0.03665
Bacteria	Proteobacteria	Bartonellaceae	Bartonella	unclassified	unclassified	0.02518
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus smithii	GCF_000238675	0.023888182
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus buchneri	unclassified	0.021059091
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus cereus thuringiensis	unclassified	0.016557273
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus vini	GCF_000255495	0.014016364
Bacteria	Proteobacteria	Enterobacteriaceae	Cronobacter	Cronobacter sakazakii	unclassified	0.011921818
Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter	unclassified	unclassified	0.010394545
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus cytotoxicus	GCF_000017425	0.009671818
Bacteria	Proteobacteria	Enterobacteriaceae	Enterobacter	Enterobacter cloacae	unclassified	0.00777
Bacteria	Firmicutes	Bacillaceae	Geobacillus	unclassified	unclassified	0.007170909
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc pseudomesenteroides	unclassified	0.005641818
Bacteria	Proteobacteria	Enterobacteriaceae	Cronobacter	Cronobacter malonaticus	unclassified	0.005528182
Bacteria	Proteobacteria	Halomonadaceae	Halomonas	unclassified	unclassified	0.005130909
Bacteria	Actinobacteria	Propionibacteriaceae	unclassified	unclassified	unclassified	0.005053636
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus otakiensis	GCF_000415925	0.004652727
Bacteria	Firmicutes	Veillonellaceae	Megasphaera	unclassified	unclassified	0.004611818
Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter	Acinetobacter	unclassified	0.004391818

				pittii calcoaceticus nosocomialis		
Bacteria	Spirochaetes	Spirochaetaceae	Borrelia	Borrelia recurrentis	GCF_000019705	0.003730909
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus amylovorus	unclassified	0.003618182
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus subtilis	unclassified	0.00342
Bacteria	Proteobacteria	Enterobacteriaceae	Escherichia	unclassified	unclassified	0.003200909
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus zeae	GCF_000260435	0.00309
Bacteria	Tenericutes	Acholeplasmataceae	Candidatus Phytoplasma	unclassified	unclassified	0.002792727
Bacteria	Proteobacteria	Enterobacteriaceae	Pantoea	unclassified	unclassified	0.002780909
Bacteria	Firmicutes	Lachnospiraceae	Butyrivibrio	unclassified	unclassified	0.002737273
Bacteria	Firmicutes	Staphylococcaceae	Macrococcus	Macrococcus caseolyticus	GCF_000010585	0.002729091
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus licheniformis	unclassified	0.002563636
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus rhamnosus	unclassified	0.002383636
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc carnosum	unclassified	0.001818182
Bacteria	Firmicutes	Enterococcaceae	Enterococcus	Enterococcus casseliflavus	unclassified	0.00164
Bacteria	Proteobacteria	Enterobacteriaceae	Klebsiella	unclassified	unclassified	0.001610909
Bacteria	Firmicutes	Bacillaceae	Bacillus	Bacillus coagulans	unclassified	0.001420909
Bacteria	Proteobacteria	Enterobacteriaceae	Klebsiella	Klebsiella pneumoniae	unclassified	0.001403636
Eukaryota	Ascomycota	Saccharomycetaceae	Saccharomyces	Saccharomyces cerevisiae	GCA_000146045	0.001360909
Bacteria	Firmicutes	Bacillaceae	Geobacillus	Geobacillus kaustophilus	unclassified	0.001093636
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc mesenteroides	unclassified	0.000714545
Bacteria	Proteobacteria	Enterobacteriaceae	Cronobacter	Cronobacter turicensis	unclassified	0.000522727
Bacteria	Proteobacteria	Enterobacteriaceae	Cronobacter	unclassified	unclassified	0.000441818
Bacteria	Firmicutes	Streptococcaceae	Lactococcus	Lactococcus garvieae	unclassified	0.000383636
Bacteria	Firmicutes	Ruminococcaceae	Subdoligranulum	unclassified	unclassified	0.000316364
Bacteria	Firmicutes	Sporolactobacillaceae	Sporolactobacillus	unclassified	unclassified	0.000169091
Bacteria	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus pentosus	GCF_000271445	0.000155455
Bacteria	Firmicutes	Streptococcaceae	Streptococcus	Streptococcus lutetiensis	GCF_000441535	0.00008
Bacteria	Firmicutes	Leuconostocaceae	Leuconostoc	Leuconostoc kimchii	GCF_000092505	3.73E-05

Table 4 displays the microbes present in 11 different coconut kefir samples and their average proportion in samples. Taxonomy was obtained using MetaPhlAn analysis of NextSeq shotgun metagenomic sequencing. All microbes shown in the table were bacteria, except one, which was classified as a strain of the Eukaryote *Saccharomyces cerevisiae.* Some previously catalogued strains of bacteria were identified, however little is known about them. The metagenomic taxonomy is consistent with the 16S classifications, with Leuconostoc and Lactobacillus the most abundant species in the final coconut kefir product.

