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Investigation of diet - microbiota interactions as affected by the carbohydrate intake of healthy human subjects

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Abstract

In relation to human health and well-being, strategies that positively influence the composition and activity of the microbiota are keenly sought, and diet is widely accepted as being a major factor for altering the gastrointestinal microbiota. The overarching aim of my Ph.D. studies was to characterize how diets with varying profiles of carbohydrate content and intake affect the gut and the oral microbiota of healthy individuals. Two key groups of subjects were used in separate studies: elite male athletes (endurance walkers) and healthy conventional subjects. During my studies, advances in DNA sequencing (and related bioinformatics methods) have enabled transition from the taxonomic profiling of microbial communities, to the production of data representing their collective genetic potential (i.e. the metagenome). As such, I have used both approaches here, and provide new insights into the nature of the diet x microbiota interactions in healthy individuals.

Chapter One reviews literature relating to diet x microbiota interactions with specific reference to how some diets (such as low FODMAP) affect the gut microbiota. Chapter Two provides the methodological details shared across the other research Chapters, and that samples either stored at sub-optimal temperatures, or that undergo repeated freeze-thaw cycles, are depleted of bacteria with a Gram-negative cell wall ultrastructure (e.g. Bacteroidetes, Proteobacteria). These results emphasized the importance of sample preservation and storage on these types of data generated from stool samples.

Chapters Three and Four presents my results and conclusions about how the dietary pattern of elite race walkers during their period of intensified training affected their oral and stool microbiota, respectively. This research was undertaken in complement to the Supernova 1 study coordinated by Australian Institute of Sports. Stool and saliva samples were collected at the beginning and end of a three-week dietary intervention period, from elite male endurance race walkers choosing to consume either a High Carbohydrate (HCHO), High Carbohydrate-periodised (PCHO) or a Low Carbohydrate High Fat (LCHF) diet, and the microbial communities were examined using 16S rRNA amplicon sequencing. The results in Chapter Three show that the LCHF diet results in substantive changes in

the oral microbiota, and in particular, reductions in the relative abundance of bacterial taxa known to be key nitrate-nitrite reducers (*Haemophilus*, *Neisseria*, and *Prevotella)* whereas increased the relative abundance of *Streptococcus* not known to be associated with nitrate reduction in the oral cavity. The Results in Chapter Four showed that the athletes could be stratified into either a *Bacteroides*-dominant or *Prevotella*-dominant "enterotypes" and while the diets consumed during intensified training did not disrupt these enterotypes, the LCHF diet significantly increased the relative abundance of *Bacteroides* and *Dorea* spp., whereas the relative abundance of *Faecalibacterium* spp. were reduced in athletes consuming the LCHF diet. Furthermore, the relative abundance of *Bacteroides* and *Dorea* following consumption of LCHF diet were found to be significantly negatively associated with fat oxidation and economy measures, respectively. Collectively, these results suggest that a ketogenic LCHF diet invokes profound changes in the oral and stool microbiota of athletes and can be associated with athlete performance measures during intensified training and simulating race conditions.

Chapter Five presents my findings of how a diet prepared from foods to provide either a low (LP 1-3 g/day oligosaccharides; 0.50 g/day polyols) or moderate (MP 6-8 g/day oligosaccharides; 3.66 g/day polyols) daily intake of prebiotic carbohydrates affected the gut microbiota of healthy adults. The parent study was a single-blinded, randomised crossover study, managed by our collaborators with the Alfred Hospital (Monash University) Translational Nutrition program. Here, I first produced both 16S rRNA and ITS-2 gene amplicon profiles to characterise the prokaryote and fungal communities, respectively. These analyses showed that the prokaryote richness is reduced and fungal richness is increased by the MP diet as compared to the LP diet. The reduction in prokaryote richness was reflected in a significant increase in the relative abundance of *Bifidobacterium* spp. with the MP diet. *Saccharomyces*-related fungal lineages were the most abundant across the cohort with both diets, I did find different prokaryote-fungal relationships with the LP and MP diets. I then used these same DNA samples for shotgun metagenome sequencing (MGS) analyses that further confirmed an expansion of Bifidobacterial spp. and revealed significant increase in gene counts for the metabolism of sorbitol and mannitol and related phosphotransferase transport systems (PTS) pathways in response to the MP diet suggesting a bifidogenic effect of moderate amounts of sorbitol and mannitol.

Chapter Six provides an integrated assessment and interpretation of the findings and potential impacts arising from my Ph.D. research. I believe my findings are novel and provide a better understanding of the diet x microbiota interactions in healthy individuals. I discuss these new insights with respect to what constitutes a healthy microbiota, and how a person's diet can be rationally managed and personalised to sustain healthy gut function, nutrition, and well-being.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and *does not include a substantial part of work that has been submitted* to qualify for the award of any *other degree or diploma* in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Published, peer-reviewed review article

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Conference abstracts

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Murtaza N, McNamara L, Hoedt E, Muir J, Gibson P, Morrison M. Diets with different prebiotic content alter the gut prokaryote and fungal microbiota. Digestive Diseases Week conference. 2018 June 5-8; Washington, America.

Murtaza N, McNamara L, Hoedt E, Ó Cuív P, Muir J, Gibson P, Morrison M. Metagenomic Analyses of the gut microbiota of healthy Australian subjects consuming diets with different levels of prebiotic content. International Human Microbiome Congress. 2018 June 26-28; Killarney, Ireland.

Murtaza N, McNamara L, Hoedt E, Ó Cuív P, Muir J, Gibson P, Morrison M. Metagenomic Analyses of the gut microbiota of healthy Australian subjects consuming diets with different levels of prebiotic content. Translational Research Institute Symposium. 2018 July 29-30; Brisbane, Australia.

Murtaza N, McNamara L, Hoedt E, Ó Cuív P, Muir J, Gibson P, Morrison M. Metagenomic Analyses of the gut microbiota of healthy Australian subjects consuming diets with different levels of prebiotic content. UQ Public Health and Clinical Symposium. 2018 November 22-23; Brisbane, Australia.

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Author Campbell K	Edited paper (1%)
Author Krause L	Statistical support and guidance (5%) Edited paper (5%)
Author Morrison M	Technical and statistical support (10%) Wrote and edited paper (15%)

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Author Charlesson B	Edited paper (1%)
Author O' Neill H	Edited paper (1%)
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No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

The study described in Chapter 3 and 4 was approved by the Ethics Committee of the Australian Institute of Sport (AIS, no. 20150802) and has been registered as a clinical trial, assigned the number ACTRN12618001529235 by the Australian New Zealand Clinical Trials Registry (ANZCTR). The microbiota research was conducted as part of UQHREC2015001965.

The study described in Chapter 5 has been conducted under ethics approvals MUHREC CF14/2904, 2014001593 and the microbiota analyses presented in Chapter 5 performed as part of UQHREC 2015000317.

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List of abbreviations used in the thesis

µl	microliter
ACE	Australian Centre for Ecogenomics
ANI	Average Nucleotide Identity
Anosim	Analysis of similarity
AXOS	Arabinoxylan oligosaccharides
BL	Baseline
BM	Body Mass
BMI	Body Mass Index
CAZyme	Carbohydrate Active enzyme
CD	Crohn's Disease
CI	Confidence Interval
CHO	Carbohydrate
DI	Diamantina Institute
EDGAR	Efficient database framework for comparative genome analysis using BLAST score ratios
EDTA	Ethylenediaminetetraacetic acid
EEN	Exclusive Enteral Nutrition
FDR	False Discovery Rate
FODMAP	Fermentable Oligo-, Di-, Monosaccharides and Polyols
g	grams
Gbp	Giga base pairs
GI	Gastrointestinal
GOS	Galactooligosaccharides
HCHO	High Carbohydrate
HMP	Human Microbiome Project
HREC	Human Research Ethics Committee
HUMAnN2	the HMP Unified Metabolic Analysis Network
IBS	Inflammatory Bowel Syndrome
IBD	Inflammatory Bowel Disease
ITF	Inulin type fructan
ITS	Internal Transcribed Spacer
LDA	Linear Discriminant Analysis

LEfSE	Linear discriminant analysis effect size
LCHF	Low Carbohydrate High Fat
LP	Low Prebiotic
MAC	Microbiota accessible carbohydrate
MAGs	Metagenome Assembled Genomes
MetaPhlAn	Metagenomic Phylogenetic Analysis
MELR	Mixed Effect Linear Regression
ml	millilitre
mM	millimolar
mg	milligrams
MP	Moderate Prebiotic
NaCl	Sodium chloride
NO	Nitric oxide
OTU	Operational Taxonomy Unit
PATRIC	Pathosystems Resource Integration Center
PCHO	Periodised Carbohydrate
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinates Analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PEP-PTS	Phosphoenolpyruvate phosphotransferase system
PT	Post treatment
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	quantitative Polymerase Chain Reaction
RDA	Redundancy analysis
16S rRNA	16S ribosomal RNA
SCFA	Short Chain Fatty Acid
sPLS-DA	Sparse PLS discriminant analysis
TRI	Translational Research Institute
UC	Ulcerative colitis
UQ	University of Queensland
VO_{2peak}	Peak aerobic capacity

Chapter 1

Chapter 1 General introduction and literature review

Diet and the Microbiome

1.1 An introduction to the gut microbiome

There is a vast diversity of microbes residing in the gastrointestinal tract (GI) of humans (Figure 1). The term “gut microbiome” in general refers to the collective genome of the microbial communities inhabiting the terminal or the large bowel region of the gastrointestinal tract (1). It has been long recognised that the gut microbiota maintains a symbiotic relationship with the host and imparts structural, nutritional and protective functions to the host (2). Fermentation of the carbohydrates by the colonic gut microbiota such as *Bacteroides, Roseburia, Bifidobacterium* and *Fecalibacterium* results in the synthesis of short chain fatty acids such as acetate, propionate and butyrate which further imparts energy and potential health benefits to the host (3). Even though it is now known about the potential health benefits of gut microbiota to the host, the ability to fully realize the importance of individual members was limited due to the conventional culture-based approaches. Isolation using the culture based approaches was an ardous task as it was partial, and only able to isolate and characterize 15-20% of the microbes thus making it more time consuming and difficult (2) to fully ascertain the roles specific microbes play in the above-mentioned ways.

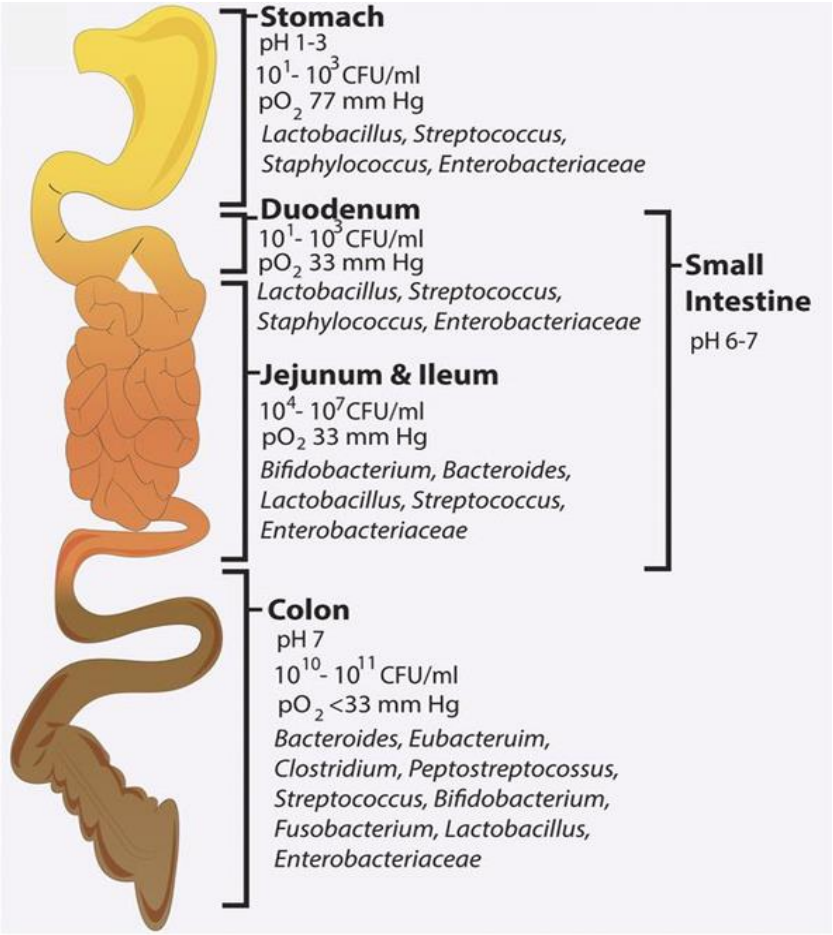

Figure 1.1 Vast diversity of microbes residing in the gastrointestinal tract of humans. Reproduced from Clarke et al. (4) :

There are several extant definitions of the term "microbiome," a field of research that has become principally associated with the technological advances in DNA/RNA sequencing and computational biology. As such, the microbiome is still commonly defined as the collective genomic content of all microbes recovered from a habitat or ecosystem (eg: saliva and stool samples, skin swabs) (5). However, although such a definition captures the functional potential inherent to the microbiota (micro-), there is a need to place this knowledge in context with the interactions and processes contingent on the physicochemical attributes of their surrounding environment (-biome). This more holistic definition of the microbiome is applied throughout this thesis, in recognition of diet as a major influence on microbiome dynamics. By doing so, the concept of nutritional ecology is introduced: how the nutrient and its variations across temporal and spatial scales affect the gut and oral microbiota. I contend that nutritional ecology will provide the mechanistic bases for understanding

"diet and the microbiome," which will translate into improved diagnoses and treatments for functional and organic diseases.

1.2 General Concepts and Approaches of Microbiome Studies

Morgan and Huttenhower present a well-structured and illustrated general overview of the techniques and approaches underpinning microbiome studies (6) (See Figure 1.2). Over the last two decades, microbiome studies have emphasised the use of polymerase chain reaction (PCR) techniques targeting regions within the gene encoding 16S ribosomal RNA (16S rRNA) in prokaryotes (i.e., bacteria principally, and archaea). When combined with the rapid advances in DNA sequencing technologies and a combination of ecologic, biostatistical, and computational methods, these 16S rRNA profiling methods have resulted in a taxonomy-based assessment of gut microbial communities' resident in different regions of the gastrointestinal tract. Importantly, these approaches have afforded the differentiation of the microbiota to reveal specific microbes and microbial consortia indicative of alterations to gut homeostasis, which are generically referred to as "dysbiosis" (7,8). During the same period the National Institutes of Health Human Microbiome Project (9) has augmented these studies by producing the "reference genomes" of individual microbial species, which has supported the inference of the functional attributes inherent to the 16S rRNA profiles by such methods as PICRUSt (10). However, and because of the continued advances in sequencing technologies, the time and cost constraints to "shotgun metagenome sequencing" are being relaxed, which affords a scale and depth of sequence coverage that provides an actual (rather than inferred) representation of the functional attributes inherent to the microbiota (6).

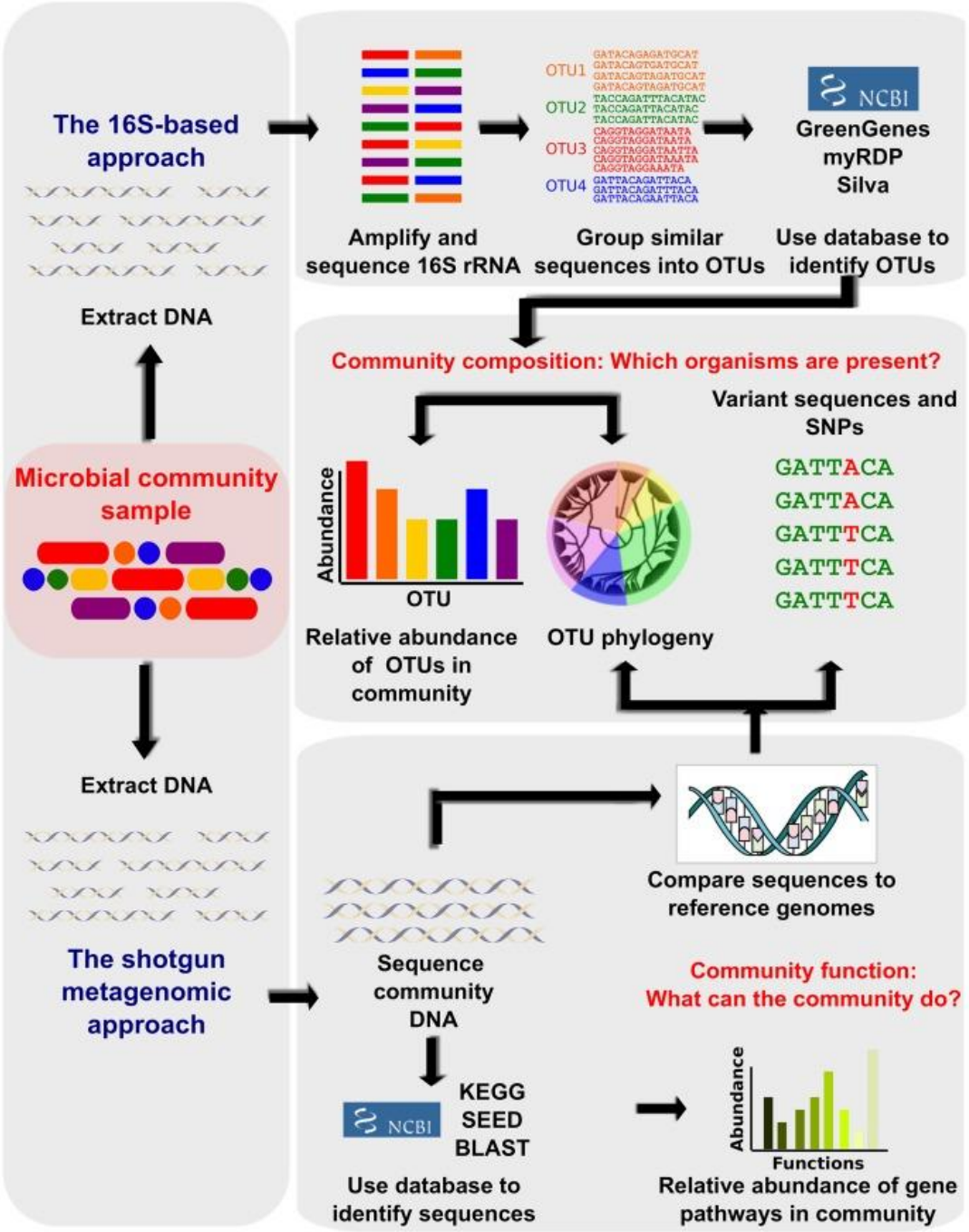

Figure 1.2 Bioinformatics methods used for analyzing the microbiota by 16S rRNA gene profiling or shotgun metagenomics approach. Reproduced from Morgan and Huttenhower (6).

These studies have also substantiated that the microbial communities of the gut are readily differentiated according to their microbial (and gene) density, diversity, and distribution; as affected by anatomic structure, host secretions, and digesta residence times at different sites. Although the

esophageal, stomach, and small intestinal microbiota have now been characterised (1), most studies that have advanced the mechanistic understanding of diet-microbiome interactions have been undertaken using stool/fecal samples and/or tissue samples collected from the large bowel. As such, the term "gut microbiome" has come to define this (terminal) region of the gastrointestinal tract. During the last 5 years in particular, shotgun metagenome sequencing of stool microbiota and the associated metagenome-wide association studies has revealed that the form and function of the stool microbiota is altered in patient cohorts with type-2 diabetes, cirrhosis, and colorectal cancer (11–14). These differences have not only provided insights of how microbial metabolism contributes to disease, but the identification of candidate gene and organismal biomarkers of health and disease (11–14). Critically, these methods have also shown the gut microbiota of humans (and other animals) is rapidly altered by changes in habitual or available diet, leading to the perception that diet may exert a stronger selective pressure on the gut microbiota than host genetics (15,16). A particular focus in the last 10 years has related to obesity research, with Turnbaugh and colleagues (17) reporting an enrichment of microbial genes involved in carbohydrate, lipid, and amino acid metabolism in the obese adult gut. More recent studies have revealed that non-obese and obese individuals are characterised by variations in gene richness: subjects with a low gene count are characterised by increased adiposity, insulin resistance, and inflammation (18,19). The difference in gene richness has been suggested to be predictive of previous weight gain and in mice, could be partially reversed following a dietary intervention for weight loss (20). The links between diet and microbiome have also been further substantiated via fecal transplant studies in animal models. For instance, Turnbaugh and colleagues were among the first to show how the transfer of an obese (or lean) phenotype to a naive host (i.e., germ-free mice) can be effectively recapitulated using diet to exert the necessary selective pressure to sustain the microbiome (21). This type of an approach is being increasingly used to establish how either specific microbes or microbial consortia contribute to the onset of non-communicable metabolic and immune-mediated diseases (22,23). Additionally, the benefits of existing and candidate next-generation probiotic strains, in terms of their capacity to attenuate inflammation and/or positively affect barrier function and host metabolism (eg, *Bifidobacterium* spp, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*), are being examined by their

introduction to either germ-free or conventionally reared mice (24–26). Table 1.1 provides a summary of some recent advances in the understanding of how diet and the gut bacteria, from ecologic, metabolic and immunomodulatory contexts, can affect gut function and health. In addition to the other contributions provided in this Chapter, there are numerous books (27) and reviews of the topic, especially as it pertains to diet-microbiome interactions during pregnancy and early life (28,29), obesity and metabolic diseases (30,31) and immune development and immune- mediated diseases (7,8,32–36). Indeed, these interrelationships are now being defined from conception to grave: from their influences on fetal and infant developmental biology and homeostatic processes, to triggering a plethora of acute and chronic (extra) intestinal diseases, through to defining rules for microbiome restoration to better treat diseases and prevent relapse.

Table 1.1 provides recent examples of diet microbiome interactions

Diet	**Ecological Effect**	**Microbiota Effect**
Breastmilk	Provides human milk oligosaccharides	Promotes growth of Bifidobacteria and *Lactobacillus* (32)
Low FODMAP diet	Mitigates IBS symptoms	Alterations in bacterial taxa (37)
Exclusive enteral nutrition	Induces remission and promotes healing in CD	Alters structure-function activity of the microbiota (33,38)
Grains, resistant starches, "fibers"	Promotes SCFA production and reduced pH	Suppresses growth of Gram-negative Enterobacteria
	Anti-inflammatory effects	Promotes growth of acetate and butyrate producers Stimulates Bifidobacteria and *F. prausnitzii* (39–41)
Dietary Metabolites	**Metabolic and/or Immunomodulatory Effects**	**Microbiota Effect**
SCFA	Metabolic effects via specific GPCR receptors Effects of butyrate on Treg cells	Variations in proportions and total SCFA produced (42) Loss of butyrate producing commensals with inflammation (eg, *F. prausntizii*) (43)
Trimethylamines	Risk factor for cardiovascular and renal disease	Microbial by-products of choline and creatinine (44)
Branched-chain amino acids	Promotes insulin resistance and diabetes	Enrichment of these pathways in microbiome of T2D (11)
Primary bile acid metabolites	Variations in host signaling via NR and GPCR	Gram-positive and sulfidogenic bacteria, *Bacteroides* (45)
Riboflavin precursors	Activation of MAIT cells	Produced mainly by Gram-negative enteric bacteria (46)
Anti-inflammatory peptides	Anti-inflammatory effects	Loss of *F. prausnitzii* associated with inflammation (47)
Polyphenols	Improve barrier function and proglucagon levels	Stimulation of *A. muciniphila* populations (48)

1.3 Fungi and the gut – the mycobiome in health and disease

In addition to Bacteria and Archaea, the fungi represent another key component of the gut microbiota. A thorough and excellent review about the gut mycobiota, insights about analysis, environmental interactions and their role in gastrointestinal diseases was recently published by Richard and Sokol (49). It is possible to target the fungal communities using culture-based or metabarcoding sequencing approaches targeting the Internal Transcribed Spacer 1(ITS1) or ITS2 sequences between the fungal ribosomal subunits. However, the low abundance of fungi in comparison to the bacterial fraction of the community have provided some constraints to the use of shotgun metagenomics sequencing (MGS), with deep sequencing depth required to produce a detectable genomic signal; and suggests that fungi might constitute ~0.1% of the gut microbiome (50). However, fungal cells are ~100 times larger in volume than most bacteria, and thereby constituting a larger biomass on a proportional basis (49). Cross-sectional studies have recently provided evidence of there being differences in the gut mycobiome in health and disease. Fungi are also well known for their secretion of a diverse variety of enzymes and other metabolites that can affect the host response and gut homeostasis, even in small concentrations. For instance, *Candida albicans* and *Saccharomyces boulardii* have been shown to have a pro and anti-inflammatory effect, respectively, using *in vitro* cultures and a mouse model of colitis (51). Mycobiome alpha diversity appears to be reduced in IBS patients compared to healthy controls (52) and Sokol et al. (53) reported that there are reductions in the abundance of the phylum Ascomycota and an increase in the phylum Basidiomycota in IBD (53). Studies on both fecal and mucosal tissue samples have reported an increase in *Candida* spp. and a reduction in *Saccharomyces cerevisiae* in IBD patients compared to healthy subjects (53–56). Decreased stool mycobiota diversity has been reported for patients with polyps, early stage tumours, and colorectal cancer, with an increase in the Ascomycota/Basidiomycota ratio and the relative abundances of both *Trichosporon* and *Malassezia* (57). Compared to non-involved tissue, fungal diversity is reduced and with the phyla Glomeromycota and Chytridiomycota detectable in colorectal adenomas, with these profiles also associated with the stage of the carcinoma development (58). Taken together these findings suggest that like bacteria, the mycobiome dysbiosis is evident in stool and mucosal tissue samples from

patients with digestive diseases or disorders, with a reduction in alpha diversity as well as taxonomic variations apparent in health and disease.