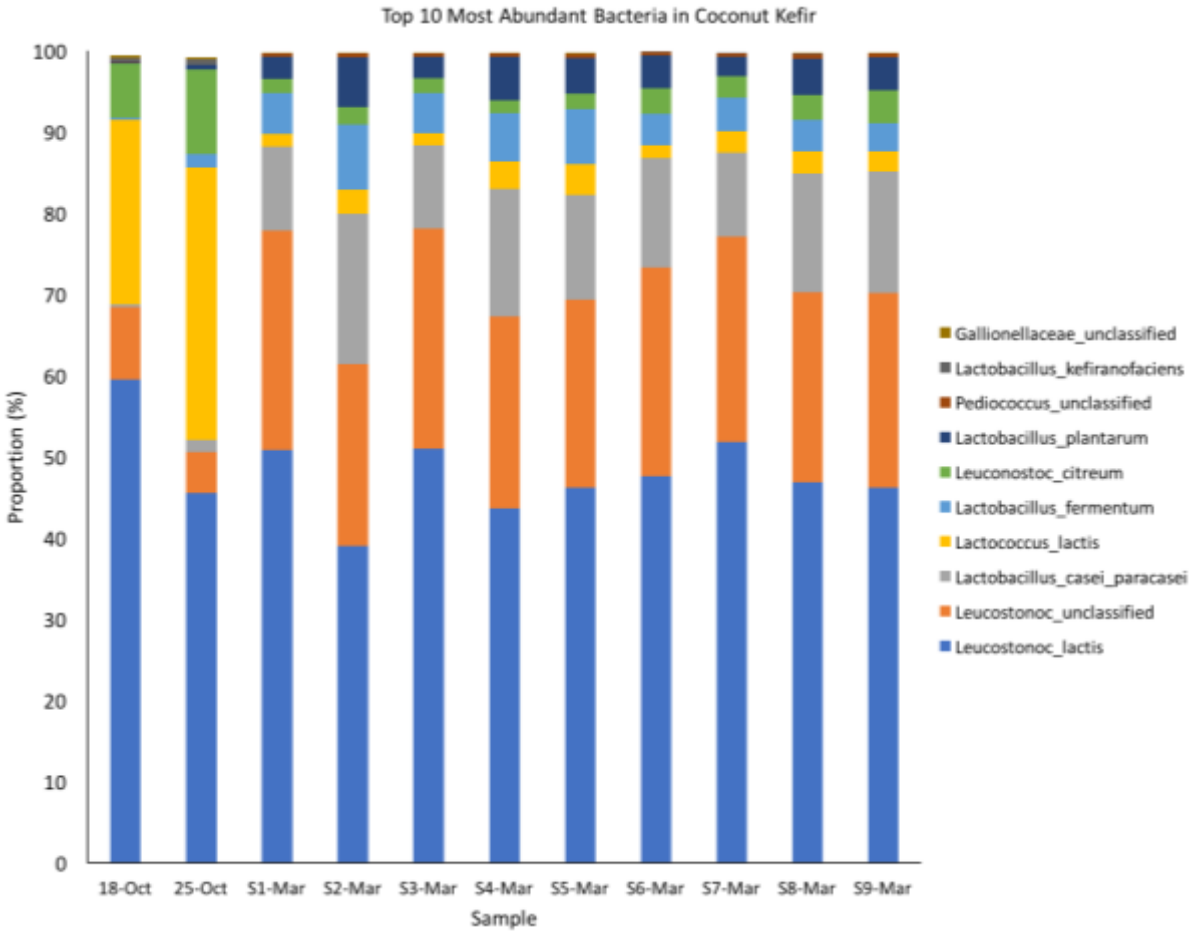

Figure 23 Stacked bar chart depicting proportions of top 10 most abundant bacterial species across 11 coconut kefir samples. Metagenomic sequencing was performed on DNA samples from 11 separately packaged bottles of coconut kefir. MetaPhlAn analysis reveals relatively stable proportions of the most abundant bacteria in the samples. Taxonomy is shown at the species level.

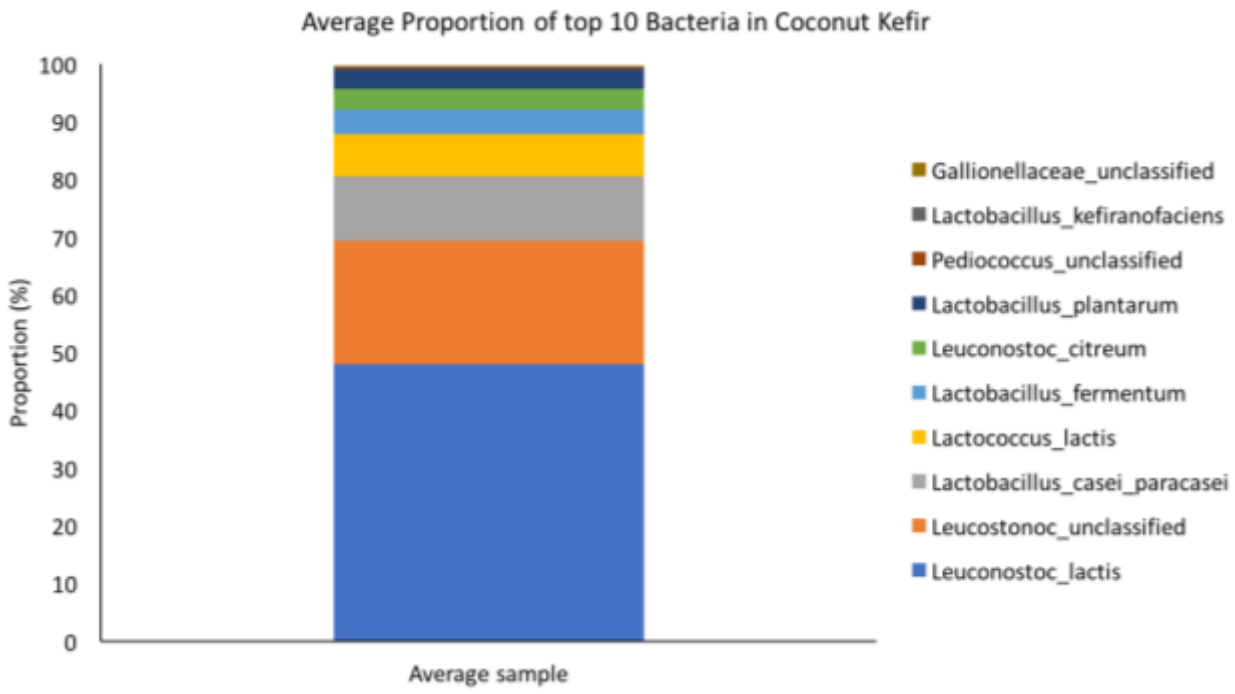

Figure 24 Stacked bar chart depicting average proportion of top 10 most abundant bacteria in 11 coconut kefir samples. Metagenomic sequencing was performed on DNA samples from 11 separately packaged bottles of coconut kefir. MetaPhlAn analysis was performed to classify taxonomy. Proportions of each of the top species was averaged across samples and shown here.

5.3 SUMMARY

In this analysis, we identified and compared the bacterial and fungal communities from several stages of kefir production. We found that the kefir grains had a consistent microbiome composition during production with some slight changes from batch to batch. These kefir grains are dominated by 1-3 species of *Saccharomyce*s and ~60 species from the genera *Lactobacillus*, *Lactococcus*, and *Leuconostoc*. The coconut milk goes through significant changes during both resting phases and during fermentation to the final product resulting in a final product containing 40-45 species primarily from the genera *Leuconostoc* and *Lactococcus*.

CHAPTER 6: DISCUSSION

6.1 MODERATE EXERCISE HAS LIMITED BUT DISTINGUISHABLE EFFECTS ON THE MOUSE MICROBIOME

The relationship between exercise and human health has been extensively studied, and it has been found that exercise makes a number of positive contributions to our health (Dangardt et al., 2013). Diet has also been shown to have a strong influence on our health and has been directly implicated in alteration of the gut microbiome (Sonnenburg & Bäckhed, 2016). Environmental factors that influence gut flora are of great interest, as recent research demonstrates that these microbes are important for normal host physiology. The effect that exercise has on gut microbial composition is an emerging field of interest, though to date, only a handful of studies have been done. Links between exercise, the gut microbiome, and disease have been observed through an increase in protective short chain fatty acids (Matsumoto et al., 2008; Perrin et al., 2001) and a decrease in bacteria associated with colorectal cancer and obesity (Choi et al., 2013; Turnbaugh et al., 2008). These studies have not provided consistent conclusions, and so this study therefore aimed to characterize the impact that moderate exercise has on gut microbial diversity in a well-controlled setting. Controlled conditions included identical housing and food type, as well as longitudinal measurements of food intake, body mass and exercise levels.

Differences in body-mass composition and food intake between control and exercise mice in the voluntary study demonstrates that this exercise protocol was indeed having a tangible physiological effect on its subjects. Voluntary exercised mice not only had significantly less fat body mass and higher lean body mass than control mice, but also maintained a higher level of

food intake throughout the study. However, this effect was not seen in the forced exercise study, suggesting that this program did not induce the same stress in the mice as the voluntary one. This idea is supported in the inflammation data, as KC (CXCL-8 in humans), an exercise-induced cytokine, was higher in the voluntary, but not forced cohort. Evidence suggests that CXCL-8, a chemokine, is released from contracting muscles and exerts a local effect to induce angiogenesis in muscles following exercise (Nielsen & Pedersen, 2007). Several studies have linked changes in inflammation to different forms of exercise, in various populations (Allen et al., 2015; Bilski et al., 2016; Cook et al., 2016; Crimi et al., 2009). Contrary to our results, some of this research has shown that both voluntary and forced exercise act to reduce pro-inflammatory cytokines. In our study there was no change in either cohort in cytokines IL-1β, IL-6 and TNFα, which are also commonly induced by strenuous exercise (Donovan et al., 2007; Nielsen & Pedersen, 2007; Pedersen, 2000). This suggests that there was no chronic effect of exercise on serum levels of inflammatory markers, however it is possible that changes were not detected due to high levels of cytokines in the control mice, due to the stress of being singly-housed.

Initial analyses of species richness and sample diversity show that neither exercise program appears to make a contribution to obvious microbiome changes. These observations contrast previous studies that did report significant differences in the microbiome of both animal models (Queipo-Ortuño et al., 2013; Zhang et al., 2013; Cook et al., 2013; Allen et al., 2015) and humans (Clarke et al., 2014) in response to exercise. There are several reasons for these observational differences, which we attempted to address further. Initially, we used female mice for the voluntary wheel running cohort because it had been reported that female mice run more than male mice (Bartling et al., 2017; Konhilas et al., 2004; McMullan et al., 2016). During the course of our voluntary exercise study, Allen et al. 2015 found microbiome differences due to

voluntary wheel running in male mice. We also noted that Allen et al. 2015 sequenced the V4 region of the 16S rRNA gene, while we had initially sequenced the V6-V8 region. Sequencing of different hypervariable regions of the 16S rRNA gene do not identify consistent taxonomy within datasets (Huse et al., 2008; Kim et al., 2011; Kumar et al., 2011). Therefore, we conducted additional sequencing of the V4 region using the same primers as Allen et al. 2015, but still found no significant differences in the microbiome in response to exercise (**Appendix – Figure 25**). Lastly, we ran the same bioinformatic pipeline as described by Allen et al. 2015 with our data and found no significant differences, but when the data from their study was analyzed with the bioinformatics pipeline from this study we replicated their published significant findings. It could be that other factors within previous studies including diet differences (Zhang et al., 2013; Mika et al., 2015), cage effects (Queipo-Ortuño et al., 2013), age of mice (Langille et al., 2014), or slight differences in housing could be contributing to some of the previously published observations. Despite the lack of major observable differences in the microbiome, it was notable that more complex methods that take into account microbial interactions like machine learning were able to distinguish subtle shifts in the mouse microbiome in response to exercise.

For both exercise cohorts, several known and novel associations with exercise were identified. The *Firmicutes* and *Bacteroidetes* phyla dominated the top 30 taxa important in differentiating between exercise and control treatments. Several other studies have linked these two phyla with response to exercise (Queipo-Ortuñoet al., 2013; Mika et al., 2015; Clarke et al., 2014), along with the other two important phyla found in the voluntary cohort, *Proteobacteria* and *Actinobacteria*. Taxa of the *Bacteroidales* and *Clostridiales* order have been shown to produce short-chain fatty acids (Baothman et al., 2016) that protect against obesity and colon

cancer (Moeinian et al., 2014; Lin et al., 2012). Within the *Bacteroidetes* phylum, at the genus level, *Bacteroides* remains an important taxon in both exercise cohorts, while the *S24-7* family is relevant in the voluntary cohort. Out of 18 *S24-7* OTUs, 16 increased with voluntary exercise. Within the *Firmicutes* phylum, taxa of the order *Clostridiales* are found to be important in differentiating between exercise and control groups in both cohorts, while *Lactobacillus* is found to be a relevant genus in the forced exercise cohort, along with the *Ruminococcaceae* and *Mogibacteriaceae* families. All five of the *Lactobacillus* OTUs and all three of the *Ruminococcus* OTUs decreased with forced exercise. *Lactobacillus* species produce short chain fatty acids such as butyrate and have been shown to be protective against pathogens (Queipo-Ortuño et al., 2013). *Lactobacillus* species are also implicated in fat storage; some increase fat storage, while others decrease it (Armougom et al., 2010; Aronsson et al., 2010; Million & Raoult, 2013). The bacterial families *Ruminococceae* and *Mogibacteriaceae* are associated with leanness (Ziętak et al., 2016).

All of these taxa have previously been described in the literature in association with exercise (Queipo-Ortuñoet al., 2013; Mika et al., 2015; Clarke et al., 2014). *Rikenellaceae*, a family under the *Clostridiales* order, was identified as a novel association within the voluntary exercise cohort, while *Lachnospiraceae*, a family under the *Bacteroidales* order was a novel association found in both cohorts. *Rikenellaceae* is associated with leanness and *Lachnospiraceae* species produce butyrate and are associated with protection against colon cancer (Clarke et al., 2013; Meehan & Beiko et al., 2014). Several studies have also described changes in the levels of *Bifidobacterium, Prevotella* and *Erysipelotrichaceae* species as a result of exercise in mice and humans (Queipo-Ortuño et al., 2013; Mika et al., 2015; Allen et al., 2015; Clarke et al., 2014). A genus of *Actinobacteria*, *Bifidobacteria* has been shown to be

effective in raising mood in CFS and IBS. It plays and important role in weight and appetite regulation and has been shown to increase with exercise (Queipo-Ortuño et al., 2013). It is associated with anti-inflammatory states (Cani et al., 2009; Round et al., 2014). *Prevotella* strains are linked with chronic inflammation and the consumption of simple carbohydrates (Larsen, 2017; Queipo-Ortuño et al., 2013) and have been shown to decrease with exercise (Clarke et al., 2014). Not much is known about the role of *Erysipelotrichaceae* species in the gut microbiome. The higher classification accuracy results within the voluntary versus forced exercise mice again support that the voluntary exercise model was more vigorous overall and that there exists a relationship between exercise and the gut microbiome.