While these microbes are now being considered as a key contributor to gut function and health, there are still relatively few published studies in terms of how dietary patterns affect the mycobiome. The current understanding of the role of diet on the gut mycobiome outlined by Richard et al. (59) is illustrated in Figure 1.3. Mice that are fed high-fat diets possess different fungal and bacterial communities than the mice on standard low fat chow diet (60) predominantly *Candida* when fed high-fat diets and *Fusarium* and *Alternaria* with standard chow. The relative abundances of *Alternaria, Saccharomyces, Septoriella* and *Tilletiopsis* spp. were also reduced in animals fed the high fat diet. In humans, a study of 98 healthy adult subjects by Hoffmann et al. (61) showed the relative abundance of *Candida* in stool samples was positively corrrelated with the consumption of carbohydrate-rich diets, and negatively correlated with the consumption of a diet with a greater content of protein and fat. Another study of 10 healthy subjects, by David et al. (16) also showed that the relative abundance of *Candida* and *Penicillium* was affected by the subject's dietary preferences for plant-based or animal-based foods, respectively. Furthermore, the subjects who consumed a diet with relatively large amounts of animal-based foods were also found to have greater amounts of fungi in their stool compared to those who consumed plant-based diets. In contrast, *Fusarium* was reported to be the most abundant genus in stool samples from healthy subjects consuming a strict vegetarian diet, followed by *Malassezia, Penicillium, Aspergillus* and *Candida* (62).

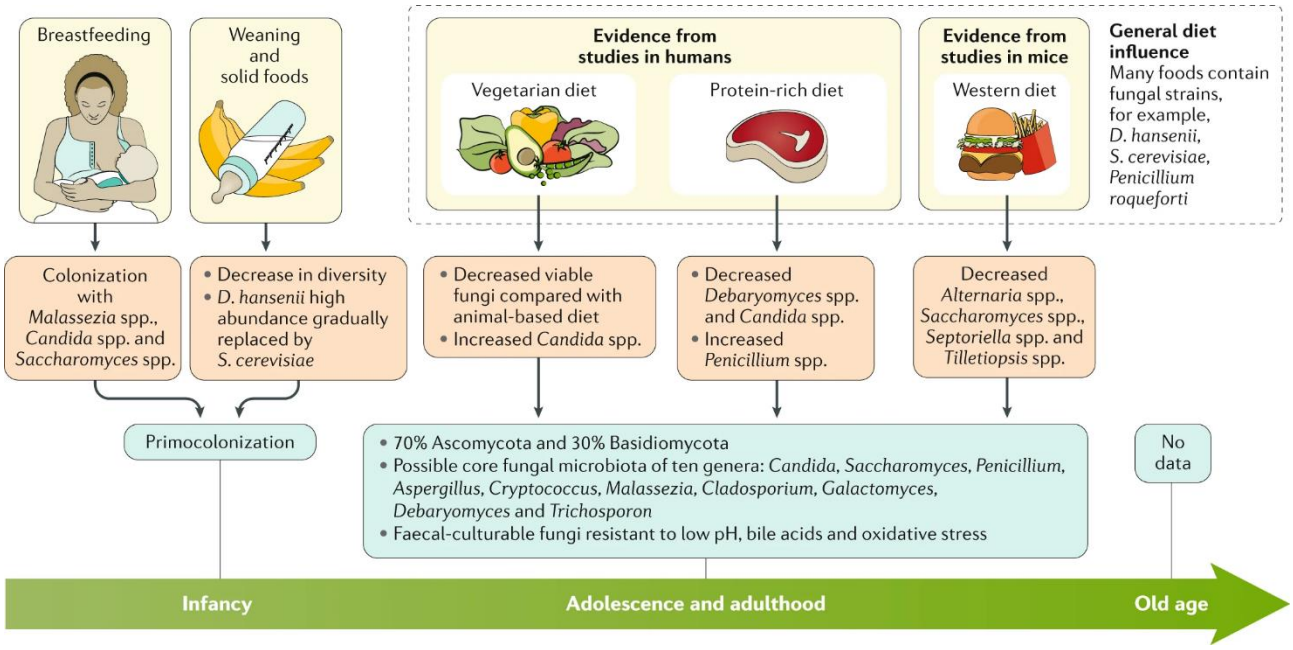

Figure 1.3 Healthy mycobiota and its evolution with environmental factor- diet. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nat Rev Gastroenterol Hepatol, The gut mycobiota: insights into analysis, environmental interactions and role in gastrointestinal diseases, Mathias L. Richard et al. (59), Springer Nature, 2019.

In summary, the available published data suggests that the gut mycobiota is dynamic and responsive to alterations in dietary pattern and/or gut homeostasis. However, more in-depth research needs to be done in order to decipher the role of the predominant fungal taxa in health and disease, and how diet might be used to better manage this important component of the gut microbiota.

1.4 Athletes and gut health

We have learned much about how changes in the microbiota at different body sites begins at conception and extends throughout our entire lifespan, and how the microbiome contributes to the onset and potential treatment of non-communicable diseases. Importantly these studies have also revealed that our so-called "commensal" microbes, many of which remain elusive to culture, contribute a myriad of previously underappreciated goods and services, and often function as a community to realize changes in our health and well-being. The overwhelming amount of this

knowledge has been produced from human subjects with an underlying health condition or drawn from the general population. There are scant reports detailing the microbiomes of elite athletes and how their dietary choice and lifestyle might impact on their microbiomes. However, given the precedents now established from studies of the general population, it is entirely plausible to expect the microbiomes of elite athletes to directly or indirectly affect their health status, resilience to infection and allergy, and performance. Clarke et al. (63) reported that the gut microbiome of professional rugby players had higher alpha diversity as compared to both the low BMI and high BMI control groups. Additionally, the rugby players compared to the healthy controls had higher relative abundance of *Akkermansia muciniphila* which has been linked with improved metabolic health in both mice and recently in overweight/obese human subjects (63–65). Further shotgun metagenomic analysis revealed that the rugby athletes had higher abundance of amino acid and antibiotic biosynthesis and carbohydrate metabolism pathways as well as higher abundance of fecal SCFA's than the control groups (66). Recent studies have also reported that an individual's microbiome can change depending on the amount of exercise they do. A study reported active women who performed at least 3 h of exercise per week to have greater abundances of *Faecalibacterium prausnitzii, Roseburia hominis,* and *Akkermansia muciniphila* as compared to the sedentary controls (67). Peterson et al. (68) revealed the presence of 3 distinct clusters or "enterotypes" characterised by either a high *Prevotella*, high *Bacteroides* or a mix of several genera, in the gut microbiome of professional cyclists. The study also revealed increased abundance of Archaea *Methanobrevibacter smithii* in a number of professional cyclists when compared to the amateur cyclists (68). In a recent study, members of the genus *Veillonella* have been associated with exercise performance in marathon runners (69). *Veillonella* was found to increase in marathon runners postmarathon and *V. atypica* isolated from the stool samples of these runners increased the exhaustive treadmill run time when inoculated into mice. The study also reported that the lactate or lactic acid produced during exercise is efficiently converted into propionate by the members of genus *Veillonella* (69). In another study, 32 sedentary adults including both lean and obese, participated in a 6-wk supervised endurance training program (30- to 60-min duration, 3x per week) with stringent dietary controls (70). Exercise increased *Faecalibacterium* in the lean subjects but reduced its abundance in the obese

subjects; unlike *Bacteroides* whose abundance was decreased in the lean subjects and increased in the obese subjects. Six weeks of endurance exercise also increased the abundance of butyrate-producing taxa and fecal acetate and butyrate concentrations only in lean subjects. Interestingly, the shifts in the bacterial taxa and SCFAs levels were subsequently decreased during the 6-wk sedentary washout period, suggesting that the effects of exercise on the microbiota were both transient and reversible (70). Taken together, all these findings suggest that the gut microbiome of athletes are different from those of mainstream healthy subjects, and diet, along with exercise, might be a principal driver behind these differences.

1.5 Oral microbiome

The oral microbiome can be defined as the collective genomes of microorganisms inhabiting the oral cavity (71,72). The oral cavity is a densely populated site of the human body comprising of more than 700 bacterial species (72). It has been well characterised in relation to infectious and periodontal diseases and dental caries as well as its association with systemic diseases such as diabetes mellitus and cardiovascular disease (73). However, the effects of diets on the oral microbiome including both the mainstream healthy subjects as well as on elite athletes are not well defined. In relation to this recent studies have shown symbiotic associations between oral microbiota, host health and the nitrate-nitrite-nitric oxide (NO) pathway contributing to the NO homeostasis (74,75). Studies in healthy human subjects have also shown the role of oral nitrate reducing bacteria in controlling the blood pressure (76). In another study, the supplementation of an antiseptic mouthwash was shown to reduce the abundance of nitrate reducing oral bacteria, reduction in the levels of circulating nitrite and the loss of gastroprotective effect against an ulcerogenic compound (77). Dietary nitrate supplementation from beetroot juice has been shown to decrease the relative abundances of bacterial taxa *Veillonella* and *Prevotella* whereas increase the relative abundances of *Neisseria* and *Rothia* in both young and old human subjects (78). Nitrate supplementation resulted in elevated plasma nitrite levels and decreased systemic blood pressure in old human subjects suggesting the association of dietary nitrate, oral bacteria and vascular health (78). Taken together, all these findings suggest the potential of the oral microbiome on host health and justify the need to investigate diet x oral microbiome interactions in humans.

1.6 The Conundrum of Diet, Microbiome and Irritable Bowel Syndrome

Thorough and excellent reviews of the diet microbiome interactions affecting IBS treatment and symptom control have been published by Rajilić-Stovanović and colleagues (79) and Staudacher and Whelan (37). Samples from IBS subjects have been used and described in Chapter 2 of this thesis. In summary, there have been much fewer studies of diagnosed patients with IBS compared with patients with IBD, and most of these published studies have used 16S rRNA gene profiling studies as part of cross-sectional and observational studies. These studies have also principally used stool samples, often from cohorts representing more than one of the major subtypes of IBS: postinfectious IBS, constipation (IBS-C), diarrhea (IBS-D), and mixed. The findings of these profiling studies are best described as variable, with some genera assigned to the Firmicutes phylum, such as *Dorea*, *Roseburia*, *Ruminococcus* and *Blautia* spp.; along with members of the Gram-negative Proteobacteria being increased compared with healthy control subjects. Conversely, "good" bacteria, such as *Bifidobacterium*, *Collinsella* and *Faecalibacterium* spp, are often observed to be reduced in patients with IBS. Other bacterial taxa show mixed responses, either increased (or reduced) according to the IBS-C or IBS-D subtypes. Remarkable in this regard are the increase in methane-producing archaebacteria reported in patients with IBS-C, and the reductions in bacteria affiliated with the Bacteroidetes phylum in IBS subtypes other than IBS-D, where these groups have been reported to increase (79). These variations are likely to be a consequence of how variations in gut transit time impose different selective pressures on the gut microbiota. For instance, bacteria with more rapid rates of growth are likely to be favored by the fast transit times associated with IBS-D, whereas microbes with slow growth rates and/or nutrient requirements including hydrogen and more reduced fermentation end-products (e.g., formate, short chain alcohols and methylated amines) predominate in microbiomes with longer transit times (IBS-C).

The reviews noted previously also highlight the conundrum associated with the use of either probiotics or exclusion/reduction diets for treatment of IBS, because these interventions can result in somewhat contradictory effects on the gut microbiota. For instance, very low carbohydrate diets have been shown to improve the symptoms and quality of life in patients with IBS-D (80), and a low FODMAP diet, compared with habitual diets in either the United Kingdom or Australia, has been

shown via randomised control and a randomised and blinded crossover trial, respectively, to be effective in controlling the symptoms of IBS (81,82). Moreover, a study of patients with IBS who received either "standard" or "low FODMAP" diet advice concluded that patients who received the low FODMAP advice reported improvement in their IBS- associated symptoms, such as bloating and flatulence, than the patients who received standard dietary advice (83). Importantly, the studies by Halmos and colleagues (84) also suggest that although diets low in FODMAPs reduce the symptoms of patients diagnosed with IBS compared with when they consume a standard diet, no changes in the symptoms scores are observed in healthy controls subjects consuming either type of diet. In relative terms, there are scant reports of the microbiome changes associated with these diets, especially in terms of using the contemporary sequencing technologies and approaches outlined previously. However, the quantification of key bacterial groups by species-specific polymerase chain reaction has been informative. In that context, Staudacher and colleagues (82) first reported a reduction in *Bifidobacterium* spp. (and IBS symptoms) in British subjects as a consequence of their intake of a low FODMAP diet, which seems contradictory to at least some of the findings linking Bifidogenic effects and IBS symptom improvement with probiotic use in IBS sufferers (see Staudacher and Whelan (37) for a detailed review). Halmos and colleagues (84) have since compared specific populations of bacteria in stool samples preserved from their previous study, and the low FODMAP diet was linked with a reduction in the absolute abundance of total bacteria and specific taxa across healthy and IBS cohorts (81). These differences also translated into statistically significant reductions in the relative abundances of a major subdivision of the Gram-positive Firmicutes (Cluster XIVa) and *A. muciniphila*, widely considered to be a beneficial mucin-associated gut bacterium. Only one bacterium measured (*Ruminococcus torques*) showed a marginal increase in total abundance and a significant increase in relative abundance in response to the period of consuming a low FODMAP diet. Intriguingly, *R torques* is also known to be mucin-associated, and other bacteria taxonomically affiliated with this bacterium have been reported to be positively associated with IBS symptom severity, and capable of mixed acid fermentation and gas production from substrates, such as FODMAPs (85,86). Collectively, these findings demonstrate that much still needs to be defined in relation to the diet x microbiome interactions for IBS symptom control and patient quality of life. In

that context, Halmos and colleagues (84) recommend that until such knowledge is available, caution should be applied with long-term adherence to a low FODMAP diet, even in patients diagnosed with IBS, and its use by asymptomatic healthy persons should be avoided.

1.7 Dietary carbohydrate microbiota interactions and microbiota accessible carbohydrates (MACs): Food for the microbiota

The part of the diet reaching the large intestine is largely composed of undigestible complex polysaccharides that gets fermented by the colonic microbiota. Fiber could broadly be divided into two types: soluble (example in oatmeal, nuts, apples, lentils, peas etc.) and insoluble (example in brown rice, whole wheat bread, whole grains). Prebiotic fibers such as inulin, FOS, GOS are examples of soluble fibers that have been reported to stimulate the growth of beneficial gut bacteria and promote host health in humans (87–90). In relation to this, the term microbiota accessible carbohydrates was coined which refer to complex carbohydrates or dietary fibres that cannot be digested by the host but are metabolised and fermented by the gut microbiota (91). In other words, MACs are indigestible short chain carbohydrates and fermentable fibres. The recommended daily intake of dietary fibre is 30 g, however the Western diet provides a daily intake of only 15 g of dietary fibres (92). Supplementation of a low MAC diet for 7 weeks in mice have been shown to significantly reduce gut diversity. Low MAC diet feeding over three generations was shown to cause irreversible loss of gut members Bacteroidales and the loss of the carbohydrate degrading enzymes glycoside hydrolases (93). Additionally, fiber deprivation or low dietary MACs have been shown to reduce the gut epithelial integrity and an enhanced pathogenic susceptibility in gnotobiotic mouse model (94). A study by Filippo et al. (39) showed that the gut microbiome of children from an African village-Burkina Faso, consuming a diet high in fiber content was significantly different from the European children consuming a Western diet. The BF children were found to have significantly higher abundance of Bacteroidetes and reduced abundance of Firmicutes with the presence of genera *Prevotella* and *Xylanibacter*- bacterial genera having genes for cellulose and xylan degradation. In addition, increased SCFA levels and decreased abundance of pathogenic microbes *Enterobacteriaceae* were found in BF compared to the European children. Resistant starch is a type

of fermentable fiber where “resistant” means its ability to resist digestion (95). Multiple studies have shown the beneficial effects of resistant starches on the colonic function and host health (96–98). Collectively, all these studies suggest that supplementation of diet with complex fermentable carbohydrates stimulates the growth of beneficial gut microbes such as *Bifidobacterium*, butyrate producing bacteria and *Lactobacillus* and enhanced SCFA production. Dietary fibers and bacterial metabolites could also affect the gut and host health by modulating inflammation. The advancements in high throughput omics approaches have now enabled us to have a better understanding of the interactions between dietary carbohydrates, gut microbiota and the host.

1.8 Summary and research goals

Diet is now recognised to have a significant impact on the structure and function of the gastrointestinal microbiota. Knowledge also needs to extend beyond the bacterial world, to less common taxa such as archaea and fungi. There is a growing need to bring (meta)genomes to life and illuminate the functional and ecologic contributions from all forms of microbial dark matter to gut function and health. Although much remains to be discovered, the development of therapeutic dietary interventions that support the rational modulation of the gut microbiota is now a much more realistic and attractive strategy. With this in mind, the overarching aim of my Ph.D. was to investigate how diets with varying carbohydrate content can affect the oral and stool microbiota of healthy individuals. I have used two key groups of subjects in two separate nutritional trials: (1) elite endurance race walkers and (2) healthy conventional subjects. My Ph.D. project is a combination of microbiology, nutritional sciences and metagenomics coupled with high throughput sequencing technologies and computational biology approaches to examine and obtain a better understanding of the taxonomic profiles of microbial communities of gastrointestinal tract in addition to understanding their functional potential in response to the carbohydrate-based dietary interventions.

While more specific aims of my project included:

1.) To investigate the effects of three-week dietary intervention with either a High Carbohydrate (HCHO), Periodised Carbohydrate (PCHO) or a Low Carbohydrate High Fat (LCHF) during intensified training on the oral microbiota of elite race walkers using 16S rRNA amplicon sequencing.

2.) To investigate the effects of the above-mentioned dietary interventions during intensified training on the stool microbiota of elite race walkers using 16S rRNA amplicon sequencing.

3.) To examine the effect of two diets; differing in their quantity of natural prebiotic carbohydrates (Low Prebiotic; LP and Moderate Prebiotic; MP) on the gut microbiome of healthy individuals to better understand the fibre-gut relationship using both amplicon-based (16S rRNA, ITS-2) and whole genome shotgun sequencing.

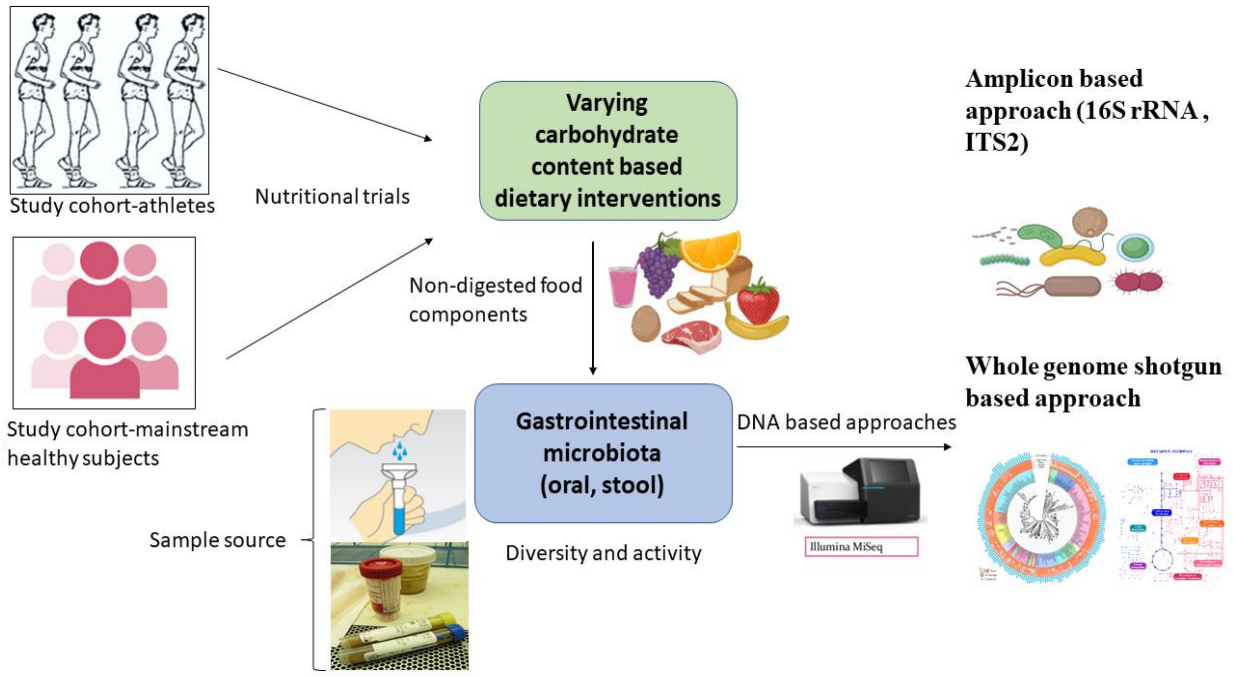

Figure 1.4 An overview of my Ph.D. research project aims and the approaches used to address these aims.

I believe my findings will not only provide a stronger understanding of diet-microbiota interactions and critical new insights of what constitutes a healthy microbiota, but also provides insights about how a person's diet can be rationally managed and personalised to sustain a healthy gut.

1.9 Published review article from Chapter 1

PubMed link of the published article: https://www.ncbi.nlm.nih.gov/pubmed/28164852

Chapter 2

Chapter 2 Research Design and Methodology

2.1 Introduction

The results and findings presented in Chapters 3-5 are derived from two separate nutritional trials, which are briefly described below. This Chapter also provides a description of the DNA extraction, PCR amplification and library construction, as well as the bioinformatics and statistical methods shared by each of these Chapters. Following the description of these methods, I present my findings using these methods with a collection of archived stool samples from our collaborators at Monash University. Here, I show how the long-term storage of the stool samples impacted on the results produced from the extracted DNA by 16S rRNA amplicon sequencing. In brief, storage at -20ºC and/or frequent freeze-thaw cycles resulted in substantive lysis of Gram-negative bacteria and produced a biased towards the Firmicutes bacteria.

2.2 Supernova 1 study (Chapters 3 and 4)

The Supernova 1 study was led by Professor Louise Burke, Australian Institute of Sport (AIS) and Australian Catholic University and undertaken in collaboration with Bond University scientists. The study was designed to examine how the diet consumed by elite endurance walkers during a period of intensive training affected athlete exercise economy and performance, and the parent publication was published in *Journal of Physiology* in 2017 (99). My contribution to this study was to separately characterise how these diets affected the oral (Chapter 3) and stool (Chapter 4) microbiomes of the athletes, using samples provided by each athlete at entry and after the period of dietary change. These samples were collected in OMNIgene preservative kits for stool and saliva samples and were shipped in dry ice and stored at -80ºC until processed for analysis. The Supernova 1 study was approved by the Ethics Committee of the Australian Institute of Sport (AIS, no. 20150802) and has been registered as a clinical trial, assigned the number ACTRN12618001529235 by the Australian New Zealand Clinical Trials Registry (ANZCTR). The DNA sequence data generation and analyses were performed under UQ-HREC 2015001965.

2.3 Using diet rich in dietary fibre to improve bowel habit and sense of well-being (Chapter 5)

This study was led by Dr Jane Muir and colleagues at the Translational Nutrition Science Group, Department of Gastroenterology, Alfred Hospital and the Monash University, Australia. The study has been conducted under ethics approvals MUHREC CF14/2904, 2014001593 and the microbiota analyses presented in Chapter 5 performed as part of UQHREC 2015000317. Subject recruitment and randomisation, diet preparations, and all clinical measures were conducted via the Alfred Hospital. The trial was conducted in two stages, each stage based on the consecutive recruitment of 9 healthy adults, and samples were provided in 2017 and 2018. The stool samples collected from the first 9 patients recruited for this were provided in two different forms: as a raw stool sample that had been subjected to thawing for subsampling and pooling, then refrozen prior to shipment to Brisbane; and a single sample of raw stool transferred to OMNIgene gut tubes (DNA Genotek). All these samples were shipped from Melbourne to Brisbane in dry ice, and immediately transferred to a -80ºC freezer.

2.4 Microbial DNA extraction

All the stool samples were handled under aseptic conditions in a Biosafety Level 2 cabinet in the Morrison Laboratory at Translational Research Institute (TRI). Genomic DNA was extracted from all the samples using the repeated bead-beating procedure for cell lysis (100) and combined with an automated column-based DNA purification procedure (Maxwell® 16MDx system, Promega Corporation, WI, USA). Aliquots (250 µL) of the mixed stool sample stored in OMNIgene tubes were transferred into 2 mL screw capped tubes containing 0.4 g of sterile zirconia beads. Then 600 µL of lysis buffer (500 mM NaCl, 50mM TRIS-HCl (pH 8.0), 50 mM EDTA and 4% [w/v] sodium dodecyl sulfate) was added and the tubes were placed within a Precellys 24 homogeniser (Bertin Corp) and subjected to 3 x 60 second intervals of bead beating at 5000 rpm. The homogenised mixture was incubated at 70 ºC for 15 minutes, with gentle shaking by hand every 5 minutes, then centrifuged for 5 minutes at 14,000 rpm. The supernatant was then transferred to a fresh 1.5 ml microcentrifuge tube and 30 µL of proteinase K was added to the supernatant and vortexed for 30 seconds. The mixture was then incubated for 20 minutes at 56ºC, and applied to the Maxwell 16 MDx cartridges, and the

DNA collected within 65 µL of elution buffer (Promega, catalogue no. AS1290). The eluted DNA solutions were placed on a magnetic stand to remove traces of magnetic particles, and the clarified samples carefully transferred to a new microcentrifuge tube. Then 4 µL of RNase (10 mg/ml) was added to each sample, followed by incubation at 37ºC for 20 minutes. The DNA concentrations were quantified using a Nano-Drop Lite Spectrophotometer (Thermo Fisher Scientific) and adjusted with elution buffer to a final concentration of 5 ng/µL for 16S rRNA amplicon library preparation. When required, a set of reagent controls were also processed to which no DNA was added. These reagent controls were processed in an identical manner to the samples for the 16S rRNA gene amplicon library construction and sequencing.

2.5 16S rRNA library preparation and sequencing

The PCR amplicon libraries (V6-V8) were produced using the Bacteria/Archaea specific primers 926-forward and 1392-reverse, which had been modified to include overhang adapters compatible with Nextera Index PCR XT kit (Illumina Corp., San Diego, CA, USA) to produce bar-coded amplicons for individual samples (101). The amplicon PCR was carried out with a total volume of 25µL comprised of 12.5 µL of Q5® (New England Biolads), 5 µL each of 10 µM forward and reverse Illumina primers and 2.5 µL of template DNA. The thermo-cycling PCR conditions for amplicon PCR were 1 cycle at 95 ºC for 3 minutes, followed by 25 cycles of 30 seconds each at 95 ºC for denaturation, 55 ºC for annealing, 72 ºC for extension, and 1 final extension cycle of 5 minutes at 72 ºC and hold at 4 ºC.