Since the forced and voluntary exercise studies were not performed together, it is difficult to compare microbiome data from both. **Appendix – Figure 29,** comparing the controls from both cohorts in week 0, illustrates how even the same strain of mice, close in age and obtained from the same breeding facility can have extremely different microbiomes. These differences could be due to factors such as exposure to different microbes from their parents, human handlers, other animals, food or even environmental surfaces. This is an excellent example of the difficulties in standardizing microbiomes studies. Comparison to other studies that appear to have used very similar methods, such as the study by Allen et al. (2015) are often not feasible.

6.2 HASKAP MODULATES THE EFFECTS OF NNK ON MICROBIAL COMMUNITY STRUCTURE IN MICE

Since its been shown that diet has a large influence on the gut microbiome (Sonnenburg & Bäckhed, 2016), food supplements have become an area of keen interest for therapeutic

modulation. It is known that plant polyphenol compounds such anthocyanins have anticancer and anti-inflammatory properties when consumed and that the microbiome plays a role in their metabolism (Faria et al., 2014). This study analyzed the shifts in the microbiome of healthy mice that had their diet supplemented with anthocyanin rich haskap berry extract. Secondly, microbiome changes were also examined in mice treated with a lung carcinogen (NNK) that also consumed a haskap-supplemented diet.

Some anthocyanins have been shown to have important benefits on metabolism. It was found that blackberry anthocyanin supplements reduced weight gain and insulin resistance in mice on a high-fat diet (Esposito et al., 2015). However, there had been no microbiome profiling done in the context of this anthocyanin-supplemented diet. Our research provides a closer look into the microbiome shifts that occur as a result of dietary polyphenol supplementation in the context of both health and disease.

Species richness was not altered by haskap supplements in either healthy or lung cancer mouse models. While we were not able to identify any single taxa that were present in significantly altered levels between either haskap and normal diet groups after multiple test correction, the Adonis tests based on beta-diversity differences between samples indicated that the haskap diet was a significant factor in explaining some of the variation in the community. It is important to note that these statistical tests do take into account the relationships and interactions between microbes in a community. Other methods of analysis such as machine learning as applied to the exercise study and network building based on co-occurrences may be more successful in identifying key bacteria that shift as a result of haskap administration. It is also possible that there are many species affected by the dietary supplement, especially if many common species have the ability to metabolize these compounds. In this experiment, we only

conducted 16S profiling, but future studies that used metagenomic sequencing may identify differences in microbial gene content that is linked to anthocyanin-metabolizing genes.

The second important finding of this study was that haskap consumption modulates changes in the mouse gut as a result of NNK administration. Not surprisingly, mice who were injected with the NNK carcinogen had slightly lower species diversity. It has been demonstrated that NNK acts to induce damage in host cells through a number of mechanisms, including DNA damage, and therefore could be toxic to bacterial cells as well (Ge et al., 2015). Although, there were community differences identified between control and NNK mice when a normal diet was administered, there were no longer significant differences between control and NNK mice when a haskap diet was administered. While it is known that phenolic compounds such as anthocyanins are metabolized by colonic bacteria, further studies are needed to further elucidate the mechanisms by which haskap alters gut bacteria (Rechner et al., 2002; Saha et al., 2016).

6.3 MICROBIAL SUCCESSION IN COCONUT KEFIR PRODUCTION IS COMPARABLE TO MICROBIAL SUCCESSION IN DAIRY MILK KEFIR

Fermented probiotics provide beneficial microbes that mediate allergies, asthma, inflammation and infection. It is thought that these bacteria and yeasts provide their health benefits through both direct interaction with the host as well as through the production of metabolites (Rosa et al., 2017, Marco et al., 2017). This study examined the succession of both bacteria and yeasts that occurred over the course of the production of coconut kefir.

Firstly, this study demonstrates that it is possible for coconut milk to sustain lactic acid fermenting microbial communities long enough to produce a fermented probiotic. Although lactose is the preferred food source for these bacteria, our results indicated that they are capable

of metabolizing the sugars in coconut milk. This is consistent with previous research that showed that lactic acid bacteria are capable of fermenting a variety of plant sugars to make sugary, instead of milk kefir (Fiorda et al., 2017).

Secondly, microbial succession in coconut kefir is comparable to that in traditional dairy milk kefir. The consistency of microbial composition of kefir grains across the fermentation process indicates that this community is very stable. This is consistent with published literature, which reports that kefir grains contain largely lactic acid producing bacteria and *Saccharomyces* yeasts (Marco et al., 2017). Examination of our liquid samples over the course of coconut kefir production shows that *Lactobacillus* was more prevalent early in fermentation and that the final product contained higher levels of *Leuconostoc.* This succession trend is consistent with that reported in a recent study analyzing the production of dairy milk kefir (Walsh et al., 2016). While metabolomics experiments would be necessary to confirm that the metabolites present in coconut kefir are the same as those in dairy kefir, the presence of these beneficial bacteria are a good indication that coconut kefir would have similar health benefits as lactose-containing fermented products.

Part of the *Firmicutes* phylum, *Lactobacillus* species, are found in a number of fermented dairy products, are abundant in the gut, and have a number of beneficial effects on the host. They are able to make intestinal tight junctions less permeable to keep potential pathogens out (Lutgendorff et al., 2008). They are known to produce butyrate, protecting against obesity and colon cancer (Moeinian et al., 2014; Lin et al. 2012). While some species are implicated in increasing fat storage, others are implicated in decreasing it (Armougom et al., 2010; Aronsson et al., 2010; Million & Raoult, 2013). In chronic fatigue syndrome (CFS) and inflammatory

bowel syndrome (IBS) clinical trials, *Lactobacillus* improves mood and reduces anxiety (Rao et al., 2009).

6.4 LIMITATIONS & FUTURE WORK

The majority of microbiome profiling conducted within this thesis, was performed by sequencing a ~450bp region of the 16S rRNA gene. There are several well-known limitations to this approach. Unfortunately this region only targets bacteria, and so there is no information gained about other microbes such as archaea, eukaryotes and viruses. To identify fungi in the coconut kefir study we had to perform a second sequencing run using the ITS2 marker region. Metagenomic techniques allow scientists to sequence all of the DNA present in samples, including other types of microbes. Secondly, the variable region and primer choice can bias amplification of particular microbes (Huse et al., 2008; Kim et al., 2010; Kumar et al., 2011). For example, it has been shown that primers for the V7-V9 region amplify higher levels of *Veillonella, Streptococcus, Eubacterium, Enterococcus, Treponema, Catonella* and *Selenomonas*, compared to other primer regions (Kumar et al., 2011).

The Microbiome Helper pipeline uses QIIME scripts for quality filtering of 16S sequencing errors and then clusters the sequences into operational taxonomic units (OTUs) if they share 97% or more sequence similarity. This is a strict cutoff and does not always allow for resolution down to the species level and definitely not the strain level. Strain identification can be biologically relevant and other bioinformatic analyses packages such as DADA2 have attempted to correct for higher resolution and cluster sequences at 100% identity (Callahan et al.,

2016). Higher resolution analyses can be leveraged in future studies, especially in probiotics research.

Taxonomic assignment is limited by the database used, and there are many from which to choose. While the database used in this study, Greengenes, links multiple curated sequence sources together, the number of "de novo" clustered OTUs identified across the studies is an example of how microbial databases are far from being complete (DeSantis et al., 2006). Furthermore, while functional predictions can be made using PICRUSt, these inferences are limited to previously annotated sequences and do not inform directly on the genes present in the sample.

A marker gene sequencing represents a snapshot of a community. It tells you who is there at that moment but does not indicate the activity of cells and whether or not they are alive. New tools such as those developed by Brown et al. (2016) are able to use metagenomics, but not targeted marker gene sequencing, to get a sense of how fast bacteria are replicating. It is important to remember that this data also represents relative abundances of microbes. Proportions can be altered based on filtering and quality-check methods (Lovell et al., 2015; Gloor et al., 2016). New tools examining raw OTU counts are being developed to circumvent this problem.

While we are able to identify trends in microbial communities, correlations of microbial shifts do not demonstrate causation. Determining the mechanisms of action of interactions will be required to definitively show that factors directly impact the gut microbiome. Much more detailed studies are required in the future, and the development of methods to examine the dynamic interactions of these microbes with their hosts.

6.4.1 COMPLEX ANALYSES TO IDENTFY RELEVANT MICROBES AND INTERACTIONS

Since sequencing methods have allowed further investigation in the microbiome, human health studies have been looking for bacteria that perform key functions. While some have been identified and termed 'probiotics', healthy individuals have been shown to have wide taxonomic variation (Shafquat et al., 2014). Although conserved functions can be identified in the healthy human microbiome, there still exists a therapeutic challenge as to which microbes to promote.

Human microbiome communities are complex and it is not always easy to identify trends by examining levels of individual taxa. Methods are need that can account for the relationships that exist between microbes. Recently, more complex analyses using machine learning and network building help to address this. Our lab hopes to shed light on the effectiveness of machine learning and network analyses on human microbiome analysis.

6.4.2 HUMAN STUDIES

While mammalian models of the gut microbiome share many physiological traits, experimental models of disease cannot completely capture disease progression as it would occur in humans, limiting the clinical relevance of studies done in rodents. Secondly, while mice share the same major bacterial phyla as humans, at a species level their gut microbiomes have limited overlap (Nguyen et al., 2015).

In order to demonstrate the therapeutic potential of factors such as exercise, prebiotics and probiotics, it is necessary to administer these regimens in healthy humans to study their full

effects. While metadata such as weight and diet can be obtained from both humans and animals, it is easier to evaluate effects on quality of life in human studies, through interviews and questionnaires.

There are also several pertinent limitations to note on mouse models. Several of our experiments rely on the single housing of mice, which is contrary to their social nature and causes stress (Gonder & Laber, 2007). This stress could impact gut physiology and therefore microbiome data. Secondly, one of our studies involves measuring the amount of food consumed by mice, based on the weight of the food added to the cage, and the weight of the food left at the end of the week. We acknowledge that this measurement may be slightly inaccurate due to food lost to the cage, however we assume that this volume is consistent for each mouse. Lastly, coprophagy (the consumption of one's own feces) is a behaviour performed by mice, which could also influence the composition of the gut microbiome through the ingestion of large amounts of colonic bacteria (Groen et al., 2006).

6.4.3 MECHANISTIC STUDIES

While human studies are vital, they can be time consuming and environmental factors are difficult to control for. It is therefore important to identify which microbiome factors hold the most clinical promise. Mechanistic studies evaluating the interactions of bacteria with the host on a molecular level are scarce, but crucial. For example, it was reported that a bacterial lyase found in the gut was capable of converting choline into trimethylamine, a precursor to trimethylamineoxidase, a potent promotor of atherosclerosis. By identifying the bacterial source

of the enzyme and performing a large-scale drug screen, researchers were able to identify a small molecule inhibitor of the lyase (Wang et al., 2015).

This brings up a second point, which is determining whether it is through the direct interactions of microbes with host cells or indirectly through their metabolic products that microbes influence host physiology. While short-chain fatty acids to date are the best-known example of important microbial metabolites, it is known that gut bacteria have the ability to produce a number of compounds, including phenolic acids and Vitamin K (Rechner et al., 2002; LeBlanc et al., 2013). Metabolomics is becoming a popular, albeit expensive method of studying microbial processes that happen in the gut, through the evaluation of metabolites. In the future we can combine metabolomics with the methods used in this study to develop a more complete model of host-microbial interactions.

6.6 CONCLUSIONS

The human microbiome is critical in human health. While microbiome research is a relatively young field, it is clear that factors influencing these gut communities is essential in uncovering their potential as a therapeutic target. In these studies, we have explored several of these factors and their connection to shifts in the microbiome. Our examination of microbial composition has illuminated shifts that occur as a result of exercise and the consumption of a prebiotic. In addition, we have revealed the succession of microbes in an alternative probiotic. There is still much research needed to uncover mechanisms by which microbes affect our health and to be able to translate this knowledge into clinical application to treat human diseases.