The Q5® Hot Start High-Fidelity (New England Biolabs, Massachusetts, USA) polymerase enzyme was used for the PCR instead of the recommended KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Massachusetts, USA) polymerase enzyme, as it showed higher amplification efficiency with a lower error rate when used by other members of the lab. The resulting PCR products were purified using Agencourt AMPure XP beads, and a subsequent PCR (Index PCR) was performed using the Nextera Index PCR XT kit. The index PCR was carried out with a total volume of 50 µL comprised of 25 µL of Q5, 5 µL each of the Nextera forward and reverse primers with the overhangs, 5 µL of cleaned amplicon PCR product and 10 µL of ultra-pure PCR grade water. Thermo-cycler

conditions for index PCR were: 1 cycle at 95 ºC for 3 minutes, followed by 8 cycles of 30 seconds each at 95 ºC for denaturation, 55 ºC for annealing, 72 ºC for extension, and 1 final extension cycle of 5 minutes at 72 ºC and hold at 4 ºC. The PCR product was again purified using Agencourt AMPure XP beads and quantified using the Quantus Fluorometer dye kit. The bar-coded amplicons were purified, quantified and subsequently pooled in equimolar quantities for Illumina Miseq sequencing performed at the Australian Centre of Ecogenomics (ACE), University of Queensland.

2.6 Bioinformatics and statistical analyses

Analysis of the microbiome data produced by Illumina sequencing platforms requires specialised packages and softwares. Throughout my thesis, I have used the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9.1 on an Ubuntu Linux virtual machine (v5.0.12) (Canonical, London, UK). I used this software to demultiplex and perform quality control checking, paired-end-merging and filtering of the sequence data, with a minimum quality score of 20 set as the acceptability threshold (102). The sequences were then clustered into Operational Taxonomic Units (OTUs) using a threshold setting of 97% sequence identity and the open reference OTU picking method was used to assign sequences to their respective OTUs according to the Greengenes database version 75 (103). The OTU tables containing taxonomic and abundance data were generated for each individual sample, and any OTUs that were not identified as Bacterial or Archaeal, and/or OTUs that comprised ≤0.01% of the total sample sequence count, were removed from further analysis. USEARCH 6.1 was also used for reference-based chimera detection and any candidate chimeric sequences were removed (104). Samples less than 1000 reads were removed during QIIME analysis and the coverage of the biodiversity present in each sample was then assessed by rarefaction analysis; with a rarefied subsampled OTU table generated by random sampling to the minimum read count. The “Core_diversity_analyses” script in QIIME was applied to the rarefied OTU table to generate taxonomy plots from phylum to genus levels and used to calculate alpha- and beta-diversity metrics. The resulting profiles were then displayed as rarefaction plots for alpha diversity. Alpha diversity which is a measure of species richness or diversity within a sample was measured by Shannon’s, Simpson’s and Good’s coverage indices. Shannon index takes into account both the species richness

and evenness of the species present. Richness measures the number of species/taxa present in a sample whereas evenness represents how evenly distributed the species are within a sample. Good's coverage is an estimate of the recovery of the total number of taxa present in each sample.

Beta diversity assess the diversity of the microbial communities between samples. The QIIME workflow uses a "UniFrac analyses" as its key metric for measuring and comparing the beta diversity represented within a dataset. In brief detail, a phylogenetic tree is generated from the OTU tables and the branch lengths are used to quantify microbial diversity and compare how similar or different the microbial communities between different samples are. There is also a weighted and unweighted form of the analysis: the weighted (quantitative) takes into account the abundance of the observed OTUs, while the unweighted (qualitative) compares samples solely by the presence or absence of OTUs. The Unifrac distance metrics can be graphically displayed by Principal Coordinates Analysis (PCoA) plots, which uses the distance matrix to plot the samples in 2D or 3D space (105). Beta diversity was examined by weighted and unweighted UniFrac and Bray-Curtis distance matrices and used to ordinate samples by Principal Coordinate Analysis (PCoA). All the QIIME scripts used for processing the data are listed in the Appendix section 7.4 below.

I also learned to use the Calypso software for mining, interpreting and statistical comparisons of the taxonomy profiles generated from my datasets (106). Multivariate methods such as Redundancy Analysis (RDA) and Analysis of Similarity (Anosim) were used to identify associations between microbial community composition and diet. RDA examines associations between the community composition and the study variables (for example in my thesis the explanatory variable studied is diet). Anosim uses dissimilarity matrices to investigate whether the microbiota profiles are significantly different between the groups. It generates a p value by comparing the intra-group distances with between-group distances.

The nutritional studies described above afforded the collection of multiple samples from the same subject, and repeated measures over time. The "mixOmics" R package offers multivariate analysis of these types of datasets (107) including sparse Partial Least Squares Discriminant Analysis (sPLS-DA) analysis via the Mixomics mixMC: multivariate data analysis framework in Calypso software.

The sPLS-DA is an extension to the sPLS program and enables the selection of most discriminative features between groups in a one-step procedure (108). I also used Linear Discriminant Analysis (LDA) Effect Size (LEfSe) within Calypso to identify individual taxa discriminatory for the different dietary patterns (109). Mixed effect linear regression (MELR) analysis was used to analyse the data with repeated measures between the dietary intervention groups. GraphPad Prism (version 7, GraphPad Software, San Diego, CA, USA) was used to perform the Spearman's correlation between the individual taxa and athlete's performance measures described in Chapter 3. GraphPad Prism (version 8) was also used to plot and perform Wilcoxon paired signed rank tests performed in Chapter 5. The significance level was set to $p < 0.05$ for all the analysis. Correction for multiple testing by false discovery rate (FDR) with values < 0.05 were considered significant in MELR analysis.

2.7 Effect of sample storage on the microbiota profiles

At the start of my Ph.D., I first used a collection of stool samples provided by our collaborators at Monash University. A total of 123 stool samples from 6 healthy (H), 27 irritable bowel syndrome (IBS) and 8 Crohn's disease (CD) patients were provided, collected as part of the nutritional trials examining the effects of either their habitual diet (B) or diets with either a low or high FODMAP content (denoted as Y and P, respectively) on these persons, and published by Halmos et al. in 2014 and 2015 (81,84). These samples had been stored at both -20ºC and -80ºC for extended periods of time and subjected to repeated freeze-thaw cycles in support of other chemical analyses, as outlined by Halmos et al. (81,84). Genomic DNA was extracted and 16S rRNA gene amplicon libraries were prepared and sequenced, and the resulting data analysed according to the protocols described above.

Table 2.1 shows the bacterial/archaeal profiles produced from these stool samples at the phylum-level of taxonomic classification. While all the major Phyla outlined in Chapter 1 are detected, these profiles were both remarkable and highly uniform, irrespective of the person's health or dietary status. Specifically, OTUs assigned to the Firmicutes phylum account for as much as 85-90% of the biodiversity recovered, and there was a coordinate and very substantial decrease in the relative abundances of OTUs assigned to the Bacteroidetes (0.5–6.0%) and Proteobacteria (0.1-4.3%). These

microbiota profiles suggested that those bacteria with a Gram-negative cell wall were "lost" during the long-term storage of these samples, which will occur if stool samples are stored for long periods at suboptimal temperatures (-20ºC) and/or, undergo repeated freeze-thaw cycles. Unfortunately, both of these circumstances had arisen as part of the sample archiving since their collection and initial analyses (Muir and Gibson, pers. comm.) and I decided not to perform more detailed analyses of these specific samples.

Table 2.1 Operational Taxonomic Unit distributions at the Phylum-level of classification from the stool samples of Crohn's disease (CD), healthy (H) and Irritable Bowel Syndrome (IBS) patients. Stool samples were provided at study entry (B, habitual diet) and following their consumption of a diet containing either a high (P) or low (Y) FODMAP content, as described by Halmos et al. (81,84). While the taxa detected from these stool samples are typical of those reported from other human subjects and patients, the profiles are all remarkable for their extremely large proportion of Firmicutes and small relative abundances of Bacteroidetes and Proteobacteria.

	Total	**CDB**	**CDP**	**CDY**	**HB**	**HP**	**HY**	**IBSB**	**IBSP**	**IBSY**
Taxonomy	%	%	%	%	%	%	%	%	%	%
Euryarchaeota	2.6	2	2.6	1.8	1.8	1.9	2.4	4.2	2.7	3.8
Actinobacteria	4	4	3.5	4.3	2.8	5.6	5.1	4.1	3.5	2.8
Bacteroidetes	3.3	0.5	1.2	0.8	6.2	3.1	5.3	3.6	4.4	4.7
Cyanobacteria	0	0	0	0	0	0	0	0	0	0
Firmicutes	87.9	89	88.7	90.4	88.1	89.2	85	86	87.8	87.3
Proteobacteria	1.6	4.3	3.6	2.4	1.1	0.1	1.2	0.7	0.3	0.4
Tenericutes	0.4	0.1	0.2	0	0	0	0	1.3	1	0.6
Verrucomicrobia	0.3	0.2	0.2	0.3	0.1	0.1	0.9	0.1	0.2	0.3

As noted above, stool samples from the Monash University study presented in Chapter 5 were provided in two forms: as a pooled sample for each subject, prepared from thawed stools collected on consecutive days from each patient and then stored frozen at -80ºC until use (hereafter referred to as M1 samples); or a single sample of stool that on collection was preserved immediately by its transfer to OMNIgene gut tubes, and stored at -80ºC (hereafter referred to as M2 samples). Given the

results obtained above, I decided to prepare stool DNA extracts from all these samples and produce 16S rRNA gene amplicon libraries that were sequenced and analysed according to the protocols described above. Table 2.2 shows there were remarkable differences between the profiles generated from the M1 and M2 samples, and in particular the relative abundance of OTUs assigned to the Firmicutes, Bacteroidetes, Actinobacteria and Euryarchaeota. Figure 2.1a shows the results of the PCoA analysis of the weighted Unifrac metrics for the M1 and M2 samples. There is a clear separation of the microbiota profiles generated from the two types of samples, further confirming that the method of stool preservation influenced the microbiota profiles. Figure 2.1b shows the Wilcoxon rank-test of the abundances scores for Gram-positive phyla (Actinobacteria and Firmicutes) which are greater for the M1 sample type, and the Gram-negative Bacteroidetes phylum, which is greater for the M2 sample. All these differences were found to be statistically significant.

Table 2.2 The relative abundance of OTUs assigned at the phylum-level of classification recovered from stool samples that had either been thawed and pooled (M1) or individual samples transferred immediately to OMNIgene gut tubes (M2). Both types of samples were stored at -80ºC until processed for analysis.

	Total	**M1**	**M2**
Taxonomy	(%)	(%)	(%)
Euryarchaeota	3.4	5.5	1.4
Actinobacteria	6.8	10.7	2.9
Bacteroidetes	35.2	18.7	51.6
Firmicutes	51	61.5	40.6
Proteobacteria	1.5	1	2.1
Tenericutes	0.4	0.5	0.3
Verrucomicrobia	1.6	2.1	1

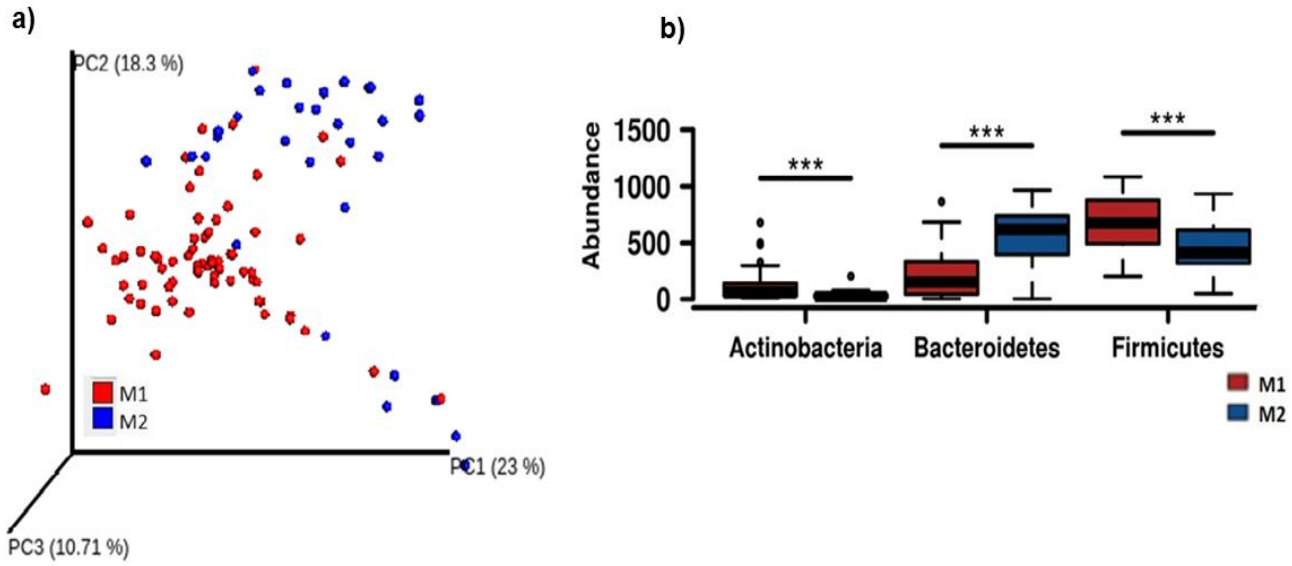

Figure 2.1 Effect of sample storage methods (M1 and M2) on the microbiota profiles. **(a)** The PCoA plots of the weighted UniFRAC beta diversity metrics for the samples stored using either the M1 (red) and M2 (blue) method further confirms that method of storage impacts on between sample (beta diversity) profiles. **b)** Wilcoxon rank tests of the relative abundance scores for the Actinobacteria, Bacteroidetes and Firmicutes phyla from the two types of stool sample storage. The M2 storage method appears to better protect the Gram-negative Bacteroidetes present in these samples (All differences are significant at $p<0.001$).

These results were produced during the first year of my Ph.D. program and highlight how important stool sample collection methods and storage are to the microbiota profile data that is produced from such samples. Based on these results, we recommended that all the stool samples collected by the Monash University group be immediately transferred to OMNIgene gut tubes and were kept frozen at -80ºC until processed. Any subsampling should be done prior to the first freezing of samples at -80ºC, to ensure the samples are thawed once, and prior to DNA extraction.

Chapter 3

Chapter 3 Analysis of the Effects of Dietary Pattern on the Oral Microbiome of Elite Endurance Athletes

3.1 Abstract

Although the oral microbiota is known to play a crucial role in human health, there are few studies of diet x oral microbiota interactions, and none in elite athletes who may manipulate their intakes of macronutrients to achieve different metabolic adaptations in pursuit of optimal endurance performance. The aim of this study was to investigate the shifts in the oral microbiome of elite male endurance race walkers from Europe, Asia, the Americas and Australia, in response to one of three dietary patterns often used by athletes during a period of intensified training: a High Carbohydrate (HCHO; n=9; with 60% energy intake from carbohydrates; ~8.5 g $kg^{-1}day^{-1}$ carbohydrate, ~2.1 g $kg^{-1}day^{-1}$ protein, 1.2 g $kg^{-1}day^{-1}$ fat) diet, a Periodised Carbohydrate (PCHO; n=10; same macronutrient composition as HCHO but the intake of carbohydrates is different across the day and throughout the week to support training sessions with high or low carbohydrate availability) diet or a ketogenic Low Carbohydrate High Fat (LCHF; n=10; 0.5 g kg^{-1} day^{-1} carbohydrate; 78% energy as fat; 2.1 g kg^{-1} day^{-1} protein) diet. Saliva samples were collected both before (Baseline; BL) and after the three -week period (Post treatment; PT) and the oral microbiota profiles for each athlete were produced by 16S rRNA gene amplicon sequencing. Principal coordinates analysis of the oral microbiota profiles based on the weighted UniFrac distance measure did not reveal any specific clustering with respect to diet or athlete ethnic origin, either at baseline (BL) or following the diet-training period. However, discriminant analyses of the oral microbiota profiles by Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and sparse Partial Least Squares Discriminant Analysis (sPLS-DA) did reveal changes in the relative abundance of specific bacterial taxa, and particularly, when comparing the microbiota profiles following consumption of the carbohydrate-based diets with the LCHF diet. These analyses showed that following consumption of the LCHF diet the relative abundances of *Haemophilus, Neisseria* and *Prevotella* spp. were decreased, and the relative abundance of *Streptococcus* spp. was increased. Such findings suggest that diet, and in particular the LCHF diet can induce changes in the oral microbiota of elite endurance walkers.

3.2 Introduction

Recent technological advances have enabled a more holistic definition and characterization of the microbes that colonise the human body, the "microbiomes". The human oral cavity serves as the habitat for a numerically large and diverse microbiome (72), which has been extensively characterised with respect to infectious and periodontal diseases, and caries, as well as for its contributions to the onset and progression of chronic conditions such as diabetes, cardiovascular disease and cancer (73). However, the impacts of dietary pattern on the oral microbiome are not well defined, neither for the general population nor for cohorts who may follow specialised diets, such as elite athletes. In that context, recent studies have revealed a positive symbiotic association between the oral bacteria and host with respect to an enterosalivary nitrate-nitrite-nitric oxide pathway, which contributes to nitric oxide (NO) homeostasis (74,75). Here, facultative anaerobic bacteria in the mouth reduce salivary gland concentrated nitrate to nitrite, which is then swallowed and absorbed into the bloodstream before further reduction to NO. The critical role of the oral microbiota in this effect has been demonstrated, where a seven-day period of antiseptic mouth wash treatment was shown to disrupt the oral microbiota of healthy non-athletes and, in the absence of any dietary modifications, was associated with reductions in plasma and oral nitrite levels and an increase in blood pressure (77). These findings raise the spectre that diet may also invoke changes in the oral microbiota that manifest in alterations of this enterosalivary pathway and NO homeostasis but remains unexplored.

A recent investigation (99) of the effect of diet and training on exercise metabolism and performance in elite endurance athletes provided an opportunity for pilot work on this theme. The "Supernova 1" study investigated parameters around endurance capacity in a cohort of elite endurance race walkers who followed one of the three popular dietary approaches during a three-week period of intensified training: a ketogenic Low Carbohydrate High Fat diet (LCHF), or a diet high in carbohydrates consumed either ad libitum (HCHO) or at specific periods on a daily/weekly basis (PCHO). While the HCHO diet is focused on optimal muscle and brain carbohydrate (CHO) stores for each training session, the PCHO diet involves a strategic combination of sessions with such dietary support as well as other which are undertaken with low muscle glycogen availability to promote greater metabolic

stress and cellular adaptation (110,111). Finally, the LCHF diet involves severe CHO restriction to promote adaptations that increase muscle capacity for fat oxidation. Details on the rationale for these radically different types of nutrition (99,110) and the actual protocols employed in this study can be found elsewhere (99,111). In summary, the Supernova 1 study found that each group of athletes achieved a significant improvement in their aerobic capacity over the training block, which was undertaken during the base phase of the annual training plan. However, while this was associated with improved economy and real-world race performance in the two groups who trained while consuming the HCHO and PCHO diets, the LCHF group experienced an increase in the oxygen cost of exercise supported by high rates of fat oxidation, and thereby failed to improve their race performance despite the gain in aerobic capacity (99). Based on these differential results, the overall aim of this study was to examine whether and how the oral microbiome of these athletes was affected by their diet during intensified training. This appears to be the first study that provides an in-depth investigation of diet x oral microbiome interactions in elite athletes.

3.3 Materials and Methods

3.3.1 Study Design and Sample Collection

The group of world-class race walkers and the design of the "Supernova 1" study are described in detail by Burke et al. (99) and Mirtschin et al. (111). In summary, these male race walkers (aged 20–35 years, BMI range 16–23 kg/m^2) were from Australia, Canada, Japan, Italy, Poland, Sweden, Chile and South Africa, and all met International Association of Athletics Federations (IAAF) standards for international competition, with more than 75% participating in the major championships during the year of the study (i.e., 2016 Rio Olympic Games and 2016 World Walking Cup). Twenty-nine study experiences were gained from 21 elite athletes who participated in either one (n = 13) or both (n = 8) of the Supernova 1 research camps conducted at the Australian Institute of Sport. Each camp involved three weeks of intensified training and rigorously supervised dietary interventions. Saliva samples were collected from the athletes prior (baseline, BL) and after the three-week training-diet

intervention using the OMNIgene saliva collection and preservative kit and according to the manufacturer's instruction (fasted collection, saliva collected by spitting into the tube).

3.3.2 Allocation to Dietary Interventions

The athletes were involved over several months of planning and received education about the range of likely effects of the diets on various aspects of health and performance. Each had ample time to choose a diet(s) according to his beliefs of the performance benefits from the chosen diet. Although this type of assignment was non-random, given that all athletes choose freely to be in the study and to be fed their diet of choice, this approach both promoted adherence to the intervention and controlled for the random effects of such rigorous dietary control (e.g., feeling anxious about losing personal freedom of dietary choice). Therefore, any effects on the oral microbiome could be attributed to the diet, including any additional intrinsic biochemical, physiological or psychological overlay that belongs to the diet itself.

Three diets were compared: (i) a diet high in carbohydrate availability (HCHO; n = 9) comprised of 60% of energy intake from CHO (~8.5 g/kg body mass (BM)/day), 16% protein (~2.1 g/kg BM/day), 20% fat; (ii) a diet with periodised carbohydrate availability (PCHO; n = 10) of similar overall macronutrient composition as HCHO but consumed at different intervals across the day and throughout the week to support different training sessions with high or low CHO availability and (iii) a ketogenic low carbohydrate-high fat diet (LCHF; n = 10) comprised of 78% fat, 17% protein (~2.2 g/kg/day) and 0.50 g/kg/day carbohydrate (3.5% energy). All the meals were prepared taking into consideration the nutritional requirement within the allocated dietary intervention. Mirtschin et al. reported the detailed nutritional information and meal plans for the above three dietary interventions of this study (111).

3.3.3 Oral Microbiota Analysis

Genomic DNA was extracted and 16S rRNA gene amplicon libraries were prepared and sequenced, and the resulting sequence data was analysed according to the protocols previously described in Chapter 2.

3.4 Results

As mentioned previously, 21 elite race walkers were recruited in the study (with eight athletes recruited in both the camps). Table 3.1 provides the anthropometric details of the subjects enrolled in the study. According to Tukey's multiple comparisons test, there were no significant differences in the age (p = 0.53 for HCHO vs. PCHO; p = 0.28 for HCHO vs. LCHF and p = 0.87 for PCHO vs. LCHF) and the BMI scores (p = 0.36 for HCHO vs. PCHO; p = 0.84 for HCHO vs. LCHF and p = 0.67 for PCHO vs. LCHF) when athletes were grouped according to the dietary intervention they received.

Table 3.1 Athlete cohort characteristics

	High Carbohydrate (HCHO) Diet	**Periodised Carbohydrate (PCHO) Diet**	**Low Carbohydrate High Fat (LCHF) Diet**
Sample size	n = 9	n = 10	n = 10
Age (years)	25.4 ± 4	27.4 ± 4.6	28.3 ± 3.5
BMI (kg/m^2)	20 ± 1.6	21 ± 1.3	20.4 ± 1.8
Country of origin	Australia, Canada, Japan, South Africa	Australia, Canada, Japan, Poland, Sweden, Italy	Australia, Canada, Japan, Poland, Sweden, Chile, South Africa
Gender	Male	Male	Male

Note: Data for Age and body mass index (BMI) are shown as mean ± standard deviation

The Shannon alpha diversity was reduced following the diet-training interventions when compared to their subject-matched BL measures; however, these reductions were not statistically significant (p

= 0.1 for BL vs. HCHO and BL vs PCHO; p = 0.62 for BL vs. LCHF). The PCoA analysis of the weighted UniFrac distances are shown in Figure 3.1 and did not reveal any distinct clustering of the saliva microbiome profiles, either with respect to the ethnic origin of the athletes or the dietary intervention. Similarly, the supervised analyses by RDA and Anosim did not identify any significant differences between the microbiota community composition at BL and following any of the three dietary interventions (data not shown). Taken together, these results suggest that the dietary interventions do not result in dramatic changes in the overall biodiversity of the oral microbiome, but rather more subtle changes in community composition. As mentioned previously in 3.3.1 above, eight athletes were recruited in both the camps, and the two baseline profiles (B1 vs. B2) of these eight athletes were compared. No substantive differences between the two microbiota profiles were apparent, as assessed by Shannon alpha-diversity and Anosim beta-diversity analyses. These tests indicate that the time between the study camps was sufficiently long to ensure a "washout" between the two camps, and, thereby, no potential carryover effects from the previous diet on the subsequent results/profiles.

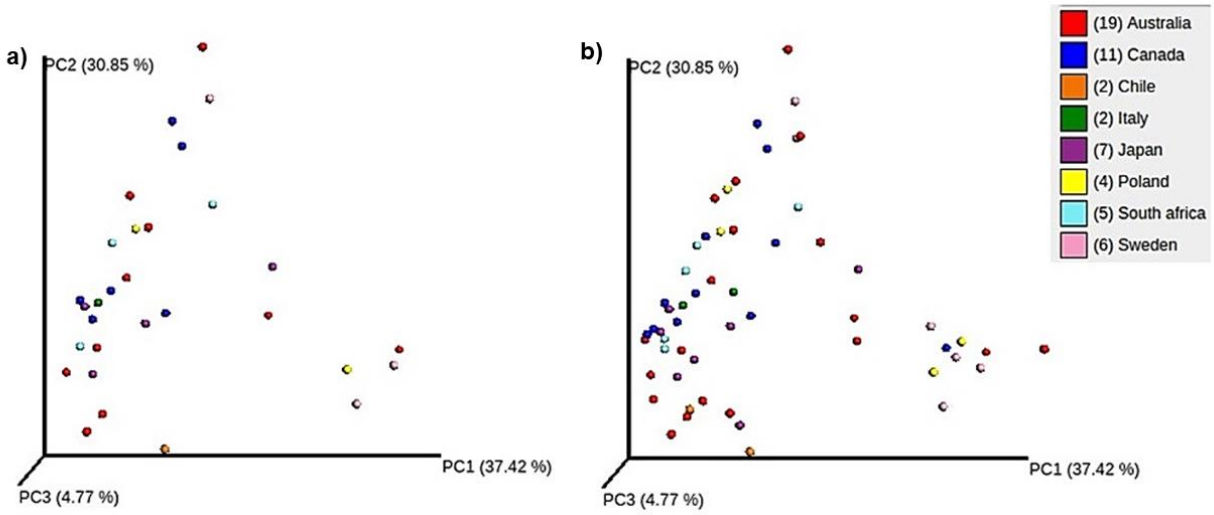

Figure 3.1 Principle component analysis of weighted UniFrac distances for the oral microbiomes of athletes at Baseline only (BL, **a**); and when combined with their profiles obtained after the diet-training intervention period (**b**). Samples are colored based on the athlete's country of origin and show no significant clustering indicative of a dietary and/or ethnic effect on the oral microbiomes.