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APPENDIX

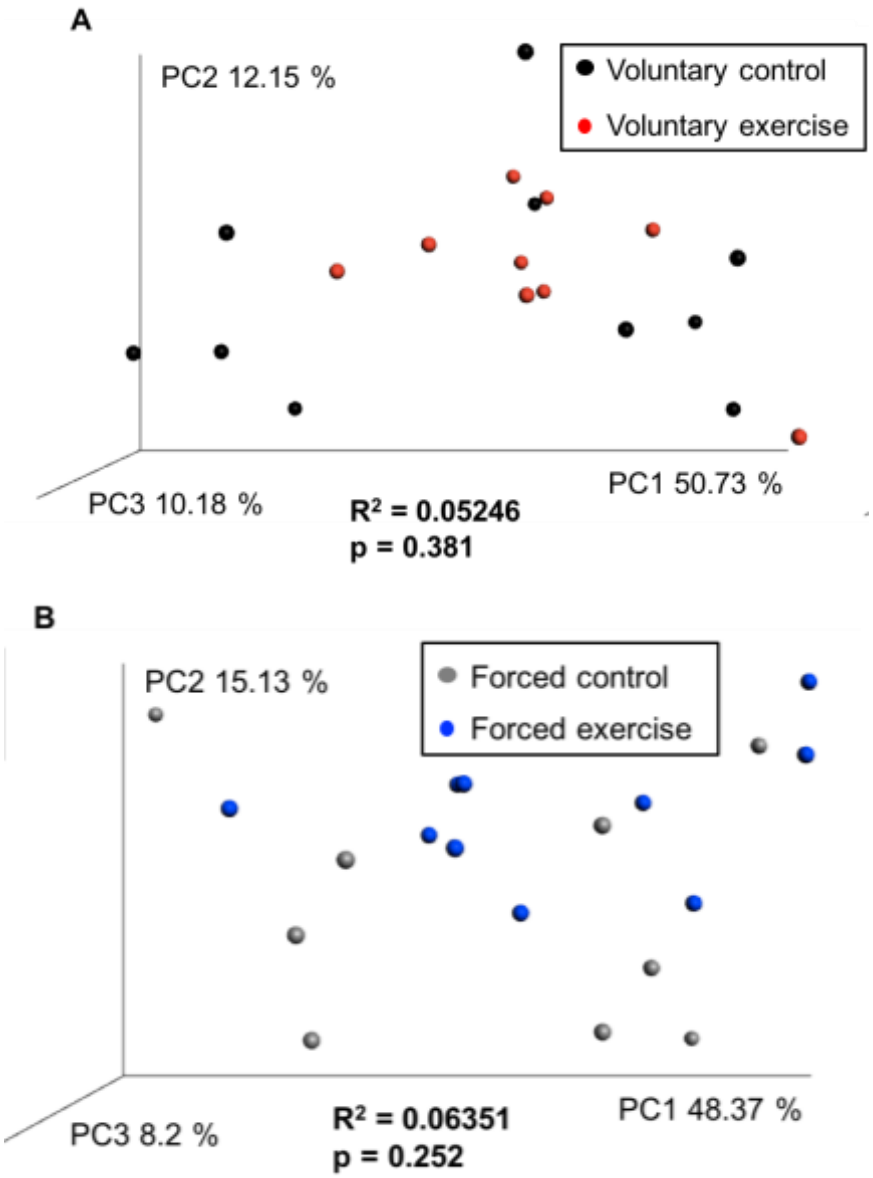

Figure 25 Weighted gut microbial diversity of voluntary and forced exercise mice using V4

Weighted UniFrac principal coordinate axis plots compare gut microbial diversity of exercise and control mice from (A) voluntary (W8: $R^2 = 0.05246$, $p = 0.381$ and (B) forced (W6: $R^2 = 0.06351$, $p = 0.252$) exercise cohorts. Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. VE: n = 9; VC: n = 10; FE: n = 9; FC: n = 10.

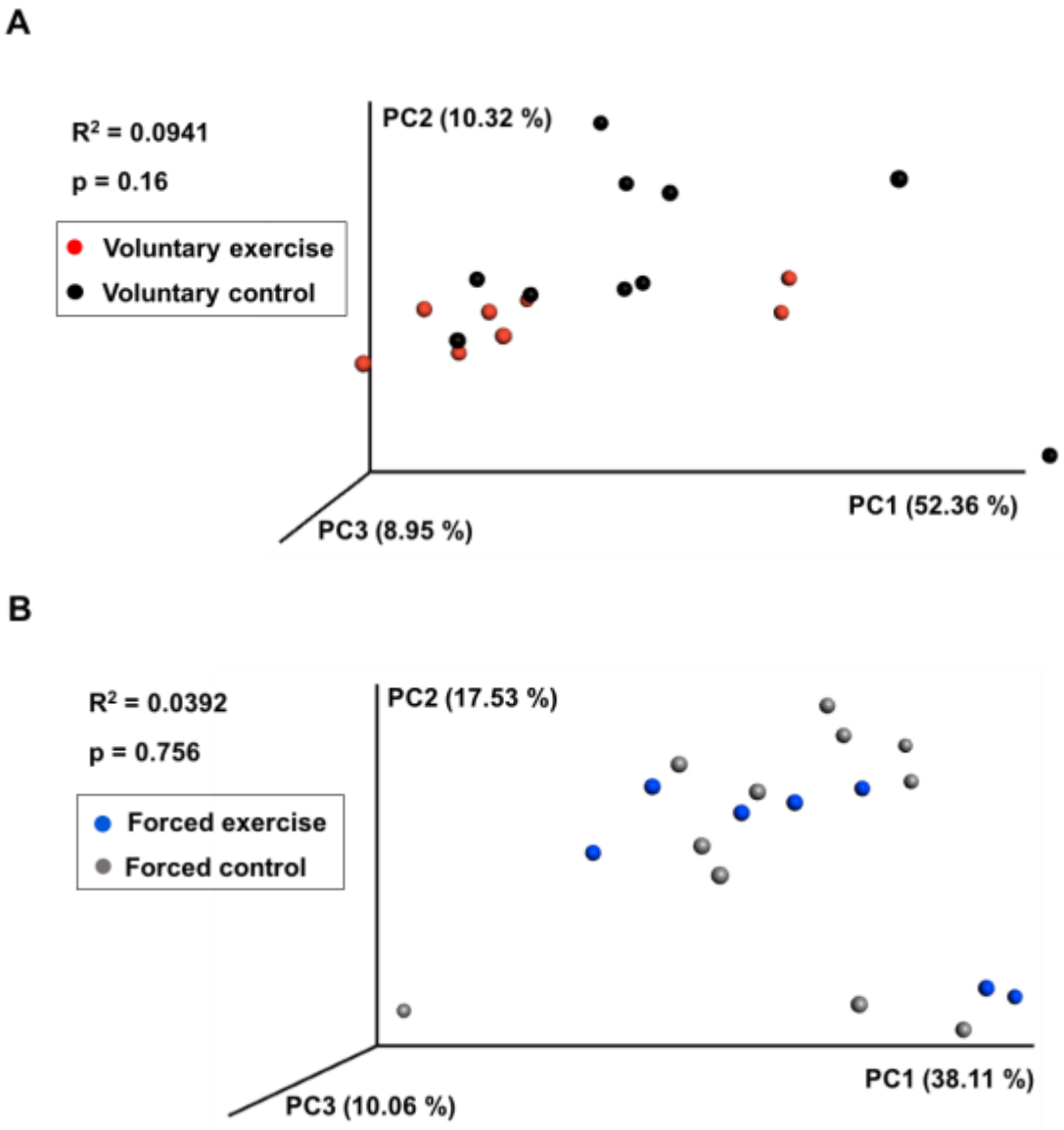

Figure 26 Weighted gut microbial diversity of mucosal samples in voluntary and forced exercise mice. Weighted UniFrac principal coordinate axis plots compare the colonic mucosal gut microbial diversity of exercise and control mice from (A) voluntary (W8: $R^2 = 0.0941$, $p = 0.16$ and (B) forced (W6: $R^2 = 0.0392$, $p = 0.756$) exercise cohorts. Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: VE: n = 8; VC: n = 10; FE: n = 7; FC: n = 11.

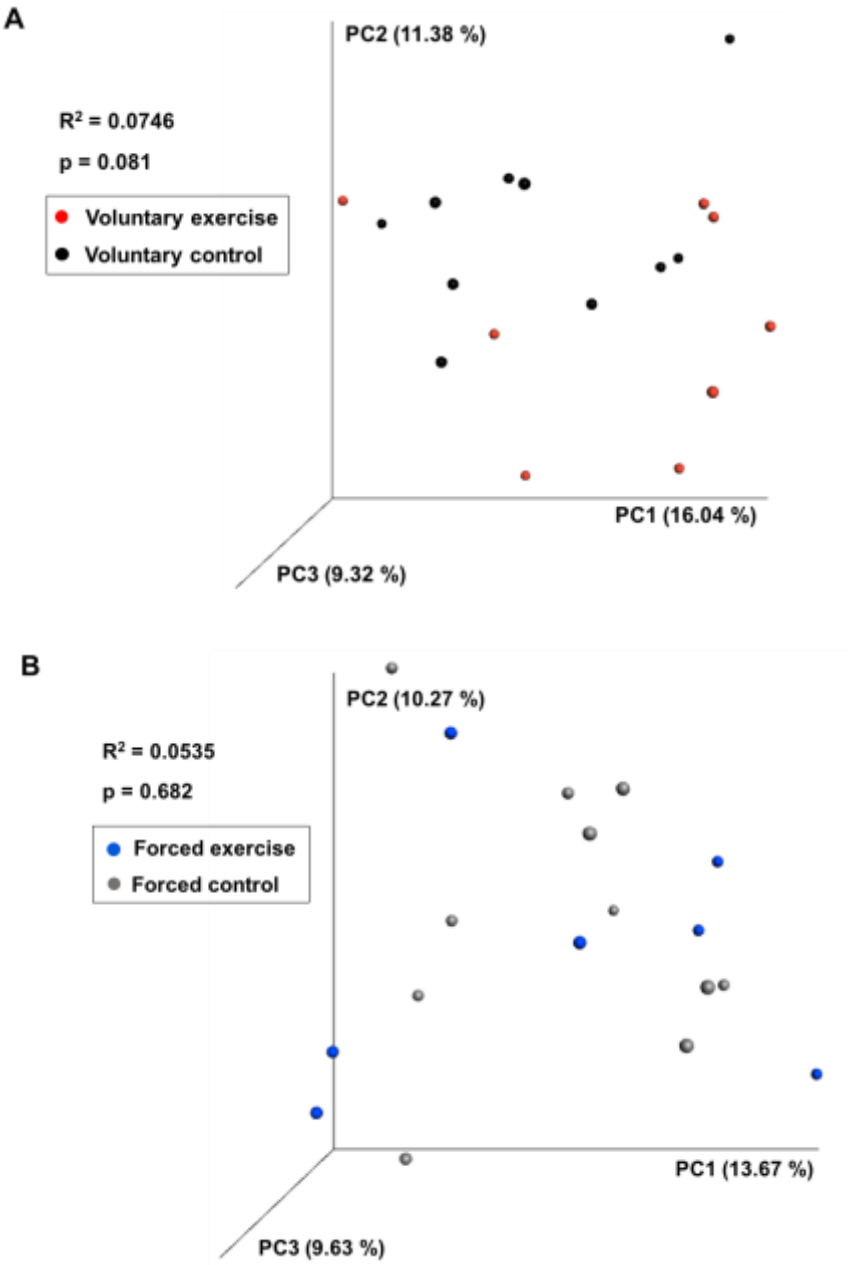

Figure 27 Unweighted gut microbial diversity of mucosal samples in voluntary and forced exercise mice. Unweighted UniFrac principal coordinate axis plots compare the colonic mucosal gut microbial diversity of exercise and control mice from (A) voluntary (W8: $R^2 = 0.0746$, $p = 0.081$) and (B) forced (W6: $R^2 = 0.0535$, $p = 0.682$) exercise cohorts. Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: VE: n = 8; VC: n = 10; FE: n = 7; FC: n = 11.