3.4.1 Comparisons of Community Profiles of Saliva Samples between Baseline and Post Interventions

LefSe analyses was used to identify discriminating taxa between baseline (BL) and post diet-training interventions. OTU's affiliated to *Streptococcus*, *Peptostreptococcus*, *Actinomyces*, *Granulicatella*, *Atopobium*, *Veillonella* and *Prevotella* were found to be enriched following the consumption of HCHO diet, whereas *Parvimonas* was discriminatory and enriched for the BL samples from these same athletes (Appendix Figure 7.1). Analysis by the sPLS-DA of the same athlete samples identified *Prevotella*, *Actinobacillus*, *Fusobacterium*, *Haemophilus* and *Gemella* to be associated and increased in BL samples (Figure 3.2 below).

MELR analysis of the oral microbiota profiles at BL and following HCHO diet training intervention was also examined using mixed effect linear regression, and the relative abundance of *Atopobium* was found to increase (p = 0.015), whereas *Capnocytophaga* (p = 0.027) and *Porphyromonas* (p = 0.03) were decreased after consumption of HCHO diet, when compared to the BL (Appendix Figure 7.2). However, no significant differences were observed once correction for multiple testing using false discovery rate was applied (FDR = 0.49).

LefSe analysis was then used to compare the microbiota profiles between BL and PCHO diet and revealed that the OTU's affiliated with *Leptotrichia, Neisseria, Moryella and Actinomyces* to be discriminatory and enriched for BL, whereas OTU's affiliated with *Streptococcus*, *Kingella*, unclassified members of *Neisseriaceae* and *Prevotella* were increased and discriminatory in the same athletes after consumption of the PCHO diet (Appendix Figure 7.3). Analysis by sPLS-DA further showed that the relative abundances of *Haemophilus*, *Neisseria*, *Porphyromonas*, *Leptotrichia*, *Kingella*, *Prevotella*, *Unclassified Neisseriaceae*, *Rothia*, *Selenomonas* and *Tannerella* were increased following the consumption of the PCHO diet whereas *Unc. Aerococcaceae*, *Unc. CW040*, *Lautropia* and *Parvimonas* were distinct and increased in the BL samples of the same athletes who

later received the PCHO dietary intervention (Figure 3.3 below). The x axis in the sPLS-DA plot refers to the contribution (or importance) of the taxa on component 1(i.e. the x axis).

MELR analysis showed that the genus *Actinomyces* (p = 0.04), *Moryella* (p = 0.05), *Oribacterium* (p = 0.04), *Peptostreptococcus* (p = 0.009) and some unclassified *Erysipelotrichaceae* (p = 0.04) were reduced in response to the PCHO diet as compared to BL (Appendix Figure 7.4). However, the statistical significance of all these differences was lost once correction for multiple testing using the false discovery rate was applied (FDR = 0.4) (Appendix Figure 7.4).

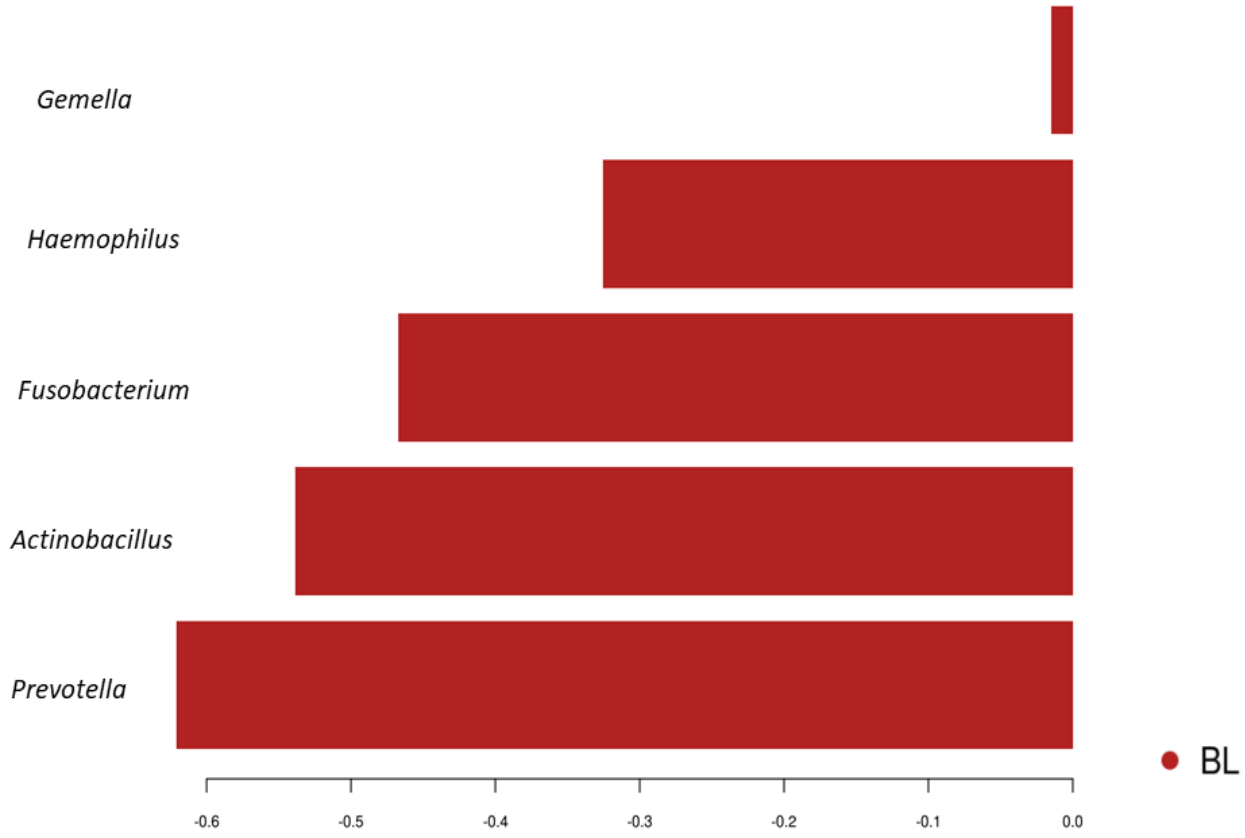

Figure 3.2 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the High Carbohydrate diet (HCHO) identified by sparse Partial Least Squares Discriminant Analysis (sPLS–DA).

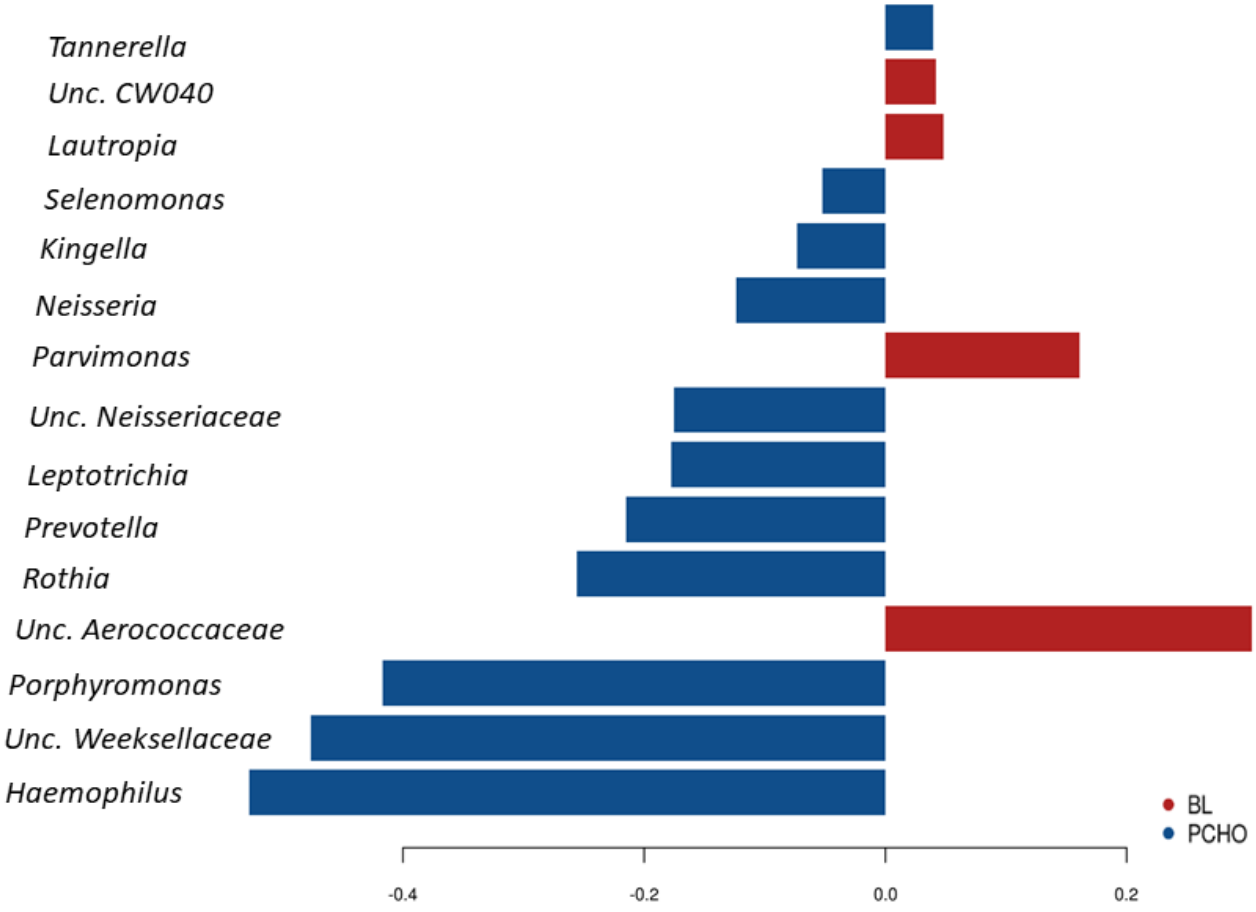

Figure 3.3 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Periodised Carbohydrate diet (PCHO, blue) identified by sparse Partial Least Squares Discriminant Analysis (sPLS–DA).

Discriminating taxa for BL and for the same samples following the LCHF diet training intervention was also identified using LefSe and sPLS-DA. LefSe analysis revealed *Leptotrichia*, *Lachnospiraceae* and *TM-7* affiliated OTU's to be increased in the BL samples, whereas *Lactobacillales*, *Streptococcus*, *Neisseria* affiliated OTU's were discriminatory and increased in the LCHF group (Appendix Figure 7.5). The sPLS-DA identified *Selenomonas*, *Unc. Planococcaceae*, *Unc. Enterobacteriaceae*, *Peptostreptococcus*, *Gemella*, *Granulicatella*, *Parvimonas*, *Unc. Clostridiaceae* to increase following LCHF diet training intervention, whereas Unc. F16, *Unc.*

Neisseriaceae, *Leptotrichia*, *Lactobacillus*, *Lautropia* and *Kingella* to be distinct and enriched in the BL samples of same athletes (Figure 3.4 below).

According to MELR analysis, the genus *Fusobacterium* (p = 0.02), *Lautropia* (p = 0.05), *Aggregatibacter* (p = 0.04), *Leptotrichia* (p = 0.040) and some unclassified *F16* (p = 0.04) were reduced, whereas *Granulicatella* (p = 0.03), some unclassified *Planococcaceae* (p = 0.03) and *Streptococcus* (p = 0.048) were increased in response to LCHF diet when compared to microbiota profiles at their BL (Appendix Figure 7.6). However, no significant differences were observed once correction for multiple testing using false discovery rate was applied (FDR = 0.4).

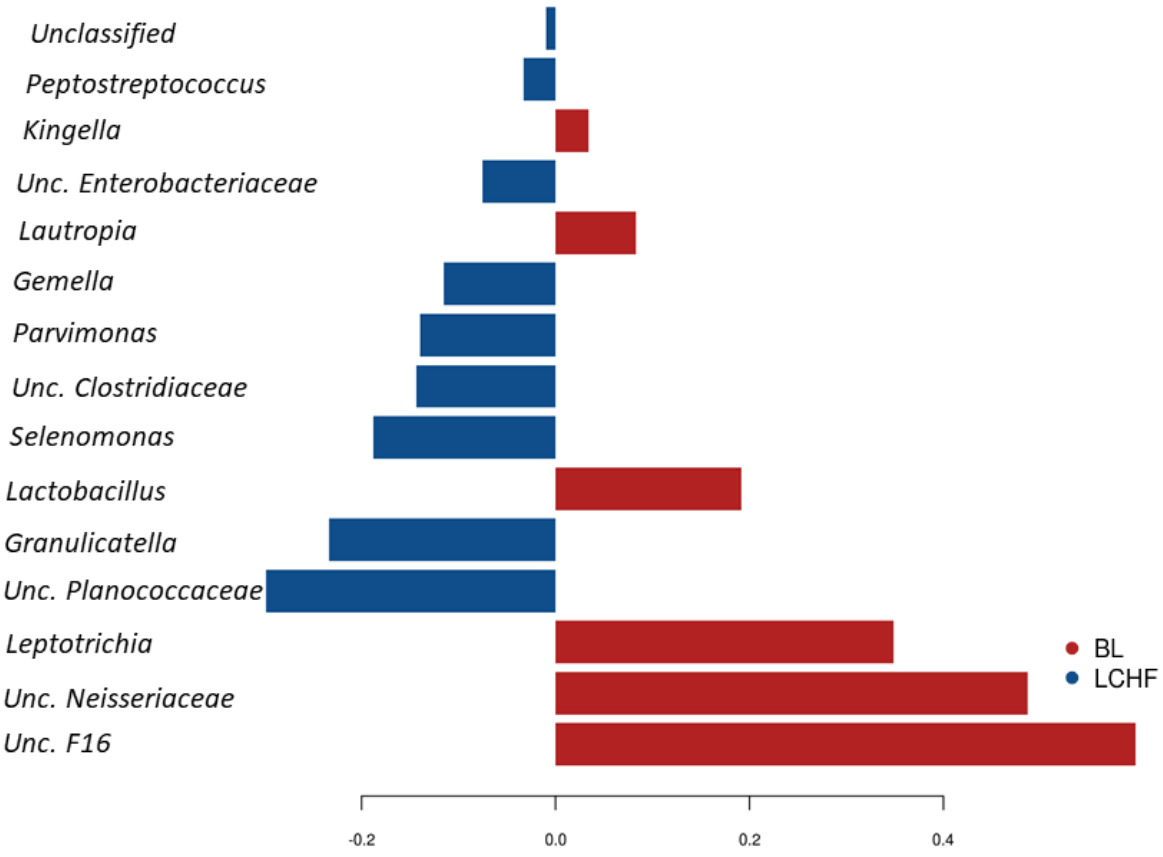

Figure 3.4 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Low Carbohydrate High Fat diet (LCHF, blue) identified by sparse Partial Least Squares Discriminant Analysis (sPLS–DA).

3.4.2 Comparisons of Community Profiles of Saliva Samples at the Conclusion of Dietary Interventions

Figure 3.5 below summarises the results of these analyses, showing the community profiles present in saliva samples at the conclusion of the dietary intervention periods, with annotations around some key genera and their inferred nitrate reductase capacity (Figure 3.5a). These profiles were compared with each other using sPLS-DA, which can be used to extract those taxa that most strongly discriminate the community structure between treatment groups (Figure 3.5b). Longitudinal comparison of the taxonomic profiles in the samples using sPLS-DA showed the strongest effect of the LCHF dietary intervention and in particular increase in the abundance of Gram-positive (Firmicutes) bacteria such as *Streptococcus*, *Peptostreptococcus*, and *Rothia* (Figure 3.5b)

LefSe analyses was also used to examine the differences in oral microbiomes post intervention and these analyses showed that the discriminating and enriched taxa (at the OTU level) were *Streptococcus* affiliated OTUs for the LCHF diet intervention, whereas Gram-negative bacteria (e.g., *Haemophilus* and *Leptotrichia* spp.) were among the enriched and discriminating taxa for the HCHO/PCHO diets (Figure 3.6)

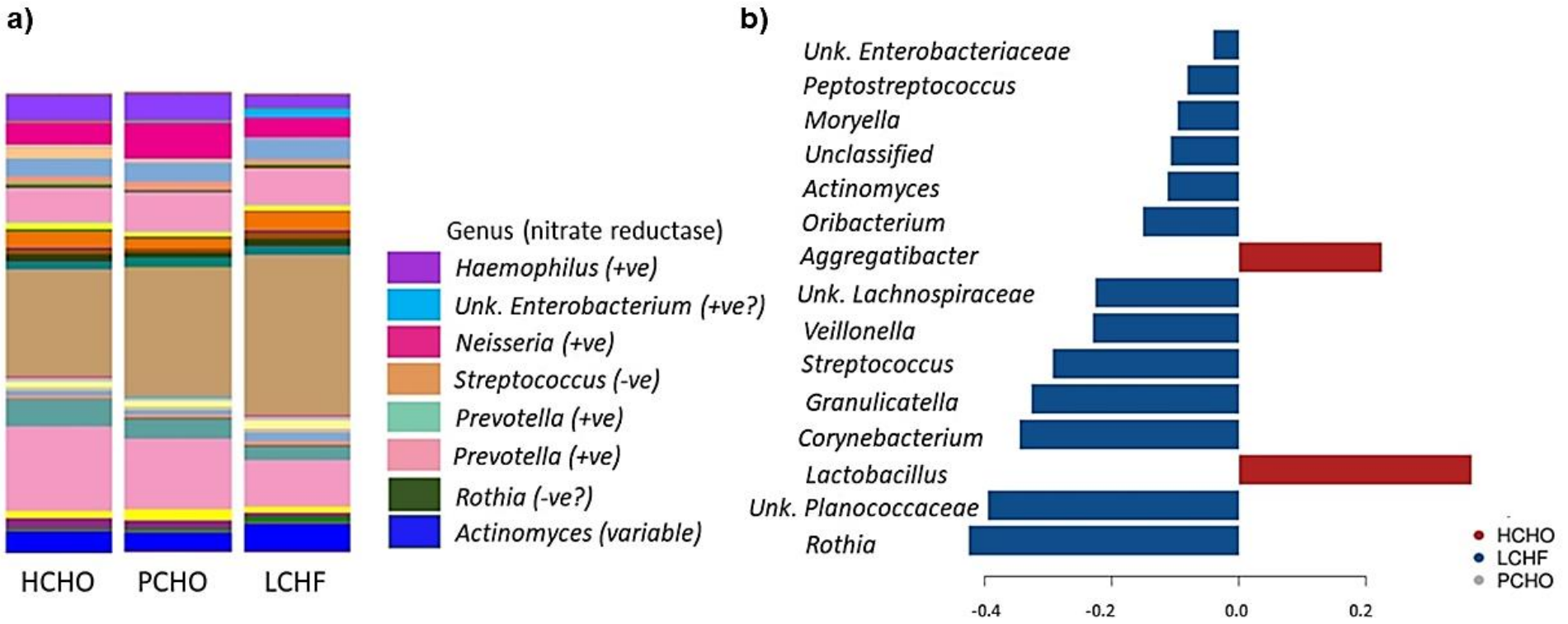

Figure 3.5 Oral microbiome profiles (genus-level) of athletes consuming either a high carbohydrate (HCHO), periodised carbohydrate (PCHO) or a low-carbohydrate high-fat diet (LCHF) after the diet-training intervention where bar plots represent: (**a**) relative abundance of genera in saliva samples after dietary interventions and their inferred nitrate reductase activity; (**b**) microbial families associated with different diets as identified by sparse Partial Least Squares Discriminant Analysis (sPLS-DA).

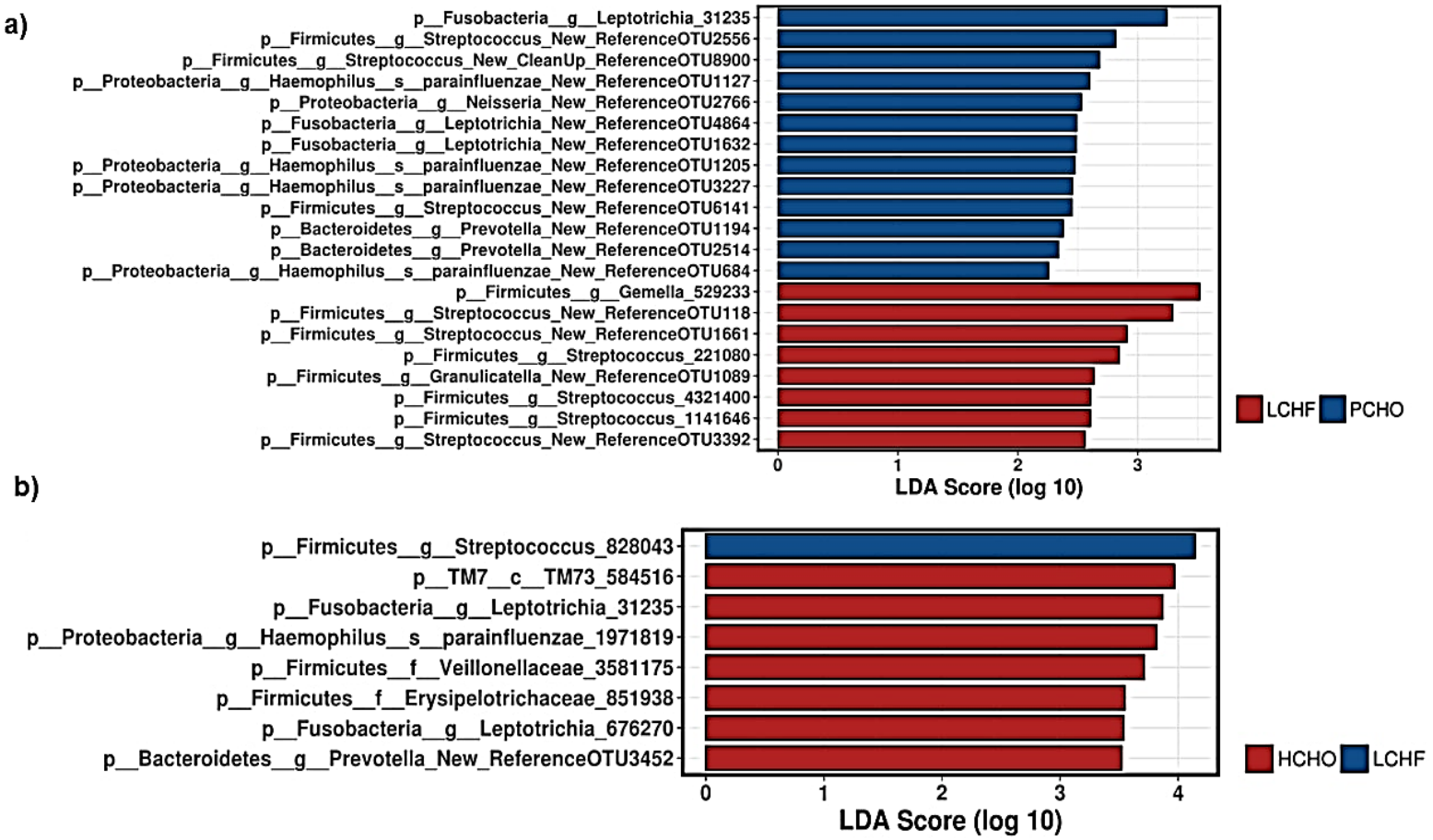

Figure 3.6 Linear Discriminant Analysis (LDA) Effect Size (LefSe) analysis at Operational Taxonomic Unit (OTU) level to compare the oral microbiome profiles of athletes post training diet interventions between Periodised Carbohydrate/Low Carbohydrate High Fat diet (PCHO/LCHF) **(a)** and High Carbohydrate-Low Carbohydrate high-fat diet (HCHO/LCHF) **(b)**, respectively.

3.5 Discussion

Despite the relatively small number of participants in this study, the dietary pattern consumed by these elite athletes during intensified training was shown to invoke remarkable effects on specific oral bacterial taxa- the bacterial communities in the mouth. The lack of a matching cohort of non-athletes (non-race walkers) and the lack of comprehensive data on the habitual dietary intake of athletes (i.e., BL samples) are acknowledged but were beyond the logistical and financial scope of the trial design. Furthermore, but understandably, the elite nature of the athletes ensured the group size is quite small, which also reduces the power needed for stringent statistical tests of significance, or the further subgrouping of the athletes according to ethnicity, etc. However, and despite these limitations, this is the first study of its type with elite endurance athletes, and one of the very few studies of the diet/nutrients × oral microbiota interactions affecting the physiology and/or metabolism of healthy human subjects. Furthermore, while fluctuations in the abundance and/or activities of nitrate-reducing bacteria in the oral microbiome are recognised to affect an individual's responsiveness to nitrate supplementation (112), this is the first study of the effects of dietary manipulation on this specific microbiome.

The bacterial taxa found in this study are similar to those represented in the Human Oral Microbiome Database (HOMD), as well as those typically reported in other studies of mainstream human subjects (71,113). The beta-diversity UniFrac principal coordinates analysis showed no apparent clustering of the oral microbial communities based on the ethnicity of the athletes nor any distinct effects of the dietary interventions under investigation in this study. This is similar to the findings of other studies of healthy individuals in which no significant clustering and bacterial taxa changes in the oral cavity have been reported (113). Nevertheless, more subtle changes within the bacterial communities in association with the diets were observed, with some of these representing potential alterations in community–host symbiosis. Here, the comparisons of the oral microbiome collected after three weeks of consuming one of three widely used diets by elite athletes during intensified training revealed that, unlike the CHO-rich diets, a ketogenic-LCHF diet appears to shift the balance of bacterial taxa that are widely considered to be key governors of the enterosalivary nitrate-nitrite-nitric oxide (NO) axis

within the oral cavity. This is an important finding since previous studies have demonstrated functional effects on host health when alterations to the oral microbiome interfere with this pathway (78). Facultative anaerobic bacteria in the mouth reduce salivary gland concentrated nitrate to nitrite, which is then swallowed and absorbed into the bloodstream, before further reduction to NO (74,75,78).

The critical role of the oral microbiome in this effect has been recently demonstrated in healthy non-athletes, where a seven-day period of antiseptic mouth wash treatment was shown to disrupt the oral microbiota and, in the absence of any dietary modifications, was associated with reductions in plasma and oral nitrite levels and an increase in blood pressure (114). Taken together, the changes seen following consumption of the LCHF diet, with respect to reductions in the relative abundances of well-known Gram-negative nitrate/nitrite reducers such as *Haemophilus*, *Prevotella* and *Neisseria*; and an increase in *Streptococcus* spp., which are not recognised to be directly involved in nitrate/nitrite reduction; raises the spectre that consumption of the LCHF diet can impair the enterosalivary nitrate-nitrite-NO axis. Indeed, further indirect support for this hypothesis can be found in a recent brief report that a three-day LCHF diet was associated with an impaired plasma nitrate/nitrite conversion following supplementation with potassium nitrate, compared with the response observed when people consumed a HCHO diet (115). This suggests that a LCHF diet might alter the baseline contribution of the nitrate-nitrite-NO pathway to NO-related health and performance benefits in athletes, as well as reduce their responsiveness to nitrate/beetroot juice supplementation as a performance aid (116). Further supporting evidence comes from the major outcome of the Supernova 1 study, which is the primary study to the current project and from which these saliva samples were derived. The study found a reduction in exercise economy (i.e., an increased oxygen cost of exercise) across a range of walking speeds in the LCHF group. It was originally hypothesised that this contributed to the failure of the LCHF group to improve their performance of a 10,000 m race walking event, despite the improvement in aerobic capacity that was seen across each of the study groups in response to the three-week block of intensified training It is plausible to attribute this loss of economy to the substantial increase in the contribution of fat oxidation to exercise substrate needs in the LCHF group, noting the longstanding recognition that CHO oxidation is slightly more

economical in generating ATP than fat oxidation per unit of oxygen utilization (117). Additionally, the increase in exercise tolerance and performance following acute and/or chronic nitrate supplementation include improved oxygen delivery to the muscle via the vasodilatory effects of NO, as well as a direct effect on mitochondria to reduce proton leak (116). However, these benefits are not universally observed across and within studies, and this variability is partially attributed to individual responsiveness, in addition to the more obvious contribution of unsuitable study protocols in relation to both the supplementation and exercise elements. However, based on the findings reported here, it is also plausible that part of the reduced exercise efficiency observed in the Supernova 1 study might be attributed to an altered oral microbiome, resulting in a reduction in nitrate/nitrite reducing activity and NO generation, with coordinate effects on circulation and mitochondrial function.

In conclusion, the results presented here are the first direct comparison of the oral microbiota profiles of elite athletes, and the effects of the dietary pattern consumed during intensified training for race-walking. The LCHF diet resulted in the most dramatic effects on the oral microbiota, with reductions in the relative abundance (*Haemophilus*, *Neisseria* and *Prevotella)*, and with a coincident increase in the relative abundance of *Streptococcus* spp. The athletes participating in this study following consumption of the LCHF diet also showed a loss of exercise economy (i.e., an increased oxygen cost of exercise) across a range of walking speeds compared to athletes consuming the carbohydrate rich diets. The findings reported here therefore justify the need to examine how diet x oral microbiome interactions affect elite athlete performance; and, particularly, NO homeostasis, and any coordinate impacts on cardiovascular and circulatory physiology.

3.6 Published journal article detailing work carried out in Chapter 3

PubMed link of the published article: https://www.ncbi.nlm.nih.gov/pubmed/30871219

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Article

Analysis of the Effects of Dietary Pattern on the Oral Microbiome of Elite Endurance Athletes

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Abstract: Although the oral microbiota is known to play a crucial role in human health, there are few studies of diet x oral microbiota interactions, and none in elite athletes who may manipulate their intakes of macronutrients to achieve different metabolic adaptations in pursuit of optimal endurance performance. The aim of this study was to investigate the shifts in the oral microbiome of elite male endurance race walkers from Europe, Asia, the Americas and Australia, in response to one of three dietary patterns often used by athletes during a period of intensified training: a High Carbohydrate (HCHO; n = 9; with 60% energy intake from carbohydrates; ~8.5 g kg^{-1} day^{-1} carbohydrate, ~2.1 g kg^{-1} day^{-1} protein, 1.2 g kg^{-1} day^{-1} fat) diet, a Periodised Carbohydrate (PCHO; n = 10; same macronutrient composition as HCHO, but the intake of carbohydrates is different across the day and throughout the week to support training sessions with high or low carbohydrate availability) diet or a ketogenic Low Carbohydrate High Fat (LCHF; n = 10; 0.5 g kg^{-1} day^{-1} carbohydrate; 78% energy as fat; 2.1 g kg^{-1} day^{-1} protein) diet. Saliva samples were collected both before (Baseline; BL) and after the three-week period (Post treatment; PT) and the oral microbiota profiles for each athlete were produced by 16S rRNA gene amplicon sequencing. Principal coordinates analysis of the oral microbiota profiles based on the weighted UniFrac distance measure did not reveal any specific clustering with respect to diet or athlete ethnic origin, either at baseline (BL) or following the diet-training period. However, discriminant analyses of the oral microbiota profiles by Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and sparse Partial Least Squares Discriminant Analysis (sPLS-DA) did reveal changes in the relative abundance of specific bacterial taxa, and, particularly, when comparing the microbiota profiles following consumption of the carbohydrate-based diets with the LCHF diet. These analyses showed that following consumption of the LCHF diet the relative abundances of *Haemophilus, Neisseria* and *Prevotella* spp. were decreased, and the relative abundance of *Streptococcus* spp. was increased. Such findings suggest that diet, and, in particular, the LCHF diet can induce changes in the oral microbiota of elite endurance walkers.

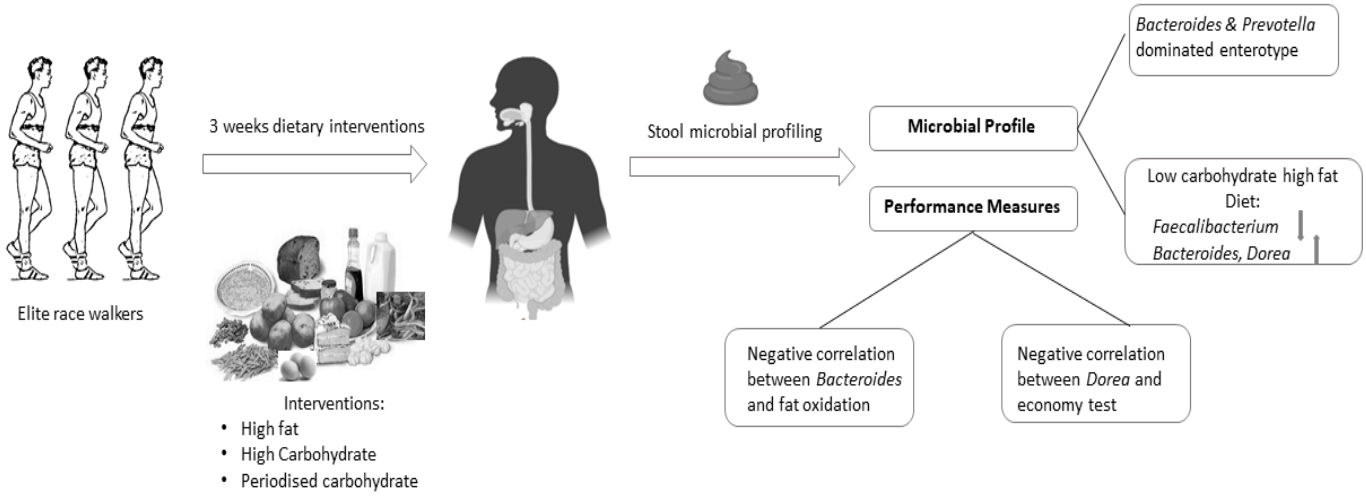
Keywords: oral microbiome; elite athletes; diet

Chapter 4

Chapter 4 The Effects of Dietary Pattern during Intensified Training on Stool Microbiota of Elite Race Walkers

4.1 Abstract

I investigated extreme changes in diet patterns on the gut microbiota of elite race walkers undertaking intensified training and its possible links with athlete performance. Numerous studies with sedentary subjects have shown that diet and/or exercise can exert strong selective pressures on the gut microbiota. Similar studies with elite athletes are relatively scant, despite the recognition that diet is an important contributor to sports performance. In this study, stool samples were collected from the cohort at the beginning (baseline; BL) and end (post-treatment; PT) of a three-week intensified training program during which athletes were assigned to a High Carbohydrate (HCHO), Periodised Carbohydrate (PCHO) or ketogenic Low Carbohydrate High Fat (LCHF) diet (post treatment). Microbial community profiles were determined by 16S rRNA gene amplicon sequencing. The microbiota profiles at BL could be separated into distinct "enterotypes," with either a *Prevotella* or *Bacteroides* dominated enterotype. While enterotypes were relatively stable and remained evident post treatment, the LCHF diet resulted in a greater relative abundance of *Bacteroides* and *Dorea* and a reduction of *Faecalibacterium.* Significant negative correlations were observed between *Bacteroides* and fat oxidation and between *Dorea* and economy test following LCHF intervention.



What are the new findings?

➢The gut microbiota profiles of endurance race walkers could be separated into two principal "enterotypes."

➢The LCHF diet is associated with a significant reduction in the relative abundance of *Faecalibacterium* spp. and an increase in the relative abundance of *Bacteroides* and *Dorea* spp.

➢The relative abundance of some bacterial taxa was correlated with measures of athlete performance and metabolic capacity.

How might it impact on clinical practice in the foreseeable future?

Dietary patterns appear to exert a subtle but meaningful impact on the gut microbiota of elite endurance race walkers. In particular, there appears to be a plausible link between a LCHF diet, the gut microbiome and impairments in exercise capacity, which may be monitored and managed to improve exercise economy and performance

4.2 Introduction

The gut microbiome is now widely recognised to be a functional and dynamic interface between host genetics, environmental and lifestyle choices. A virtual plethora of observational and case-control studies have investigated and reported on the variations in the gut microbiome of healthy subjects compared to those afflicted with acute, chronic and non-transmissible diseases (118–121). But relatively few studies to date have investigated the structure-function relationships of the gut microbiome of elite athletes, despite their remarkable physiology and metabolism compared to mainstream (regular healthy) members of the community. Clarke et al. (63) compared the gut microbiome of professional Irish rugby players with mainstream subjects of either matched BMI (>25) or average BMI (<25). Rugby players possessed greater bacterial diversity compared with both non-athlete groups, which could be readily linked to the greater intake of dietary protein measured

for these athletes. More recently, metagenomic and metabolomics analyses of these same cohorts have shown that the gut microbiota of rugby players were enriched for pathways involved in amino acid and carbohydrate metabolism and possessed greater amounts of faecal short-chain fatty acids (SCFA) as compared to the sedentary controls (66). Peterson et al. (68) have also compared the gut microbiota of professional cyclists and category 1 level (amateur) cyclists. These studies revealed the presence of three taxonomic clusters or "enterotypes" for all the cyclists: either *Prevotella* or *Bacteroides* dominated or a mixed taxa cluster. An increased abundance of *Methanobrevibacter smithii* was also evident in professional cyclists. Collectively, these studies confirm that the gut microbiota profiles of elite athletes are different to those of mainstream and/or non-elite control subjects and that diet might be a principal driver of these differences.

Typically, elite endurance athletes follow special dietary practices, particularly during periods of specialised training or competition preparation, to benefit from exercise-nutrient interactions that underpin adaptation and performance (110). In the parent 'Supernova study 1, Burke et al. (99) investigated aspects of endurance capacity in a cohort of elite race walkers who followed one of three popular dietary approaches to a 3-week block of intensified training: a ketogenic LCHF or diets with continuous or periodised exposure to high carbohydrate availability (HCHO or PCHO, respectively). Athletes consuming HCHO and PCHO diets were found to have improved exercise economy (increased speed achieved for a given oxygen utilisation) and race performance (event lasting ~40 min) compared to those athletes consuming a LCHF diet. This suggests that the latter dietary pattern, which achieved significant alterations in host physiology (i.e., a major increase in capacity for fat oxidation) is not conducive for performance of endurance exercise conducted at sustained higher intensities, where oxygen delivery to the muscle becomes limiting.

Given the scarcity of information on baseline (BL) gut microbiome profiles of elite athletes and the effect of dietary changes on the gut microbiota in such cohorts, the aim of the current investigation was to characterise the stool microbiome profiles elite race walkers that participated in the Supernova 1 study, using samples collected before and after undertaking three weeks of intensified training while following different dietary programs under rigorous study control. I hypothesised that the BL profiles

might share features of the microbiota previously described in endurance athletes (cyclists) but that this might change in response to the radical dietary changes implicit in a ketogenic LCHF diet.

4.3 Materials and Methods

4.3.1 Study Design and Sample Collection

A detailed description of the experimental design and physiological measures performed as part of the Supernova 1 study was provided by Burke et al. (99) along with justification for each of the dietary interventions (111). Briefly, a total of 21 male participants (aged 20–35 years), all of whom met International Association of Athletics Federations (IAAF) standards for international race experience, were accepted into the study. The dietary interventions were conducted over two separate training camps in November 2015 (n = 10) and January 2016 (n = 19) with 8 athletes recruited into both camps. During their 3-week training camp, the athletes were assigned to the specific diets according to their beliefs in the potential effect on their performance to either a diet high in carbohydrate availability (HCHO; n = 9) comprised of 60% of caloric intake from carbohydrate, CHO (~8.5 g/kg body mass (BM)/day), 16% protein (~2.1 g/kg BM/day), 20% fat; a diet with periodised carbohydrate availability (PCHO; n = 10) of the same macronutrient composition as HCHO but periodised in consumption across the day and throughout the week, so to support different training sessions with a high and low CHO availability; and a ketogenic low carbohydrate-high fat diet (LCHF; n = 10) that was comprised of 78% fat, 17% of protein (~2.2 g/kg BM/day) and <50 g/day of carbohydrate content (~3.5% energy). Stool samples were collected from the athletes at the beginning and end of the 3-week training-diet intervention period using the OMNIgene stool collection and preservative kit. Physiological measures such as VO_{2peak} and walking economy, 10 km race time, 25 km long walk time, respiratory exchange ratio and fuel oxidation rates were measured in the Supernova 1 study by Burke et al. (99). These data are used for the correlation analysis.

4.3.2 Stool Microbiota Analysis

Genomic DNA was extracted and 16S rRNA gene amplicon libraries were prepared and sequenced, and the resulting data analysed according to the protocols previously described in Chapter 2.

4.4 Results

Burke et al. (99) reported that race walkers who consumed the LCHF diet during intensified training achieved a substantial increase (~tripling) in their rates of whole-body fat oxidation during exercise compared with BL , with peak rates of ~1.6 g/min (and up to 2 g/min in some individuals) being the highest reported values in the literature. However, this was associated with a reduction in exercise economy (increased oxygen cost to achieve the same walking speed) and a failure to improve 10 km race performance (change = 1.6% slower (90% CI = −8 to +5%) despite achieving an equal increase of 3–7% in aerobic capacity over the training block as the other groups. Conversely, athletes consuming either the HCHO (n = 8) or PCHO (n = 10) diets showed improvements in exercise economy and athlete performance as reported by Burke et al.(99) (mean race improvements = 6.6% (4–9%) and 5.3% (3–7%), respectively). These differences in athlete physiology and performance in response to diet provide a unique opportunity to examine whether and how the gut microbiome of these athletes is affected by diet and/or can be associated with changes in athlete physiology and performance.

4.4.1 *Bacteroides*- or *Prevotella*-Enterotypes are Predominant in Elite Race Walkers

Sample ordination by PCoA revealed that 28/29 athletes at BL could be separated into two distinct clusters, while one athlete showed a clear separation from both (Figure 4.1 a). The genus-level taxonomic profiles for the three clusters at BL are shown in Figure 4.1 c, with 7/29 athletes found to possess a "*Prevotella*-predominant" cluster and 20/29 athletes being "*Bacteroides*-dominant." The remaining two points on the PCoA plot were from the same athlete who participated in both training periods and was a "Firmicutes-dominant" cluster with a remarkable level of methane-producing and

succinate-utilizing microbes (*Methanobrevibacter* and *Succinivibrio*). Furthermore, these clusters were sustained throughout the intensified training and diet-intervention period, as shown when these profiles were included in the beta diversity analysis (Figure 4.1 b). As noted in the Methods, a number of athletes participated in both camps, so the stool microbiota profiles of the two BL samples for these athletes were compared to each other. A combination of RDA and Anosim analyses as well as the Shannon diversity measures for the matched samples showed no remarkable or significant differences in these profiles. As such, the length of time between camps and the return of these athletes to their habitual diet was considered to be sufficient to ensure there were no carryover effects from the prior camp (and dietary intervention). In summary, the stool microbiomes of virtually all these elite athletes could be differentiated into either a *Prevotella* or *Bacteroides*-dominant enterotype and the dietary interventions and intensive training period had limited influence on the stability of these enterotypes.

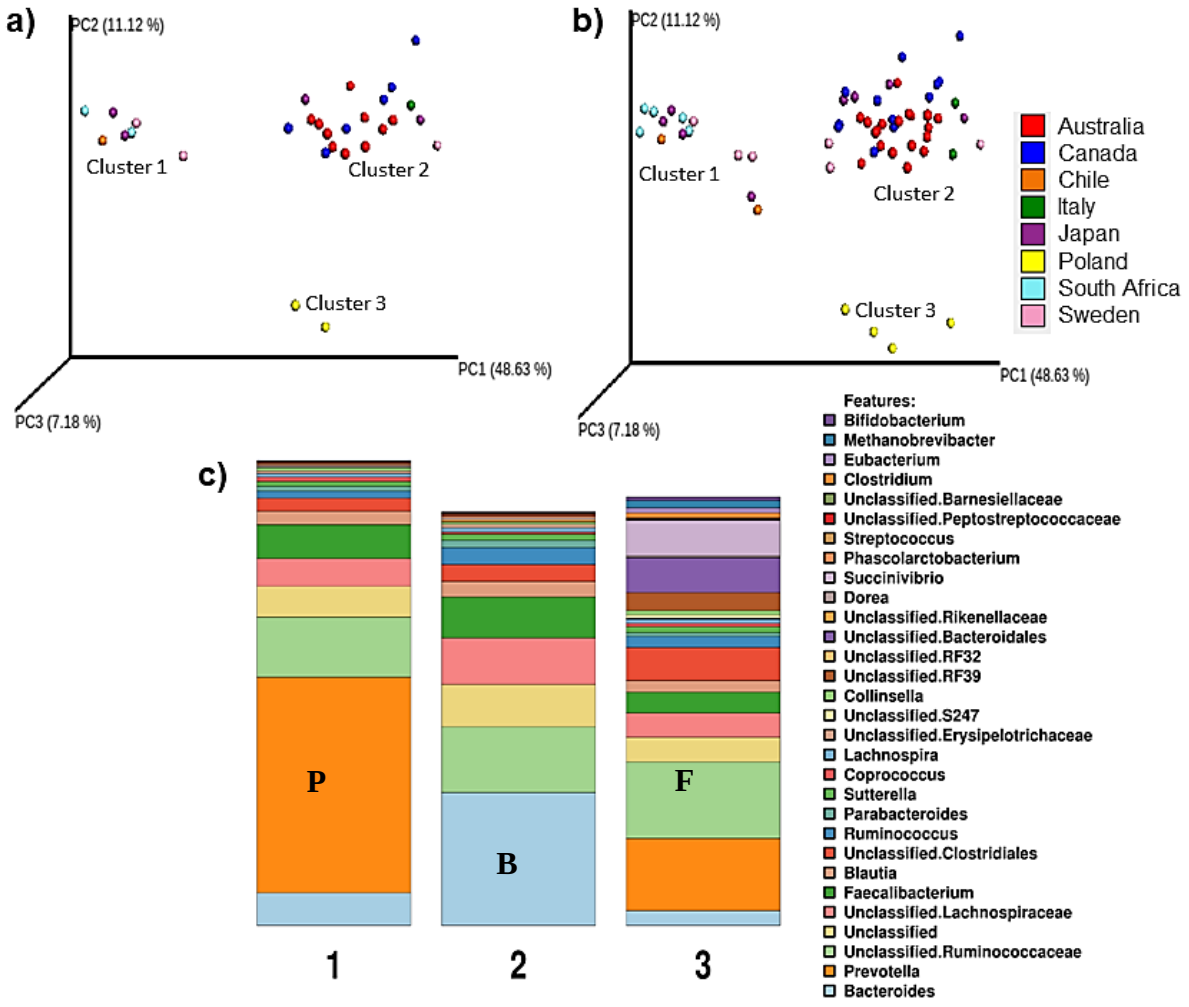


Figure 4.1 Principal coordinates analysis (PCoA) of the weighted Unifrac distances produced from the stool samples of athletes collected at baseline only (**a**); and combined with their matching stool samples collected after the 3-week diet-training intervention period (**b**). The individual samples are coloured coded according to the athlete's country of origin. The athletes are separated into three distinct clusters and importantly, this clustering did not appear to be disrupted in response to the diet consumed during the training period. The first three Principal Coordinates and the amount of variation each explains are shown (PC1, PC2, PC3). (**c**) The profiles of the predominant taxa present in the baseline stool samples of athletes with either a *Prevotella*-dominant (P, cluster 1; n = 7), a *Bacteroides*-dominant (B, cluster 2; n = 20) or a Firmicutes-dominant (F, cluster 3; n = 2) "enterotype".

4.4.2 HCHO and PCHO Diets Result in Subtle but Distinct Alterations in Firmicutes Lineages

HCHO (*n* = 8 athletes) and PCHO (*n* = 10 athletes) diets resulted in only minor, subtle changes in microbial community composition. RDA and Anosim did not identify any significant associations between microbial community composition and HCHO and PCHO diets and no differences in alpha diversity were observed between BL and post dietary interventions. Furthermore, the HCHO and PCHO diets did not result in any significant changes in the relative abundance of any specific taxa once adjustments were made for multiple testing using false discovery rate according to MELR analysis.

The less stringent tests LefSe and sPLS-DA did identify a restricted range of bacterial taxa affected by these diets. LefSe found an increase of *Clostridiales,* in particular, *Ruminococcaceae*, *Coprococcus* spp. and *Akkermansia muciniphila* in athletes consuming the PCHO diet relative to their BL profiles and an increase of *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae* in athletes consuming the HCHO diet (Appendix Figure 7.7a and b). The sPLS-DA suggested that OTU's assigned to *Unclassified (Unc.) YS2*, *Akkermansia, Bifidobacterium* and *Streptococcus* spp. were increased whereas *Bilophila* was decreased following consumption of the PCHO diet (Figure 4.2).

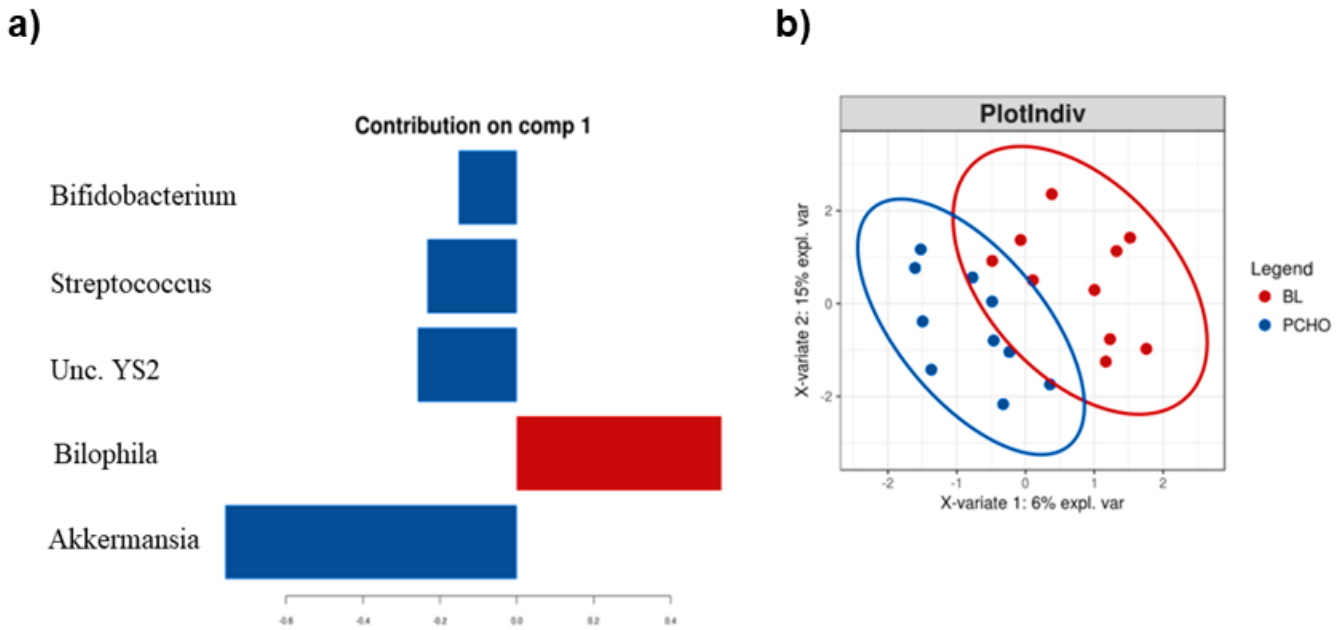


Figure 4.2 Genera differentiating between the stool microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Periodised Carbohydrate diet (PCHO, blue) identified by sPLS–DA **(a);** The sPLS-DA ordination plot of these same data for each athlete, the ellipsoids represent 95% confidence intervals for each sampling period **(b).**

The relative abundances of *Unc. YS2*, *Streptophyta* and *Clostridiaceae* were all increased in athletes who consumed the HCHO diet compared to their BL samples, whereas OTUs assigned to *Unclassified RF39* and *Sutterella* spp. were enriched and discriminatory of their BL samples (Figure 4.3). Collectively, these results show that the effects of consuming a carbohydrate-rich diet in these athletes was subtle and principally restricted to members of Firmicutes lineage.