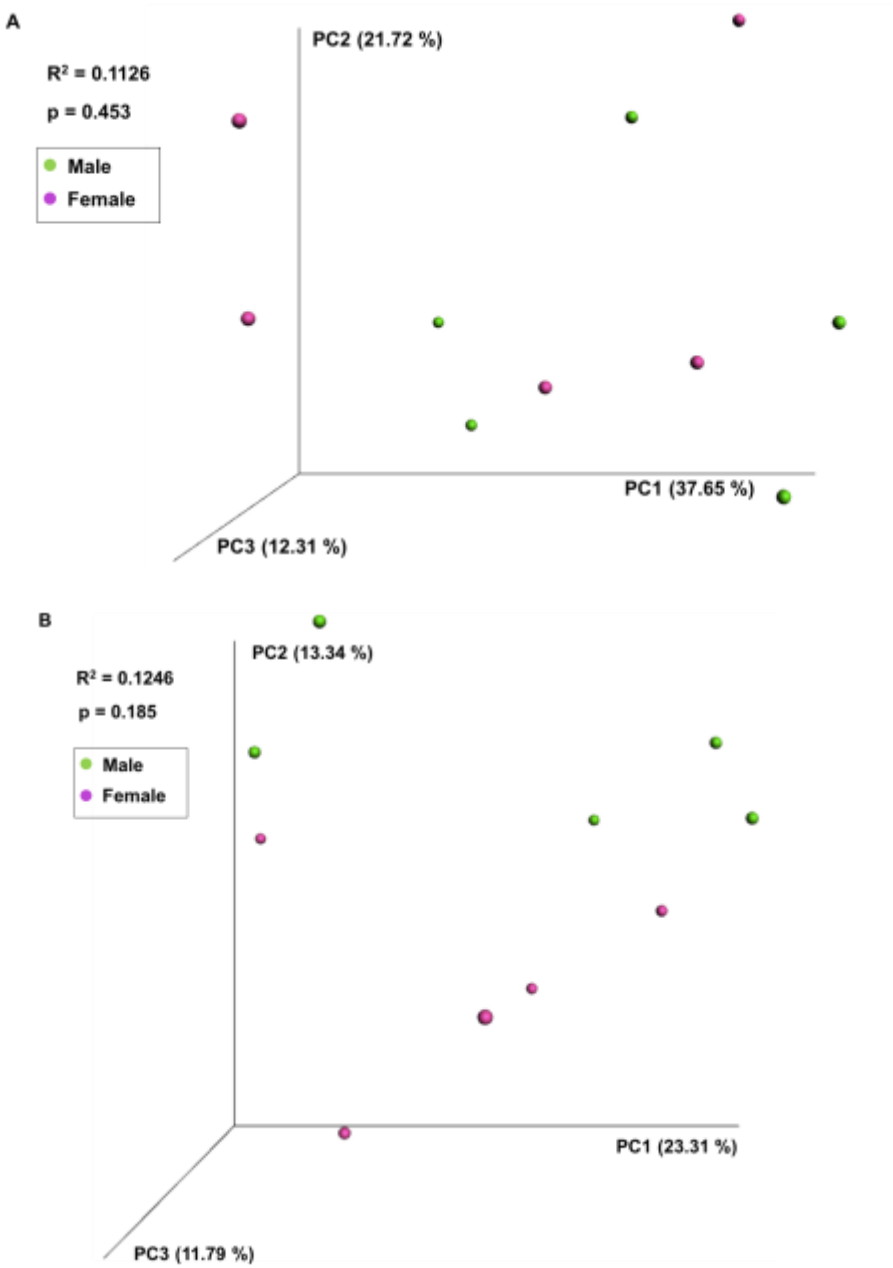

Figure 28 Comparison of male and female gut microbial diversity in forced exercise mice.

Fecal gut microbial diversity male and female mice from the forced exercise cohort is compared at week 6 by (A) weighted UniFrac principal coordinate axis plot ($R^2 = 0.1126$, $p = 0.453$) and (B) unweighted UniFrac principal coordinate axis plot ($R^2 = 0.1246$, $p = 0.185$). Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: male: n = 5; female: n = 5.

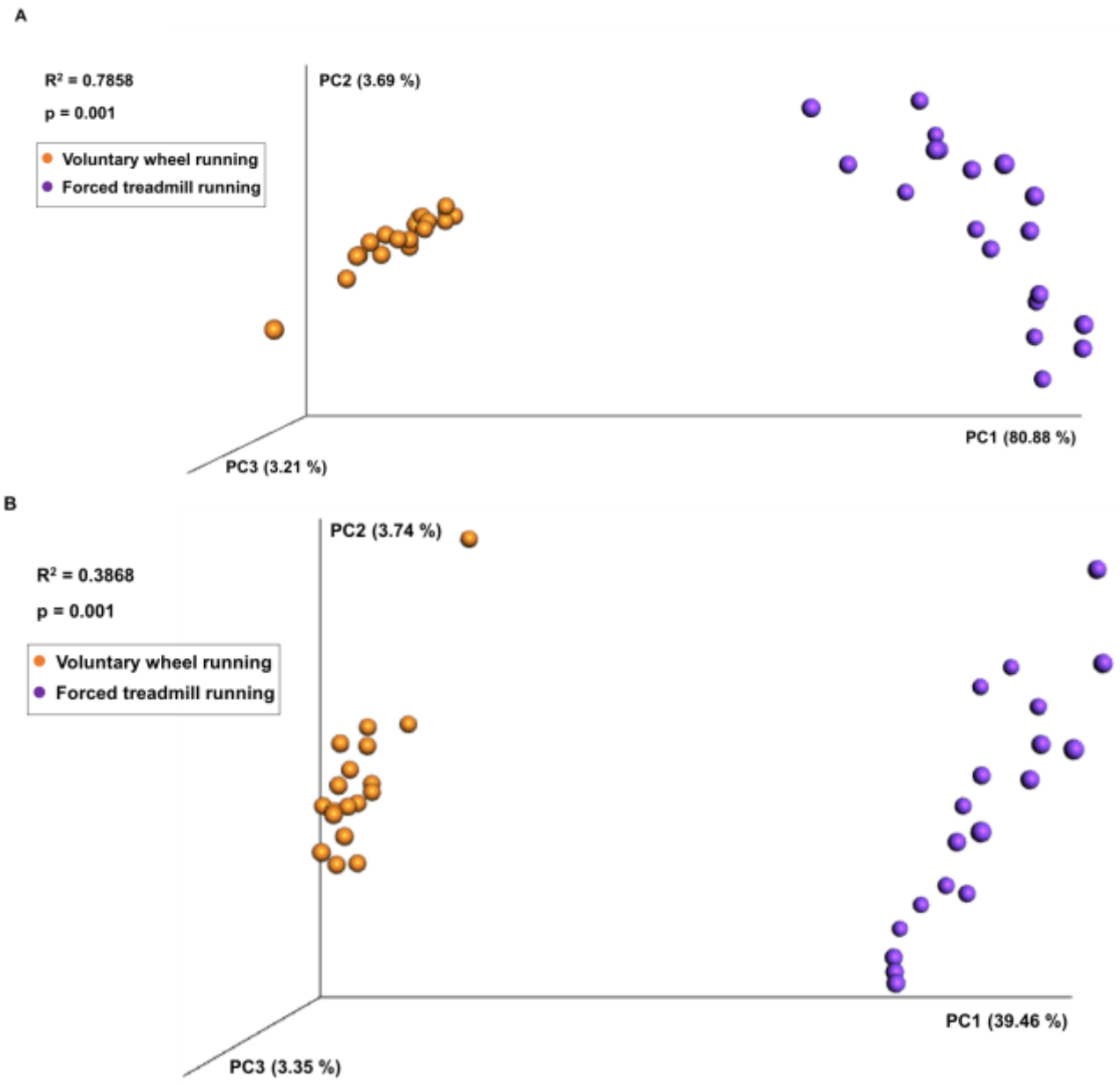

Figure 29 Comparison of gut microbial diversity in voluntary and forced exercise controls. Fecal gut microbial diversity control mice from the voluntary and forced exercise cohort is compared at week 0 by (A) weighted UniFrac principal coordinate axis plot ($R^2 = 0.7858$, $p = 0.001$) and (B) unweighted UniFrac principal coordinate axis plot ($R^2 = 0.3868$, $p = 0.001$). Beta-diversity of fecal samples was compared using an Adonis test with a significance cutoff of $p < 0.05$. Number of mice in each cohort: voluntary wheel running: n = 19; forced treadmill running: n = 19.