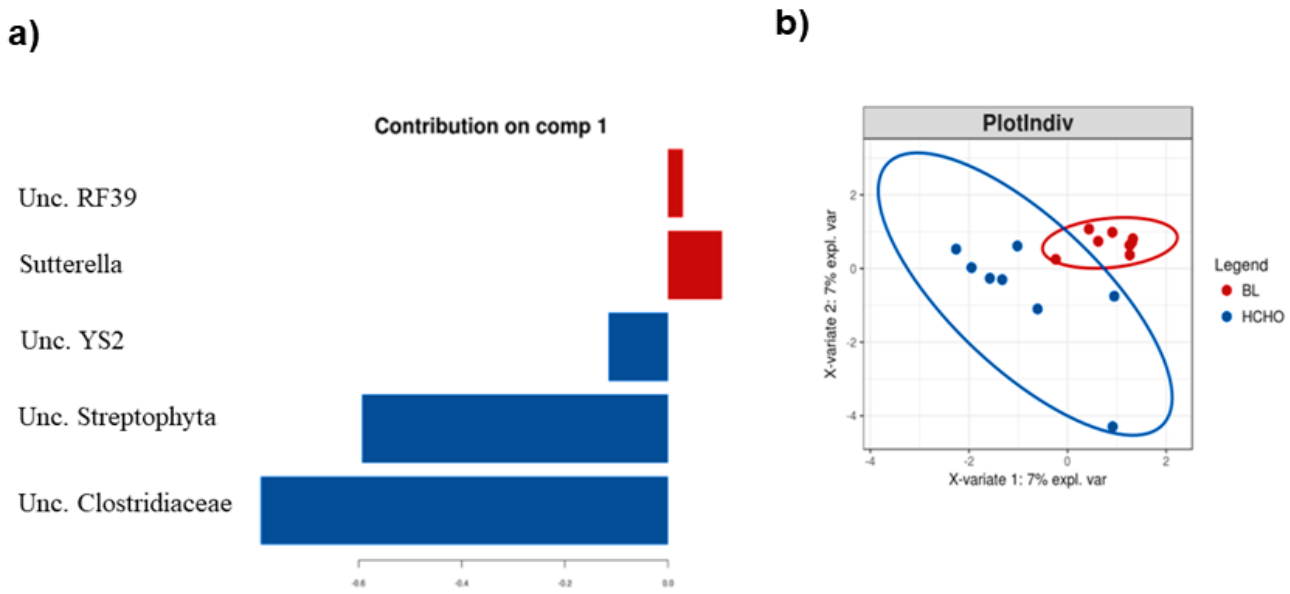


Figure 4.3 Genera differentiating between the stool microbiota profiles of athletes at baseline (BL, red) and after their consumption of the High Carbohydrate diet (HCHO, blue) identified by sPLS–DA **(a)**; The sPLS-DA ordination plot of these same data for each athlete, the ellipsoids represent 95% confidence intervals for each sampling period **(b)**.

4.4.3 The Low Carbohydrate High Fat Diet Results in More Profound Effects on the Gut Microbiota

The LCHF diet (n = 10 athletes) had a stronger impact on the stool microbiota profiles of the athletes than the HCHO and PCHO diets. RDA and Anosim both identified significant differences (p = 0.020 and p = 0.029 respectively) between the stool microbiota profiles of athletes at BL and following the consumption of the LCHF diet (Figure 4.4a and b).

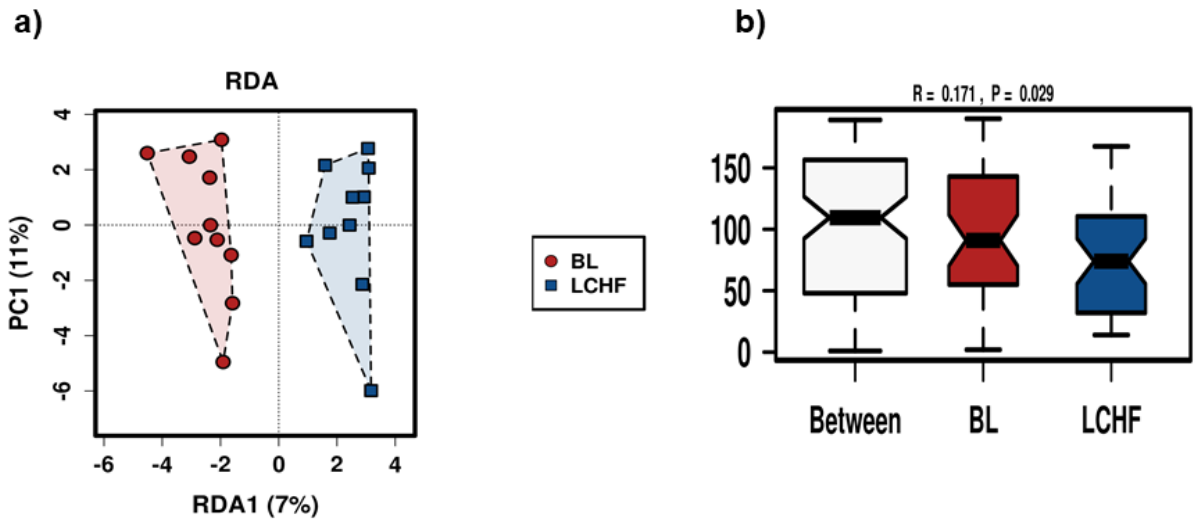


Figure 4.4 (a) Redundancy analysis (RDA) and (**b)** Anosim plots of the stool microbiota profiles for athletes either at baseline (BL, red) or after consuming the Low Carbohydrate High Fat (LCHF) diet (blue). In panel (**b**) the between plot is a measure of the magnitude of difference in profiles between the samples collected at BL and after consumption of the LCHF diet. Both analyses showed that the differences in the stool microbiota profiles between the two sampling periods are statistically significant (p= 0.005 and 0.029, for RDA and Anosim analyses, respectively).

However, there was no significant change in the alpha diversity between BL and post LCHF consumption (Appendix Figure 7.8). Furthermore, the LCHF diet resulted in a significant reduction in the relative abundances of *Faecalibacterium* spp. (p = 0.0003), an increase in *Dorea* spp. (p = 0.007) and several OTUs assigned to the genus *Bacteroides* (p = 0.002) (Figure 4.5 a-c). These results were further verified using Wilcoxon rank *t*-test (p = 0.002, p = 0.01, p = 0.003 respectively) (Appendix Figure 7.9).

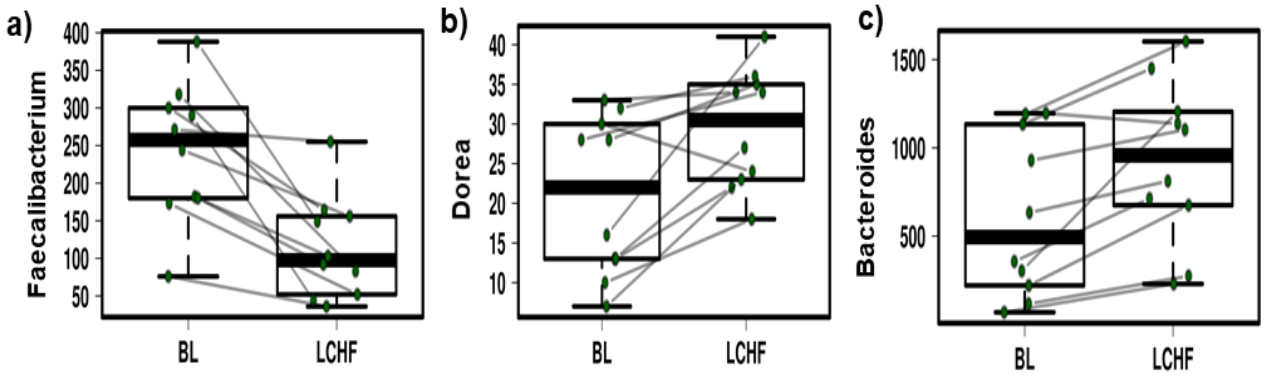


Figure 4.5 Mixed effect linear regression (MELR) analysis after consumption of the Low Carbohydrate High Fat (LCHF) diet identified significant reductions in the relative abundances of *Faecalibacterium* ((**a**), $p = 0.0003$) and significant increases in the relative abundance of both *Dorea* ((**b**), $p = 0.0068$) and *Bacteroides* ((**c**), $p = 0.0022$) Here, sampling time point was set as a fixed effect and athlete as a random effect. Baseline (BL) and LCHF refer to the relative abundances of these taxa measured at baseline and after consumption of the LCHF diet, respectively. Those data collected from the same athlete are connected by the lines.

Analysis using LefSe also identified an increase of *Dorea* as well as *Enterobacteriaceae* in response to the LCHF diet and a reduction of *Faecalibacterium* and *Bifidobacterium* spp. (Appendix Figure 7.10a and b). The sPLS-DA further found an increase in the relative abundance of OTUs assigned to *Unc. Peptostreptococcaceae, Unc. RF39, Unc. Enterobacteriaceae* and *Unc. Barnesiellaceae* and *Akkermansia;* while the relative abundances of *Bifidobacterium, Veillonella, Streptococcus, Faecalibacterium, Succinivibrio, Odoribacter* and *Lachnospira* spp. were reduced after consumption of the LCHF diet (Figure 4.6). In summation, these results suggest that the LCHF diet results in a stronger selective pressure on the gut microbiota of these athletes than the HCHO or PCHO diets, which were closer to their typical diets, leading to an increase in the relative abundance of bacterial taxa with recognised capabilities for lipid metabolism.

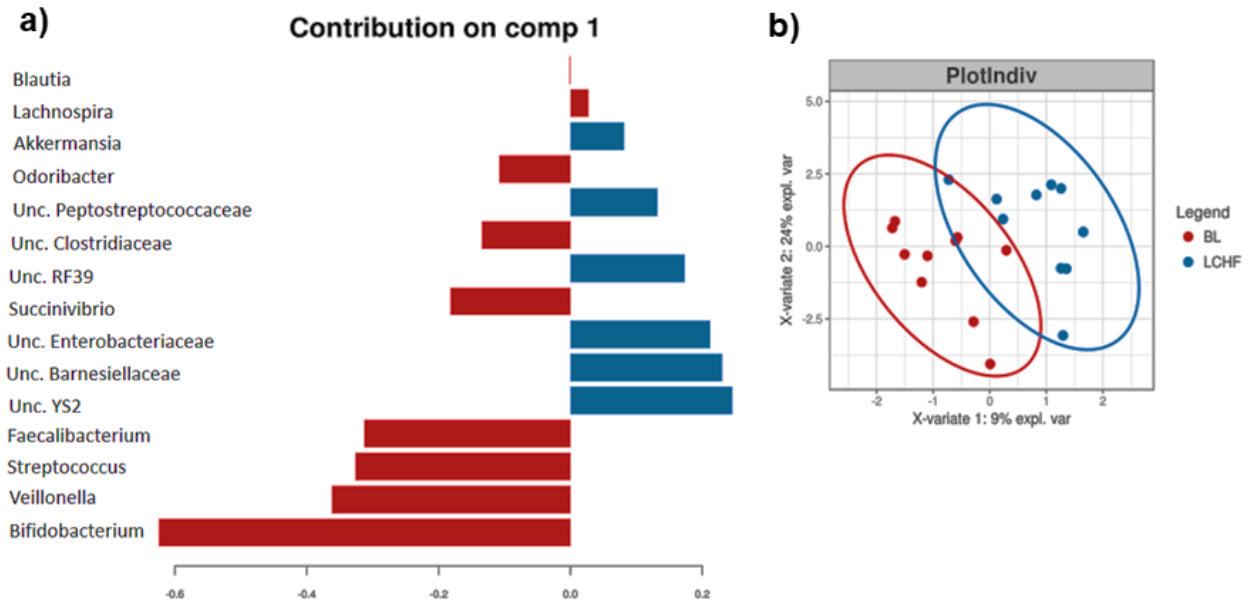


Figure 4.6 Genera differentiating between the stool microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Low Carbohydrate High Fat diet (LCHF, blue) identified by sPLS–DA **(a)**; The sPLS-DA ordination plot of these same data for each athlete, the ellipsoids represent 95% confidence intervals for each sampling period **(b)**.

4.4.4 *Bacteroides* and *Dorea* spp. Abundances are Negatively Correlated with Athlete Performance Measures Following Consumption of the LCHF Diet

Based on the statistically significant differences in *Bacteroides, Faecalibacterium and Dorea* observed between the stool microbiota profiles of those athletes consuming the LCHF diet and their BL samples, these data were compared with various physiological and performance measures also collected at the beginning and the end of the diet-training period as part of the Supernova 1 study. Although no significant correlations were found between these taxa and any performance measures at BL, significant negative correlations (Spearman test) were apparent after consumption of the LCHF diet between *Bacteroides* abundance and fat oxidation ($r = -0.72$, $p = 0.02$); and *Dorea* spp. abundance and exercise economy ($r = -0.65$, $p = 0.04$, Figure 4.7).

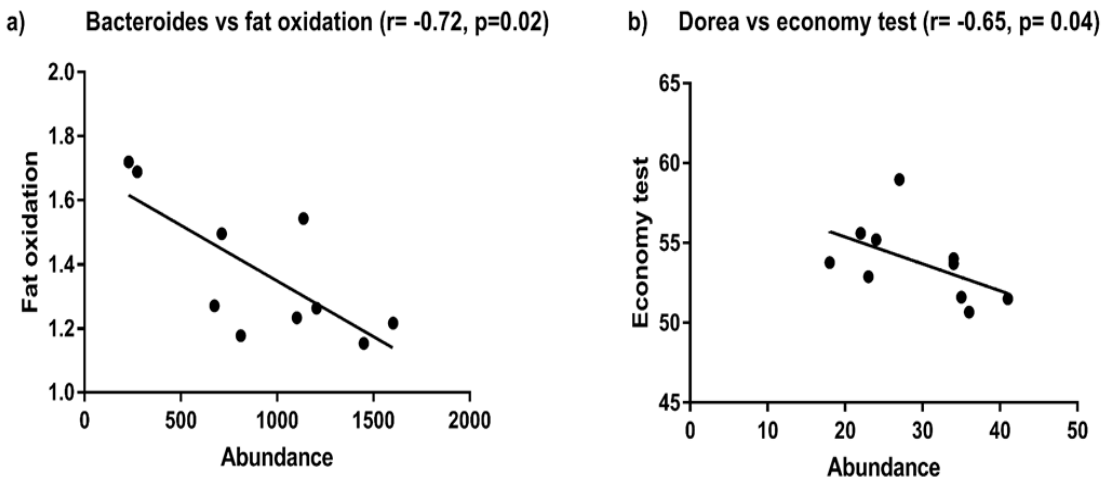


Figure 4.7 The relationship between **(a)** the abundance of *Bacteroides* spp. and fat oxidation and; **(b)** the abundance of *Dorea* spp. and economy test, for athletes after their consumption of the low carbohydrate high fat diet. The Spearman correlation coefficient and p values for each are also shown and were statistically significant.

4.4.5 Is There a LCHF × Enterotype Interaction?

Given the LCHF diet had the strongest impact on the faecal microbiome of athletes and there was a balanced distribution of athletes between the *Bacteroides* (n = 5) or *Prevotella* (n = 4) enterotype at BL, we also examined whether there was a diet × enterotype interaction evident in these athletes. Interestingly, while all the athletes did show a decrease in the relative abundance of specific *Prevotella* affiliated taxa and an increase in the relative abundance of specific *Bacteroides*-affiliated taxa (Appendix Chart 7.1 and Chart 7.2) these alterations were not sufficient to disrupt their "enterotype."

Rank tests revealed there was also a significant ($p < 0.05$) reduction in the relative abundance of *Faecalibacterium* following the LCHF diet intervention, independent of their BL enterotype. In contrast, *Bifidobacterium* was significantly reduced and *Sutterella* increased in those athletes with a *Bacteroides* enterotype, whereas *Unclassified* members of *Clostridiales* were significantly increased in the athletes with *Prevotella* enterotype (Figure 4.8). Taken together, these findings suggest there were also LCHF × enterotype interactions in this athlete cohort.

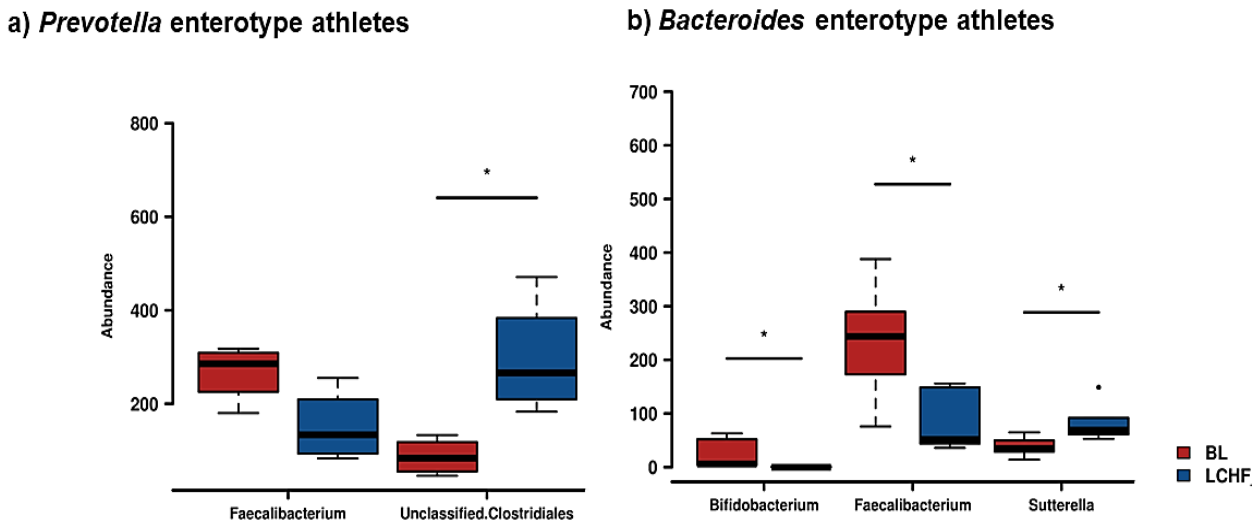


Figure 4.8 Rank test of the changes in relative abundances of specific bacterial taxa between baseline (red) and following consumption of the Low Carbohydrate High Fat diet (LCHF, blue), when athletes are stratified according to their stool enterotype being either *"Prevotella*-dominant" (**a**) or "*Bacteroides*-dominant" (**b**) In athletes assigned to either enterotype, there was a reduction in the relative abundance of *Faecalibacterium* following consumption of LCHF diet. In those athletes with the *Prevotella*-dominant enterotype, a significant increase in *Unc. Clostridiales* was observed ($p < 0.05$); whereas a significant reduction in *Bifidobacterium* and an increase in *Sutterella* was observed in athletes with the *Bacteroides*-dominant enterotype following consumption of the LCHF diet ($p < 0.05$ in both instances). * represents significant differences ($p < 0.05$).

4.5 Discussion

Diet is now widely accepted as one of the major determinants of the composition and function of the gut microbiota, with concordant impacts on our nutrition and health. However, despite the robust evidence that diet is a critical factor in the metabolism and performance of endurance exercise/sport (122), very few studies have reported on the effect of specific dietary patterns during periods of intensified training on athlete physiology and the gut microbiota. Unfortunately, logistical constraints precluded the recruitment of a matching non-athlete (non-race walkers) cohort and the provision of comprehensive data on their habitual diet (i.e., prior to baseline). However, our study does provide

the first in-depth investigations of the effect of dietary interventions for periods of intensified training on the gut microbiome of elite athletes and is empowered by the repeated measures for each athlete. This not only provides a direct comparison of their stool microbiome pre- and post-intervention but also the capacity to compare how any changes in the gut microbiome might be correlated with athlete performance measures. Thus, our study still provides valuable insights about the diet, microbiome and performance interactions.

The baseline samples from this study provided some insight into the gut microbiota profiles of elite endurance athletes (race walkers) when consuming their habitual diet ahead of intensified training. Our results show that the stool microbiota of race walkers could be clearly separated into two principal "enterotypes": one being *Prevotella*-predominant (7/29) and another *Bacteroides*-dominant (20/29). In general terms, these two "enterotypes" bear similarities with those originally proposed by Arumugam et al. (123) from their analyses of cohorts of both patient and healthy subjects drawn from the general communities of western European countries; the enterotypes were also reported in a recent study of amateur and professional level cyclists by Peterson et al. (68). From their study of healthy non-athlete volunteers, Wu et al. (124) proposed that long term dietary patterns were the primary determinant of the persistence of either a *Bacteroides* or *Prevotella* enterotype, with diets favouring animal protein and fats supporting a *Bacteroides*-dominant enterotype; whereas a carbohydrate/fibre-rich diet favoured the establishment of the *Prevotella* enterotype. In that context, while Clarke et al. (63) did not report there being similar enterotypes to those noted above, the stool microbiota profiles of professional rugby players could be differentiated from the BMI-matched and normal BMI non-athletes by an increased relative abundance of taxa assigned to *Akkermansia, Succinivibrionaceae,* S24-7, RC9 and *Succinivibrio*. In our study, a single athlete could be separated from the two enterotypes noted above, because of the remarkable relative abundances of methane-producing archaea *(Methanobrevibacter)* and succinate-utilizing bacteria *(Succinivibrio),* as well as unclassified members of the phylum Tenericutes, order *RF39* and *Anaeroplasmatales.* It is still unclear as to extent which environmental, genetic or lifestyle factors might contribute to the presence of enterotypes in the human gut microbiome, as host physiological factors such as BMI, age and so forth, do not seem to be strong drivers of these profiles. However, Vandeputte et al. (125) recently revealed that the total

bacterial load of the stool sample can be strongly associated with the enterotype predicted from the same stool sample. Furthermore, Korean et al. (126) have suggested that analytical factors such as the OTU picking method and the taxonomic level at which the data are studied, as well as the distance metric(s) and cluster scoring methods used, can also have some influence on data resolution and enterotype predictions. Here, our analytical methods clearly revealed the presence of enterotypes consistent with those reported for healthy mainstream subjects as well as other categories of elite athletes. Our analyses also suggest these enterotypes were resilient to change during the 3-week dietary intervention periods. Taken together, it therefore seems plausible that like in other healthy mainstream subjects, the stool microbiota enterotype of these elite athletes are resilient to short-term changes in their diet. Based on these results, future studies of elite athletes and/or where the number of subjects that can be recruited into the study might be constrained, a prospective assessment of the gut microbiome and enterotype representation be undertaken; to determine how subjects might be assigned to different interventions/treatments and to assess diet x enterotype interactions.

Clarke et al. (63) reported significantly a greater relative abundance of *Akkermansia* in Irish rugby players as compared to the non-athlete controls and Peterson et al. (68) reported the presence of *Akkermansia* in 30/33 cyclists in their study. Here, OTUs assigned to the genus *Akkermansia* were detectable in some but not all the athletes and the different diets used here also appeared to have a limited effect on the relative abundance and/or prevalence of this genus. It is plausible that the difference in the relative abundance and/or prevalence of *Akkermansia* spp. between the specialist athlete groups could be linked with the differences in their dietary protein intake (16–17% daily caloric intake in race walkers, as compared to 22% in rugby players (63) and ~33% by most professional level cyclists (68). However, future studies need to be conducted to establish this hypothesis.

The relative abundance of *Faecalibacterium* spp. was found to be decreased in athletes after their consumption of the LCHF diet. Interestingly, previous studies have not remarked on the relative abundance of this bacterium in athletes, despite it being one of the most abundant bacterial taxa present in the gut microbiota of healthy mainstream subjects. *Faecalibacterium prausnitzii* is also

widely recognised for its production of a suite of metabolites and peptides with anti-inflammatory effects (127). Studies with rodent models and human subjects of obesity and type 2 diabetes have shown reductions in the relative abundance of *F. prausnitzii* associated with these conditions. High-fat diets are likely to change the both the amounts and profile of bile acid secretions reaching the large intestine (128), which could also result in reductions in the relative abundance of *Faecalibacterium* spp., as it is known to be a bile sensitive bacterium (129,130). Taken together, the significant reduction in the relative abundance of *Faecalibacterium* spp. in response to the consumption of the LCHF diet is both plausible and the potential effects of this change should be further investigated.
The comparative analyses also showed there was an increase in the relative abundance of *Dorea* spp. in response to consumption of the LCHF diet. This finding is consistent with those reported from rodent-based studies of obesity and lipid metabolism using high-fat diets (131–133). Interestingly, positive associations have been reported between the relative abundance of *Dorea* spp. with serum total cholesterol and LDL concentrations in high fat-induced hyperlipidaemic rats (134). Furthermore, while the relative abundance of *Dorea* spp. is consistently increased by high-fat diets, this change can be counteracted in rats by the supplementation of their diets with either mono- or tributyrin; and both compounds are also associated with reductions in liver and serum biomarkers of hyperlipidaemia (134,135). Presently, much less is known about the possible roles of *Dorea* spp. in human obesity and lipid metabolism, but our findings provide further evidence that such studies are warranted.

Despite the resilience of an athlete's stool enterotype to short-term dietary change, there was a notable increase in the relative abundance of *Bacteroides* spp. in those athletes that consumed the LCHF diet. As noted above, high fat diets tend to increase bile acid secretion into the gut and David et al. (16) have reported an increase in the abundance of bile-tolerant bacteria in human subjects who consume a diet rich in animal-based proteins and fats and *Bacteroides* spp. are well recognised for their resistance to these host secretions. In that context, Wu et al. (124) have previously reported strong positive correlations between members of this genus and the intake of dietary fat- and protein-based nutrients as reported by questionnaire. Similarly, Shankar et al. (136) reported the gut environment of the group of US children studied was rich in metabolites arising from animal proteins and fats and *Bacteroides*-dominated; as compared to Egyptian children with greater concentrations of short chain

fatty acids and fibre-degrading genes and a *Prevotella*-dominated microbiota. In the larger, parent study supporting the research presented here (Supernova 1), Burke et al. (99) found that although all dietary groups improved their aerobic capacity over the 3-week training block, the LCHF diet was associated with a negative effect on exercise economy and performance in the elite race walkers, when compared to those athletes consuming either the HCHO or PCHO diets. Interestingly but somewhat paradoxically, our analyses revealed that after the consumption of the LCHF diet, the relative abundance of *Bacteroides* spp. was significantly negatively correlated with fat oxidation; and that the relative abundance of *Dorea* spp. was significantly negatively correlated with the economy test, measured as described by Burke et al. (99). Such findings suggest that an individual's responsiveness to such a diet is complex and perhaps, can also be affected by the amount of dietary fat that actually reaches the distal gut, where it may have associative effects on the gut microbiota of the nature reported here.

In conclusion, the stool microbiota profiles of elite endurance athletes bear gross similarities to those reported for healthy mainstream and other elite endurance athletes, in so far as the representation of *Bacteroides* and *Prevotella*-dominated enterotypes. While these enterotypes appear relatively stable in response to short-term changes in diet and despite the relatively small number of subjects available for study, a ketogenic low carbohydrate, high fat diet was still found to invoke significant alterations in the relative abundances of some key bacterial taxa. Although the findings of this pilot study cannot differentiate between cause versus consequence, the findings do justify the need for more detailed longitudinal studies that examine how diet x microbiome interactions may be better understood and managed to optimize athlete training and performance.

4.6 Published journal article detailing work carried out in Chapter 4

PubMed link of the published article: https://www.ncbi.nlm.nih.gov/pubmed/30682843

nutrients

MDPI

Article

The Effects of Dietary Pattern during Intensified Training on Stool Microbiota of Elite Race Walkers

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Abstract: We investigated extreme changes in diet patterns on the gut microbiota of elite race walkers undertaking intensified training and its possible links with athlete performance. Numerous studies with sedentary subjects have shown that diet and/or exercise can exert strong selective pressures on the gut microbiota. Similar studies with elite athletes are relatively scant, despite the recognition that diet is an important contributor to sports performance. In this study, stool samples were collected from the cohort at the beginning (baseline; BL) and end (post-treatment; PT) of a three-week intensified training program during which athletes were assigned to a High Carbohydrate (HCHO), Periodised Carbohydrate (PCHO) or ketogenic Low Carbohydrate High Fat (LCHF) diet (post treatment). Microbial community profiles were determined by 16S rRNA gene amplicon sequencing. The microbiota profiles at BL could be separated into distinct "enterotypes," with either a *Prevotella* or *Bacteroides* dominated enterotype. While enterotypes were relatively stable and remained evident post treatment, the LCHF diet resulted in a greater relative abundance of *Bacteroides* and *Dorea* and a reduction of *Faecalibacterium*. Significant negative correlations were observed between *Bacteroides* and fat oxidation and between *Dorea* and economy test following LCHF intervention.

Keywords: stool microbiome; diet; race walkers; training

Chapter 5

Chapter 5 Diets with different prebiotic content differentially alter the gut prokaryote and fungal microbiota

5.1 Abstract

Background: Prebiotics are defined as materials that, upon ingestion, can stimulate the growth of "beneficial" gut bacteria. Prebiotics are often consumed as dietary supplements (e.g. as powders or capsules) and while the impacts of this modality have been extensively examined, less attention has been given to diets prepared from foods containing varying amounts of prebiotics, and how differences in the "*in situ"* consumption of prebiotics might affect the entire gut microbiome (i.e. bacteria, archaea and fungi).

Methods: In this randomised, single-blinded crossover study, stool samples were collected from 18 healthy adult subjects after their consumption for three weeks of a diet prepared to provide either a low prebiotic content (LP 1-3 g/day oligosaccharides; 0.50 g/day polyols) or moderate prebiotic content (MP 6-8 g/day oligosaccharides; 3.66 g/day polyols), with a one week washout period between these diets. Stool DNA was extracted and subjected to prokaryotic 16S rRNA, fungal ITS2, and shotgun metagenomic sequencing (MGS). The taxonomic and functional gene profiles observed for the stool microbiota were examined and compared using the QIIME2 and HUMAnN2 bioinformatic workflows, respectively.

Results: Both 16S rRNA and MGS data analysis showed that *Bifidobacterium* spp. were significantly increased with the MP diet, with commensurate reductions in bacterial richness. While *Saccharomyces* spp. were the most abundant fungal group across entire cohort and for both diets, the ITS2 profiles indicate that fungal richness is increased in response to MP. The MGS data also reflected these trends and also showed that genes encoding polyol metabolism were enriched following consumption of the MP diet. Both diets supported distinct inter-Domain networks, indicative of changes in fermentation profiles and gas production.

Conclusions: A diet comprised of foods providing a moderate daily intake of prebiotics in healthy subjects increases the relative abundance of *Bifidobacterium* but reduces bacterial and increases

fungal richness scores. The archaeal and fungal communities in these subjects were also impacted by the prebiotic content of the diet, and these inter-Domain relationships warrant further examination in the context of diet x microbiome interactions affecting gut function.

What are the new findings?

- The MP diet is associated with a significant increase in the relative abundance of *Bifidobacterium* spp. according to both 16S rRNA gene amplicon and MGS data.

- The increase in *Bifidobacterium* spp. in healthy subjects was associated with reduced bacterial richness whereas an increase in fungal richness following consumption of MP diet.

- Genes encoding polyol metabolism and phosphoenolpyruvate:sugar phosphotransferase system (PTS) pathways were enriched following MP dietary intervention.

- Diet-related inter-Domain relationships were more effectively revealed by a combination of taxonomic profiling and MGS, rather than either approach alone.

How might it impact on clinical practice in the foreseeable future?

Diet is widely accepted to exert meaningful impact on the gut bacteria of healthy human subjects, but there is limited understanding of the impacts of diet on the non-bacterial members of these communities. These studies show there is are plausible links between the prebiotic content of the diet and the gut mycobiome and methanogenic archaea in healthy individuals, and the characterization of these inter-Domain relationships are relevant to improving our understanding of the consequences of diet on gut function.

5.2 Introduction

According to the latest definition proposed by Gibson et al., prebiotics refer to "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (137). While the prebiotic concept has expanded to include non-carbohydrate compounds (e.g. plant polyphenols) as well as the site of the beneficial effects of prebiotics beyond the colon; the most common and diverse class of prebiotics are indigestible short and medium chain-length oligosaccharides, which pass undigested through the upper GI tract and impart their impacts on the colonic microbiota, and particularly, bacteria such as Bifidobacteria and *Lactobacillus* Whereas probiotics includes live microorganisms such as Bifidobacteria and *Lactobacillus*, prebiotics are substrates that serve as nutrients for beneficial microbes residing in the gut (20). Certain soluble fermentable fibres can have prebiotic effects if they are selectively utilised by the host microbiota and promote health (41,138). However, not all dietary fibres can be classified as prebiotics. A prebiotic should elicit a biased response towards health-promoting bacteria unlike dietary fibres such as pectins and xylans that favours the growth of a wide range of bacteria (137).

Prebiotics such as inulin-type fructans (ITF), as well as arabinoxylan oligosaccharides (AXOS), fructooligosaccharides (FOS) and galactooligosaccharides (GOS) have all been reported via individual studies to increase the abundance of Bifidobacteria and other lactic acid bacteria (87,88,139–141). Furthermore, the bifidogenic effects of ITF and AXOS are attributed to the ability of Bifidobacteria to take up and degrade larger oligosaccharides, and cooperative relationship with microbe(s) capable of cross feeding on its fermentation products and monosaccharides (142–144). Such a cooperative interaction of *Bifidobacterium* with butyrate producers such as *Anaerostipes* spp. and *Eubacterium* spp. would also help explain how AXOS and ITF positively affect colonic butyrate concentrations (144,145).

Although the effects of prebiotic "fibre" interventions such as inulin, FOS, GOS, AXOS on the gut microbiota have been relatively well studied, a recent systematic review and meta-analysis by So et al. (146) compared studies on fibre and prebiotics supplementation in healthy subjects, and has provided some interesting insights. This systematic review was based on 64 studies involving 2099

healthy human participants, and met the inclusion criteria of: a) randomised controlled trial (RCT), cluster RCT, or quasi-RCT; b) studies involving healthy adult participants (≥18 y of age); c) dietary interventions aimed at increasing fibre intake; d) studies that included a placebo for supplement interventions and either a low-fibre control or habitual diet group for food interventions as comparative groups and; e) studies that measured fecal microbiota related outcomes at the end of intervention. Based on this meta-analysis, So et al. (146) concluded that while prebiotics (and particularly, fructans and GOS) consistently increase the abundance of Bifidobacteria, a positive effect on other key "beneficial" bacterial taxa, including *Roseburia* spp., *F. prausnitzii*, *E. rectale*, and *Ruminococcus bromii*, as well as SCFA concentrations, was inconsistent , suggesting that the impact of prebiotics on the human gut microbiota is quite narrow and the beneficial effects arise from microbes other than the ones being routinely studied.

Although much is reported about the effect of prebiotics on human gut bacterial communities, there appears to be little information about the impacts of different prebiotic carbohydrates on the gut archaeal and fungal communities in health and disease. Fungal communities rumen microbiomes communities have long been examined via the PCR-based amplification of Internal Transcribed Spacer (ITS) regions between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA gene (23, 24) and these approaches have recently been adapted for use with human stool samples (53). In that context, recent studies suggest that the Ascomycota, Basidiomycota and Zygomycota phyla are dominant in the gut of healthy human subjects (149,150). A study by Hoffmann et al. (61) in 98 healthy adults found that *Candida* relative abundance was strongly correlated with the recent (short term) consumption of dietary carbohydrates and negatively correlated with consumption of a diet with high protein and fat content. Furthermore, multiple studies conducted on both the fecal and mucosal tissue samples have reported an increase in the abundance of *Candida* spp. and a decrease in fungal diversity in Crohn's disease patients (53–56). Few studies have revealed different fungal profiles in the healthy human gut with different types of diet (62,151). *Candida* was observed in samples from both the healthy vegetarian individuals and those on a western diet (62,151). Fungi *Fusarium*, *Malassezia*, *Penicillium* and *Aspergillus* were detected in more than 60% of the fecal samples collected from healthy vegetarian individuals whereas rarely detected in samples collected

from healthy individuals on a western diet suggesting the influence of diet on the gut mycobiome. Dysbiosis of the intestinal fungi have been associated with intestinal diseases such as IBD, colorectal cancer (CRC), colitis and obesity (24,28,33–35). Reduction in fungal diversity have also been reported in IBS subjects as compared to the healthy subjects (52). In summation, gut mycobiota seems to be an important participant in gut physiology and homeostasis and more in-depth research needs to be done to better understand the role of fungi in host health and how diet can be used to manage gut mycobiota.

The human gastrointestinal tract, and the large bowel in particular, is also colonised by methanogenic Archaea (belonging to phylum Euryarchaeota), that produce methane as the end point of their anaerobic respiration. *Methanobrevibacter smithii* is the most prevalent and numerically abundant species, with *Methanosphaera stadtmanae* and the *Methanomassiliiococcales* found in lower numbers and are less prevalent (152,153). *Methanobrevibacter* spp. favours the hydrogen-dependent reduction of carbon dioxide and/or formate to methane (154) whereas the latter species appear restricted to the use of methanol (*Methanosphaera* spp.) and/or methylated amines *(Methanomassiliiococcales* (155). As such, the populations of the methanogenic archaea will be governed by the provision of both hydrogen and specific carbon sources that are by-products of bacterial fermentation; suggesting that diet could have a potential impact on their abundance. However, the literature detailing the effect of diet on human gut methanogenic populations is scant. Hoffman et al. (61) reported in their study of healthy human subjects that there was a positive correlation between the relative abundance of *Methanobrevibacter* spp. and the intake of dietary carbohydrates, and accordingly, a negative correlation with dietary intake of proteins and amino acids. Additionally, the abundance of *Methanobrevibacter* spp. could be increased by a change in the subject's habitual diet to include a greater amount of carbohydrates (61). In the human gut, when H_2 accumulates due to bacterial catabolism, archaeal growth is stimulated associated with the utilisation of H_2 into methane (156). Thus, methanogenic archaea may play a key role via interspecies hydrogen transfer to sustain bacterial fermentation, similar to that observed in other animal gut systems (157–159).

Methanogen prevalence and positivity is characteristic of persons deemed to possess slower gastrointestinal motility, irrespective of their health status or type of measurement used (160). Conversely, the relative abundance and prevalence of *Methanobrevibacter* spp. is frequently reported to be dramatically reduced in CD and UC patients from Western countries (161); although one small study has reported an increase in *Methanobrevibacter* spp. abundance in Indian IBD patients (162). Methanogen positivity is more frequent in patients suffering from IBS-C subtype, but infrequent in patients with IBS-D. Furthermore, recent studies suggest that *Methanosphaera* spp. are not only increased in IBD patients, but unlike the other two groups of methanogenic archaea, stimulates a strong proinflammatory response from gut-associated and peripheral immune cells (163,164). While the mechanisms underlying these pathologies are still unclear, these findings support the contention that diet x methanogen interactions may be relevant in terms of both gut function and host response via both arms of the immune system.

In summary, there are only a small number of studies that have collectively examined the bacteria, mycobiome and archaeal populations within the human large bowel; although these studies suggest all three Domains of microbial life can affect the health and well-being of their host. I believe a better understanding of how diet influences these communities, and their interactions, is now possible by an integrated use of methods that provide taxonomic (i.e. 16S rRNA and ITS2) and functional (i.e. shotgun metagenomics sequencing) information of these communities. Here, I have applied this collection of methods to examine the effects of natural prebiotic diets on the gut bacterial, fungal and archaeal communities of healthy adult Australians, recruited as part of a randomised single-blinded crossover study of the impacts of these diets on other indices of colonic health. I present here the analyses of these data from the context of diet x microbiome interactions, and more specifically, how the amount and/or form of the daily intake of prebiotics impacts the diversity and inter-Domain relationships among the large bowel microbiota.

The aims of the current research are to characterize the changes in bacterial microbiota of the subjects in response to prebiotic diets through 16S rRNA analysis, study the fungal mycobiome of these subjects in response to the prebiotic diets using ITS2 sequence analysis, gain deeper insight into the taxonomic composition and metabolic potential of the gut microbiome by whole metagenome shotgun sequence analysis and studying how the natural prebiotic content of the diet affects the dynamic interrelationships of all three domains of microbial life (Bacteria, Archaea and Fungi) resident in the human gut.

5.3 Materials and methods

5.3.1 Study design and sample collection

This research has been done in collaboration with colleagues at the Translational Nutrition Science group in the Department of Gastroenterology, Central Clinical School, Alfred Hospital and Monash University (under ethics approvals MUHREC CF14/2904, 2014001593 and UQHREC 2015000317). The aim of the study was to examine the responses of healthy adults to high fibre diets (~30 g/day) prepared to provide either a low (1-3 g/day oligosaccharides; 0.50 g/day polyols) or moderate (6-8 g/day oligosaccharides; 3.66 g/day polyols) intake of prebiotic carbohydrates (hereafter referred to as LP and MP, respectively, Table 5.1). The healthy volunteers (n=18) were recruited by the Monash team and the study was conducted via this site as a randomised, single-blinded crossover study, with the LP and MP diets provided for three weeks and with a one week “washout period” in between (Figure 5.1). All the meals of the two diets LP and MP were prepared by university chefs at Monash University. The meals were delivered as frozen meals to the participant's homes weekly for the three-week interventions. Food diaries were recorded, and compliance was measured from returned food diaries. These diaries were checked by the research dietician. SCFA measurements were also made by the Monash team. Briefly, SCFA concentrations were made in triplicates from the fecal contents by gas chromatography. Total SCFA (sum of SCFA) and individual SCFA (acetate, propionate, butyrate) concentrations were reported as μmol/g of fecal matter. SCFA data has been used in Chapter 6. For microbiota analyses, stool samples were collected from the subjects using the OMNIgene stool

collection kit and provided as part of their site visits at entry, and after each of the three-week dietary intervention periods.

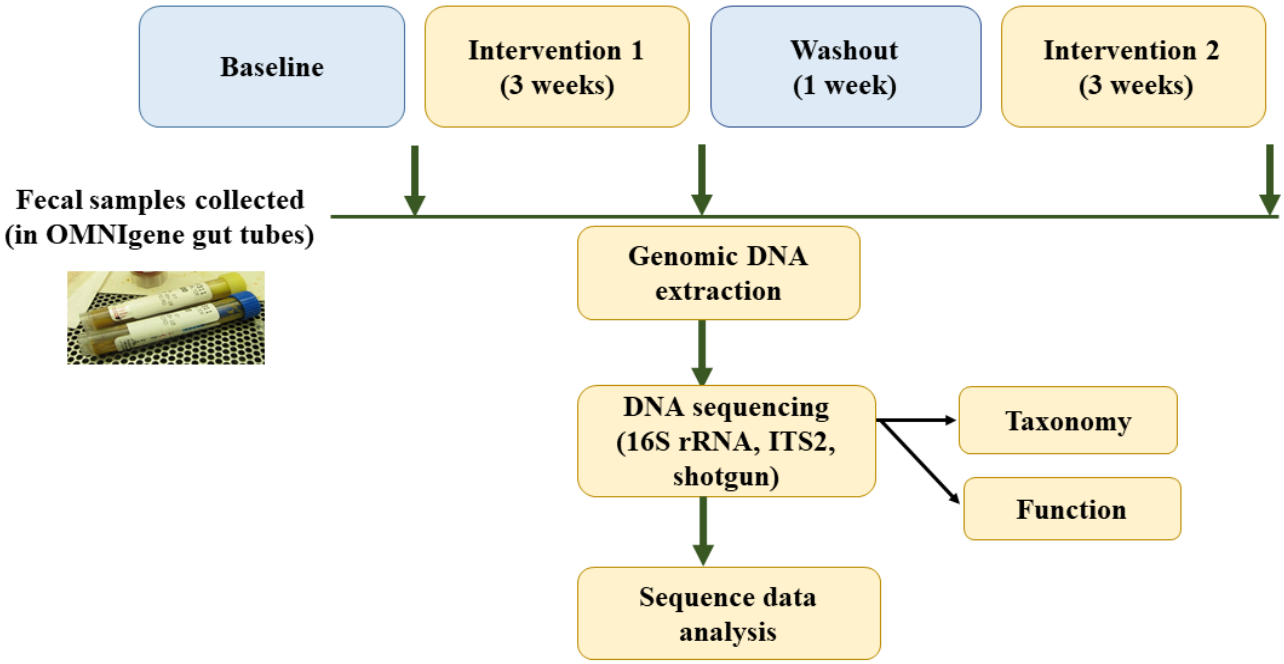

Figure 5.1 showing the study design.

Table 5.1 Compositional attributes of the LP and MP diets consumed during the study period

	Low Prebiotic (LP) diet	Moderate Prebiotic (MP) diet	p-value
Sample size	18	18	
Energy (average kJ/day)	8419.38 ± 1430.87	8432.44 ± 1346.58	0.97
Protein (g)	99.78 ± 14.40	99.30 ± 15.43	0.92
Fat(g)	78.43 ± 18.40	72.42 ± 14.65	0.28
Saturated fat(g)	27.16 ± 6.63	23.97 ± 4.90	0.1
Carbohydrates (g)	206.93 ± 32.95	220.31 ± 34.16	0.24
Sugars (g)	84.84 ± 16.77	93.67 ± 16.36	0.12
Dietary fibre (g)	29.80 ± 5.22	35.77 ± 5.64	0.002
Total oligosaccharides (g)	1.92 ± 0.62	7.02 ± 1.54	<0.001
Fructans (g)	1.63 ± 0.60	5.31 ± 1.22	<0.001
GOS (g)	0.29 ± 0.08	1.73 ± 0.39	<0.001
Excess fructose (g)	1.28 ± 0.34	6.84 ± 1.73	<0.001
Total polyols (g)	0.50 ± 0.17	3.66 ± 0.77	<0.001
Sorbitol(g)	0.34 ± 0.11	2.10 ± 0.60	<0.001
Mannitol (g)	0.16 ± 0.10	1.56 ± 0.32	<0.001

Note: Data are shown as mean ± standard deviation

5.3.2 Genomic DNA extraction and 16S rRNA library preparation and sequencing

Stool DNA extractions, preparation of the 16S rRNA bar-coded amplicon libraries and sequencing workflows followed the protocols described in Chapter 2.

5.3.3 ITS2 sequencing and library preparation

Fungal library preparation and sequencing was performed at the Australian Centre for Ecogenomics (ACE) according to the methods described by Sokol et al. (53) which targets the Internal Transcribed Spacer 2 (ITS2) using primers 5'-GTGARTCATCGAATCTTT-3' and 5'-GATATGCTTAAGTTCAGCGGGT-3'. The ITS2 amplicon library preparation protocol (METABIOTE®, Genoscreen, Lille, France) was used and stool DNA was used as the template for PCR under the following conditions: 94°C for 2 min, 35 cycles of 15 sec at 94°C, 52°C for 30 sec and 72°C for 45 sec, followed by 7 min at 72°C. The resulting PCR amplicons were purified using Agencourt AMPure XP Beads (Beckman Coulters, Brea, CA). Purified DNA was indexed using

unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002) and a second PCR of 9 cycles was then conducted under similar PCR conditions with Q5 Hot Start High Fidelity 2X Master Mix. Indexed amplicons were pooled in equimolar concentrations and sequenced on MiSeq Sequencing System (Illumina) with V.3 (300 bp) chemistry according to the manufacturer's protocol.

5.3.4 16S rRNA & ITS2 sequence bioinformatics analysis using QIIME2

The bioinformatics analysis of the 16S rRNA and ITS2 amplicon sequence data presented here have been processed using QIIME 2 (ver. 2018.8.0) (165), so those details different from those provided in Chapter 2 are described here. The raw sequence reads produced from the 16S rRNA and ITS2 amplicon libraries were processed with FastQC (version 0.11.4, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and then cutadapt (version 1.17) was used to remove those reads lacking primer sequences (166). Low quality reads were removed using Trimmomatic v0.32 (167) set with a sliding window of four bases using a Q-score criteria. All reads were then hard trimmed to 250 bases, and any with less than 250 bases were removed. The forward reads remaining following this procedure were then processed via QIIME2 for feature selection, abundance calculations and taxonomy assignments. First, the reads were de-noised (i.e. filtered, dereplicated, and chimeras identified and removed) using DADA2 (--p-trunc-len = 0) (168) and the relative frequencies of each resulting "representative feature sequence" were calculated. The taxonomies for these representative feature sequences were assigned by BLASTn alignments using the classify-consensus-blast function with default parameters, for each sequence against either the non-redundant SILVA (for 16S rRNA profiling: release 132, clustered at 99% identity, (169)) or UNITE (for ITS2 profiling: version 7.2, clustered at 99% identity (170)) databases. This feature table was then filtered to remove any sequences with a relative abundance of less than 0.01%, as well as those also deemed present in the control samples (i.e. reagents only), to produce a "filtered feature" table. Samples with less than 1000 reads were also removed from further analysis, of which 4 samples were excluded from the ITS2 dataset. The filtered feature table was rarefied to 16878 reads for 16S rRNA and to 1745 reads for fungal ITS2 sequence analysis.

5.3.5 Shotgun library preparation and sequencing

Shotgun MGS library preparation and sequencing was conducted at UQ-ACE. Aliquots (10 ng) of genomic DNA from each sample was used for library construction with the Nextera DNA Flex Library Preparation Kit (Illumina #20018705) and bead clean up via the Mantis Liquid Handler (Formulatrix) and Epmotion (Eppendorf # 5075000301) automated platforms. Each library was then quantified, and QC was performed using the Quant-iT^{TM} dsDNA HS Assay Kit (Invitrogen) as per the manufacturer's protocol. The libraries passing QC were then pooled at equimolar amounts (2 nM) to create a sequencing pool. The library was then sequenced on the NextSeq500 (Illumina) using NextSeq 500/550 High Output v2 (2 x 150bp paired end) chemistry.

5.3.6 Shotgun metagenome sequence (MGS) data analysis

The workflow I have developed and used for these analyses is illustrated in Figure 5.2, and a brief description of the different modules is presented in Table 5.2. Illumina sequence adaptors were trimmed from the raw sequences using qc.illumina.py script and then concatenated the forward and the reverse trimmed reads into a single FASTQ file per sample. Human reads were removed from all the samples by mapping the reads against the human (hg19) database using bowtie2 (version 2.2.1) (171). Sequences that were unmapped to the human genome were used for taxonomic and functional pathways profiling using the HUMAnN2 pipeline (172). Taxonomy profiles predicted from the MGS data were produced using MetaPhlAn2 (173), and functional pathways were assigned to the MGS data using ChocoPhlAn as the reference pangenome database and UniRef50 as the protein reference database (i.e. assignments made for those reads with >50% identity). The output tables for each sample (gene families, pathway abundance and pathway coverage) were then joined using the humann2_join_tables.py function, normalised to copies per million (cpm) values and then reclassified into Gene Ontology (GO) and Protein Families (Pfam) categories, using the humann2_renorm_table, humann2_regroup_table and humann2_rename_table utility scripts. The resultant table was exported to QIIME and Calypso for downstream statistical analyses. FastQC was used to check the quality and Nonpareil was used to estimate the coverage of the metagenomic data (174,175). All the commands and the shell scripts used for these analyses are provided in the Appendix section.

5.3.7 Recovery and comparative genomics of Metagenome Assembled Genomes (MAGs)

Trimmomatic v0.32 was used to trim adapter sequences and remove low-quality bases from the raw sequence shotgun sequence reads (167). These quality checked paired-end reads were then assembled using MEGAHIT (version 1.1.1) (176), and BamM (version 1.7.3) was used for read mapping and the generation of contiguous sequences (contigs). MetaBAT (version 0.32.4) was then used for binning and to recover the population genomes or Metagenome Assembled Genomes (MAGs) (177). CheckM (version 1.0.7) was finally used to check the quality of the recovered MAGs by estimating the percent contamination and completeness scores (178), and MAGs greater than 80% completeness and less than 10% contamination were used for comparative genome analysis. The MAGs were uploaded to Pathosystems Resource Integration Center (PATRIC) and the Similar Genome Finder service within PATRIC was used for taxonomic identification of the MAGs. Similar genome finder service compute genome distance estimation using MinHash (Mash) algorithm based on hierarchical clustering to the public genomes that are available on PATRIC (179,180).

MAG alignments were constructed using progressiveMauve (181,182) which is a new method to align two or more genomes that have undergone rearrangements due to recombination. As such, progressiveMauve can accurately align regions that are conserved in some, but not all, of the genomes being compared; and it can also be applied to larger number (and more divergent set) of genomes than the original Mauve algorithm. The MAGs were also uploaded onto the software platform EDGAR for generation of an Average Nucleotide Identity (ANI) matrix and genome comparison (183). The ANI matrix provides a measure of nucleotide-level genomic similarity and compares genetic relatedness among prokaryotic genomes (184). I also used PATRIC to identify the carbohydrate degrading enzymes for each MAG. These enzymes were identified using the genome annotation service available within PATRIC which generates a features.txt file listing all the features of the genome.

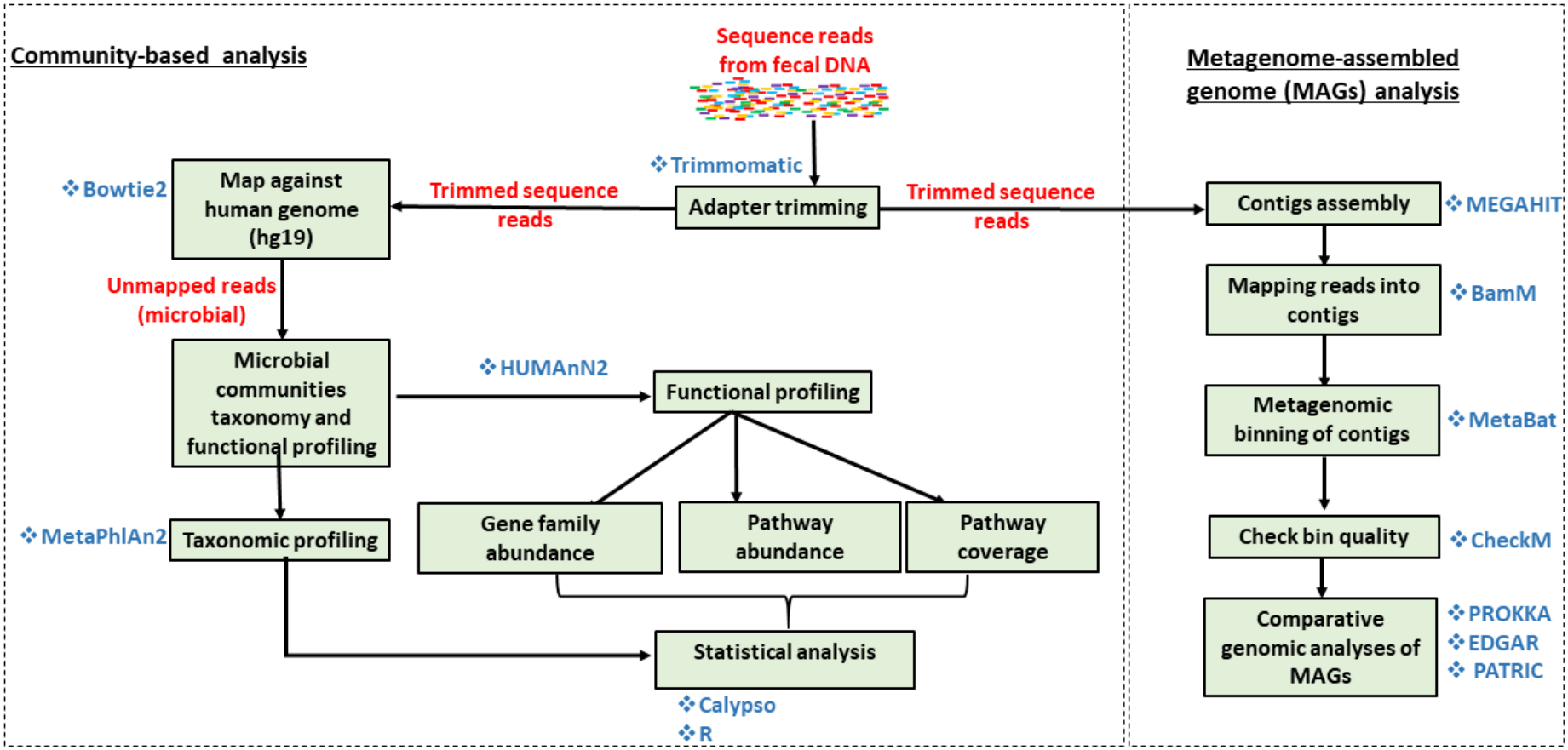

Figure 5.2 Bioinformatics workflow developed for processing the shotgun metagenomics (MGS) data; developed in collaboration with Mr. Jing Jie. MGS data can be processed in two separate workflows as shown above: **(Left panel)** Community-based analysis that allows for the taxonomic and functional profiling of the microbial reads through HUMAnN2 pipeline after quality trimming the raw reads using Trimmomatic and removal of the human host reads using Bowtie2. **(Right panel)** The MGS data is also used to generate Metagenome Assembled Genomes (MAGs) where

the trimmed sequence reads are assembled using MEGAHIT, mapping the reads using BamM, then generating metagenomics bins using MetaBat and finally quality checking of the recovered bins or MAGs using CheckM. Only high-quality MAGs (completeness ≥ 80% and contamination ≤ 5%) were then annotated using Prokka and comparative genome analysis performed using EDGAR and PATRIC.

Table 5.2 Brief description of the modules used for the MGS data analysis

Work module	Reference	Brief description
Trimmomatic	(167)	Trimming tool to remove Illumina sequence adaptors
Bowtie2	(171)	Fast gapped read alignment tool to align against hg19 database for removing human reads from the samples
BamM	Github (a)	BamM is used for mapping multiple sequencing libraries against an assembly
MEGAHIT	(176)	Ultra-fast NGS de-novo assembler for assembling large and complex metagenomics data
MetaBat	(177)	Tool for reconstructing single genomes from complex microbial communities
CheckM	(178)	Tool to check the quality of genomes recovered from isolates, single cells or metagenomes
PATRIC	(185)	Tool for curation of the genomic data or annotating the genomes
Prokka	(186)	Tool for annotation of the genomes
Metaphlan2	(173)	For taxonomic profiling of the metagenomics shotgun data
HUMAnN2	(172)	For functional profiling of the metagenomics and metatranscriptomics data

Github link: (a) http://ecogenomics.github.io/BamM/

5.3.8 Statistical analyses

Calypso (version 8.18), Prism8 and the R package were used for all statistical analyses, and all the scripts and the R-based code used are provided in the Appendix. The Shapiro-Wilk test confirmed the taxonomic count data were not normally distributed, and as such, were subjected to a square-root transformation prior to repeated-measures statistical analysis in Calypso version 8.18 (187). Spearman's correlations were also calculated from the non-normally distributed data, the correlation plots were made using the *corrplot* package, and the adjusted *p* values were calculated using the *p-adjust* function in R. The threshold for statistical significance was set to $p < 0.05$ for all the analyses. The corrections for multiple testing by false discovery rate (FDR) with values < 0.05 were also considered to be statistically significant in mixed effect linear regression (MELR) and correlation analysis.

5.4 Results

5.4.1 Bacteria/Archaea community profiles based on 16S rRNA amplicon sequencing

Table 5.3 shows the Phylum-level assignments and prevalence rates of stool bacteria present when subjects consumed either the BL, LP, or MP diets. In general terms, the profiles are unremarkable, comprised predominantly of lineages affiliated with the Firmicutes, Bacteroidetes and Proteobacteria. Members of the Actinobacteria, Euryarchaeota, Lentisphaera, Verrucomicrobia and Tenericutes were found at lesser relative abundances but high prevalence rates; while Archaeplastida, Opisthokonta, SAR, and Synergistes were detected in only some of the samples. From these profiles, I have selected the 20 most abundant bacterial genera from across the cohort, and their relative abundances with the BL, LP and MP diets, are illustrated in Figure 5.2. Of these, 10/20 bacterial genera were found in all the samples (*Bacteroides*, *Faecalibacterium*, *Blautia*, *Roseburia*, *Parabacteroides*, *Lachnospiraceae* NK4A136, *Ruminococcus1*, *Fusicatenibacter*, *Subdoligranulum* and *Collinsella).* The most abundant genus was *Bacteroides*, frequently comprising >20% of the total bacterial community profile, although 9/50 samples possessed a much lower relative abundance (1%-7%). The *Prevotella* spp. could be divided into two distinct groups: *Prevotella* 7 and *Prevotella* 9 but were detected in only

32% and 22% of the samples respectively. Interestingly, the relative abundance of *Prevotella* 9 was notably greater (11%-21%) in those samples found to possess with low *Bacteroides* spp. Another 10/50 samples were found to have *Prevotella 7* with the relative abundance in the range of 2.61%-18.30% and notably *Prevotella 9* was not detected in these samples. Taken together, these results are consistent with the "enterotype" concept, with *Bacteroides and Prevotella* communities present. Notably, the top 20 groupings as part of QIIME2 include groupings annotated as "unclassified" (3.05%-21%), "uncultured" (0.44%-3.50%) and "uncultured_bacterium" (0.01%-25.27%). These groups relate to reads that via the SILVA database are either not assigned at the Domain level (unclassified), lack a cultured reference isolate (uncultured) or can't be assigned beyond the family level of classification (uncultured bacterium). These results suggest that a notable proportion of the microbial communities of these subjects might be considered as microbial dark matter.

Although the relative abundance of Bifidobacteria spp. compared to other bacterial taxa was low and as such, not included in the top 20 genera, there were still notable changes that appears attributable to consumption of the LP diet. In that context, Bifidobacteria spp. were detectable (0.005-4.78% of the rarefied data) in all stool samples following consumption of the BL and MP diets; whereas the genus was detected in only 13/18 samples collected after the LP diet, and also at a lower relative abundance (0.005-1.2%). These findings suggest that the LP diet was rate-limiting to the growth of Bifidobacteria in comparison to the subject's habitual diet (i.e. BL) and the MP diet. Methanogenic archaea were also detectable in some of the stool samples. *Methanobrevibacter* spp. were the most prevalent and abundant, detected in 22/50 samples at 0.04%-7.03%; whereas *Methanosphaera* spp. were detected in 10/50 samples at 0.2%-2.0%.

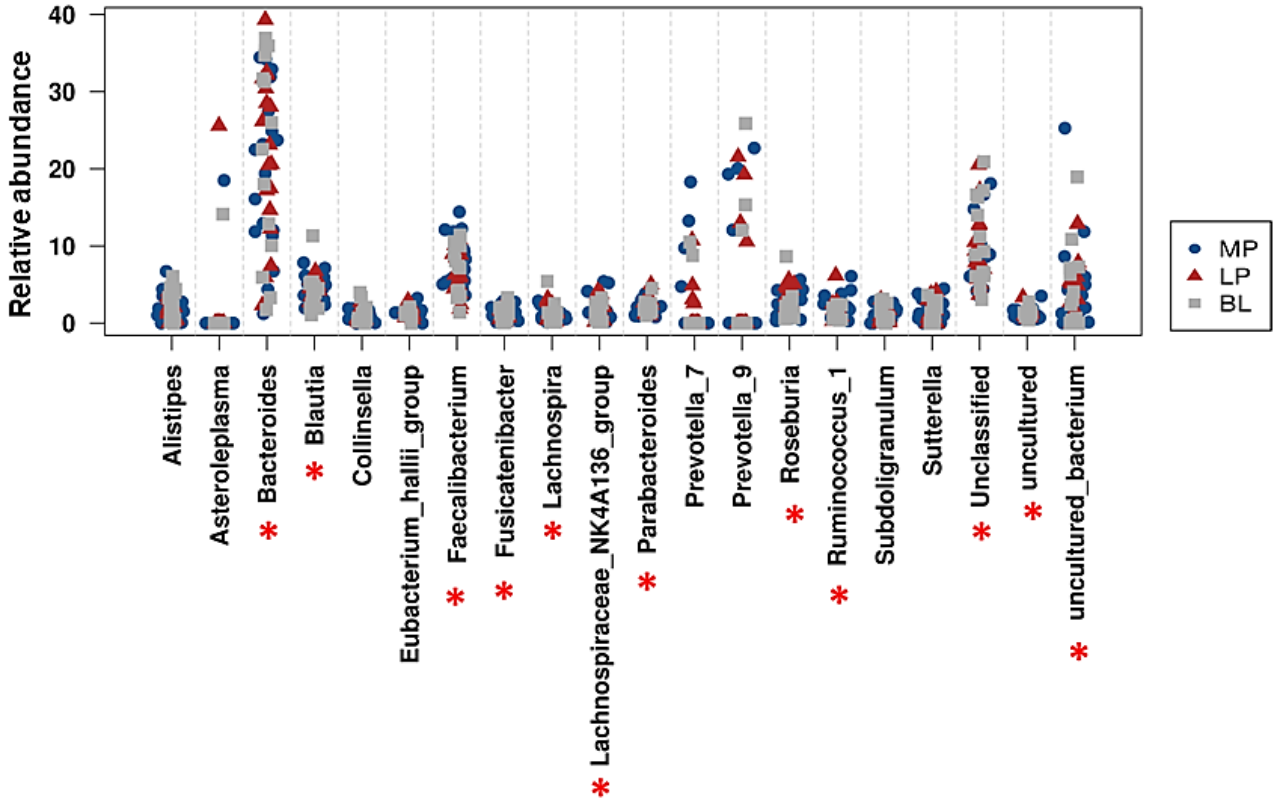

Figure 5.3 The relative abundance scores for the top 20 bacterial "groupings" present in the stool samples collected from healthy adult subjects during consumption of their habitual diet (baseline, grey squares) and following their consumption of the LP (red triangles) and MP diets for three weeks (blue circles). These genera were ranked based on the mean relative abundance scores calculated from all the samples (i.e. n=50), based on the rarefied read counts. Those genera with a prevalence rate of 100% are annotated by *. The groupings annotated as unclassified, uncultured and uncultured bacterium relate to reads that via the SILVA database are either not assigned at the Domain level (unclassified), lack a cultured reference isolate (uncultured) or can't be assigned beyond the family level of classification (uncultured bacterium).

Table 5.3 Total bacterial phyla and top 20 bacterial groupings abundance and prevalence in the samples.

Phylum	**Range (%)**	**Prevalence (%)**	**Genus**	**Range (%)**	**Prevalence (%)**
Actinobacteria	0.62-5.14	100	*Bacteroides*	1.22-39.32	100
Bacteroidetes	24.46-43.67	100	*Blautia*	1.10-11.33	100
Firmicutes	43.00-68.38	100	*Collinsella*	0.03-3.97	100
Proteobacteria	0.47-8.56	100	*Faecalibacterium*	1.4-14.42	100
Verrucomicrobia	0.01-5.1	80	*Fusicatenibacter*	0.05-3.32	100
Lentisphaerae	0.01-3.41	78	*Lachnospira*	0.08-5.41	100
Tenericutes	0.01-7.47	72	*Lachnospiraceae NK4A136*	0.14-5.40	100
SAR	0.01-12.10	48	*Parabacteroides*	0.80-5.02	100
Cyanobacteria	0.01-1.90	44	*Roseburia*	0.32-8.63	100
Euryarchaeota	0.01-7.04	44	*Ruminococcus 1*	0.27-6.19	100
Archaeplastida	0.01-9.11	24	*Subdoligranulum*	0.05-3.13	100
Opisthokonta	0.01-1.24	24	*Eubacterium hallii group*	0.50-3.23	96
Synergistetes	0.24-0.40	6	*Alistipes*	0.06-6.72	94
			Sutterella	0.47-4.47	68
			Prevotella 9	0.01-25.87	32
			Prevotella 7	0.01-18.30	22
			Asteroleplasma	0.01-25.60	16

5.4.2 Fungal community profiles based on ITS2-amplicon sequencing

The fungal communities predicted from the ITS2 amplicon data are shown in Figure 5.4 and Table 5.4. The Phylum Ascomycota was found to be the most predominant with the relative abundance in the range of 54-100% and *Saccharomyces* the most abundant genus, present in all the samples with the relative abundance of 32-100% of the mycobiome. Other members of phylum Ascomycota such as *Cladosporium, Candida, Penicillium* and *Kazachstania* were detected in 52%, 43% ,41% and 13% of the samples respectively. (Figure 5.4 and Table 5.4). In addition to Ascomycota, members of the phylum Basidiomycota were detected in most but not all the samples (80% prevalence, Table 5.4), with *Agaricus* and *Malassezia* the most abundant representatives, detected in 41% and 39% of the samples, respectively; and *Rhodotorula* and *Cystofilobasidium* detected in only 15% and 9% of the samples. In summation, the fungal profiles were dominated by members of phylum Ascomycota and particularly *Saccharomyces*, with lesser amounts of *Agaricus* and *Malassezia* (Basidiomycota) detected in some but not all samples. As such, these profiles are unremarkable relative to those found in other healthy human subjects.

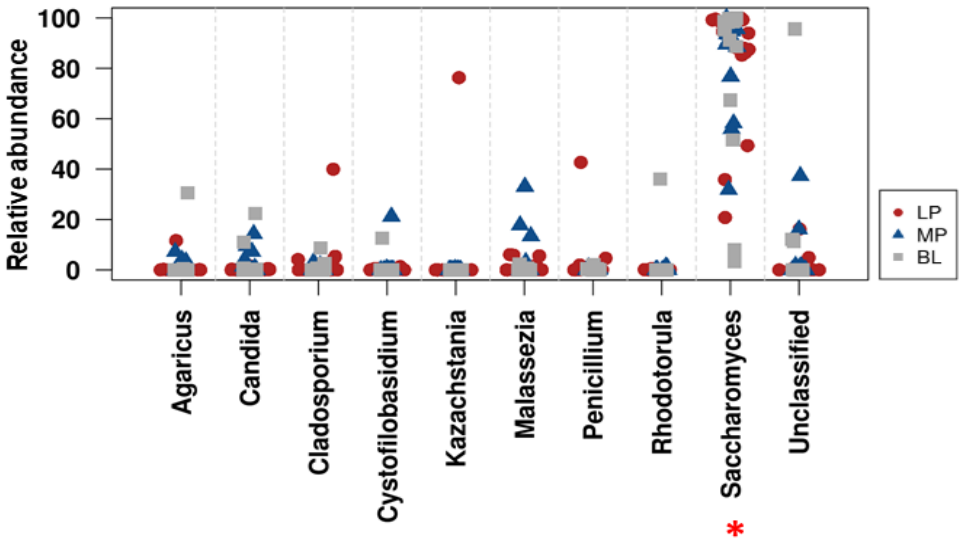

Figure 5.4 The relative abundance scores for the top 10 fungal genera present in the stool samples collected from healthy adult subjects during consumption of their habitual diet (baseline, grey squares) and following their consumption of the LP (red circles) and MP diets for three weeks (blue triangles). These genera were ranked based on the mean relative abundance scores calculated from all the samples (i.e. n=47), based on the rarefied read counts. Those genera with a prevalence rate of 100% are annotated by *.

Table 5.4 Abundance and prevalence of phyla and top 10 fungal genera in the samples

Phylum			Genus		
Taxonomy	**Range (%)**	**Prevalence (%)**	**Taxonomy**	**Range (%)**	**Prevalence (%)**
Ascomycota	54.76-100	100	*Saccharomyces* (A)	31.80-100	100
Basidiomycota	0.11-47.45	80.43478	*Cladosporium* (A)	0.06-40	52
Mucoromycota	0.01	2.173913	*Candida* (A)	0.05-22.41	43
			Agaricus (B)	0.06-30.66	41
			Penicillium (A)	0.06-42.70	41
			Malassezia (B)	0.06-33.12	39
			Unclassified	0.06-95.64	33
			Rhodotorula (B)	0.11-36.10	15
			Kazachstania (A)	0.06-76.27	13
			Cystofilobasidium (B)	0.11-21.26	9

Note: A and B refers to phylum Ascomycota and phylum Basidiomycota respectively.

The relative abundance and the prevalence data obtained from both the PCR based and MGS methods is shown from all the samples collected at BL and post LP and MP dietary interventions. However, the statistical analysis has only been performed with the data collected following the consumption of either the LP and MP diet.

5.4.3 The MP diet differentially affects bacterial and fungal richness scores but not other measures of alpha diversity.

The Shannon diversity measures for the Bacterial/Archaeal and the Fungal communities were not significantly different from each other after consumption of the LP and MP diets (Figure 5.5a and d). This alpha diversity (i.e. within sample) metric is a representation of both the richness and evenness of the respective communities, so I then attempted to compare the bacterial and fungal species evenness and richness scores independently. There were no apparent differences in the evenness scores measured for both domains following consumption of the LP and the MP diets (Figure 5.5b and e). In contrast, the richness scores for Bacteria/Archaea and Fungi groups did show significant and contrasting differences in response to the MP diet, with Bacteria/Archaea richness decreased ($p=0.05$, Figure 5.4c) whereas the fungal richness scores increased with the MP diet. ($p=0.02$, Figure 5.4f). These results suggest that the increase in dietary prebiotic intake has measurable but differential effects on the Bacteria/Archaea and fungal diversity in healthy subjects.

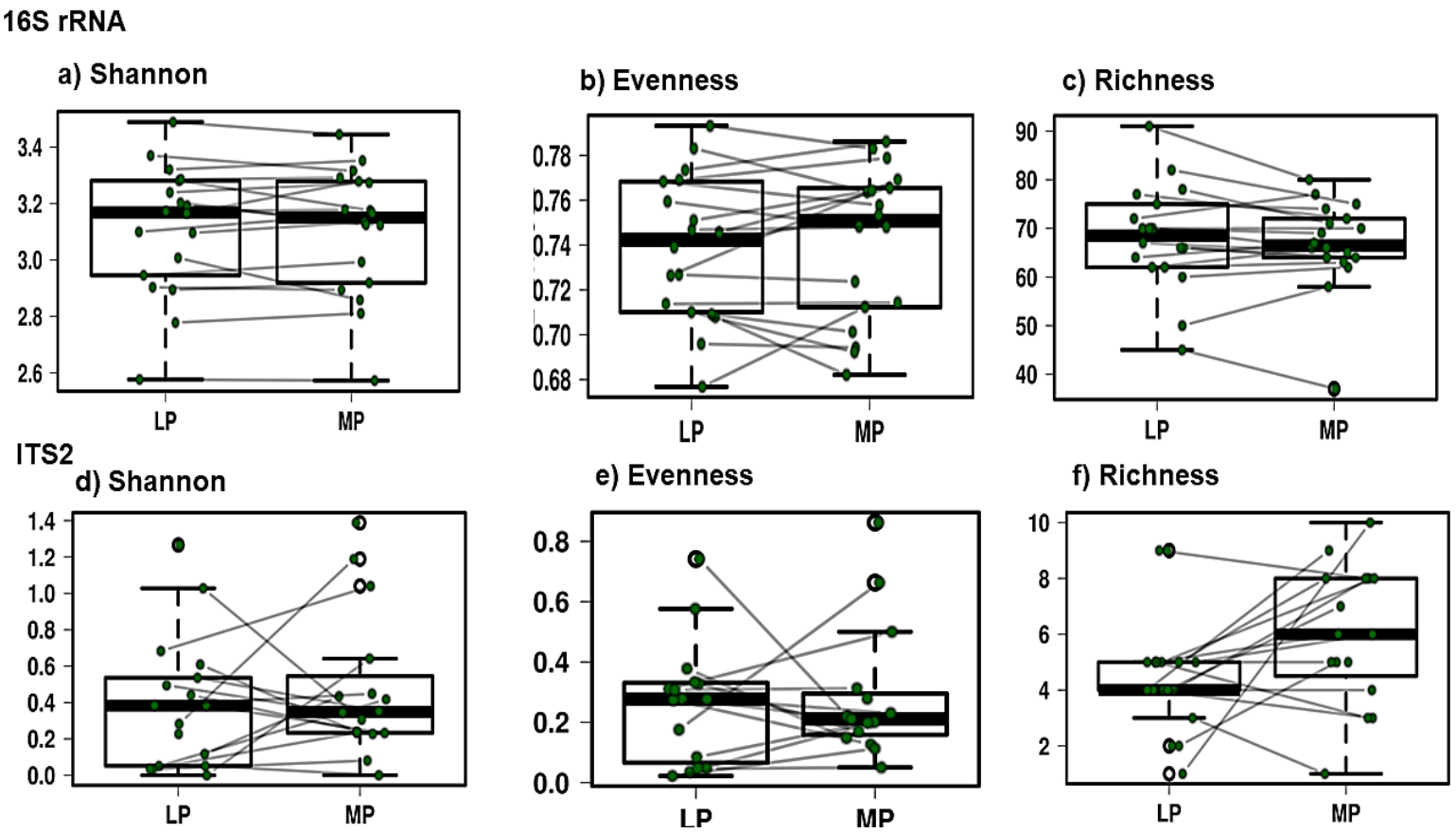

Figure 5.5 Alpha diversity measures calculated for prokaryotic (16S rRNA) and fungal (ITS2) data. There is a reduction in prokaryotic richness (p=0.052) and significant increase in fungal richness (p=0.025) when subjects consumed a diet with moderate prebiotic content.

5.4.4 MELR, LefSe and sPLS-DA analysis shows that both prokaryote and fungal taxa are specifically affected by the prebiotic content of the diet

MELR analysis of the 16S rRNA and ITS2 data comparing the LP and MP diets (Figure 5.6) showed there were significant increases in the relative abundances of *Anaerostipes* ($p < 0.001$, FDR < 0.001) and *Bifidobacterium* ($p < 0.001$, FDR < 0.001) and a decrease in the relative abundance of *Butyricoccus* ($p= 0.001$, FDR$= 0.022$). The MELR analysis of the fungal ITS2 data also identified shifts in response to the prebiotic diets, with consumption of the MP diet associated with increases in the relative abundance of *Candida* ($p=0.003$, FDR$= 0.087$), *Agaricus* ($p=0.033$, FDR$= 0.2$) and *Aspergillus* ($p=0.027$, FDR$= 0.2$) (Figure 5.6). However, these changes in fungal taxa were not statistically significant once FDR correction was applied. Interestingly, the bacterial taxa noted above were not included as part of the top 20 most abundant genera (Table 5.3) suggesting that the lesser abundant/dominant bacterial communities are more sensitive to the prebiotic content diets. In contrast, both *Candida* and *Agaricus* were among the top 10 most abundant fungal genera (Table 5.4).

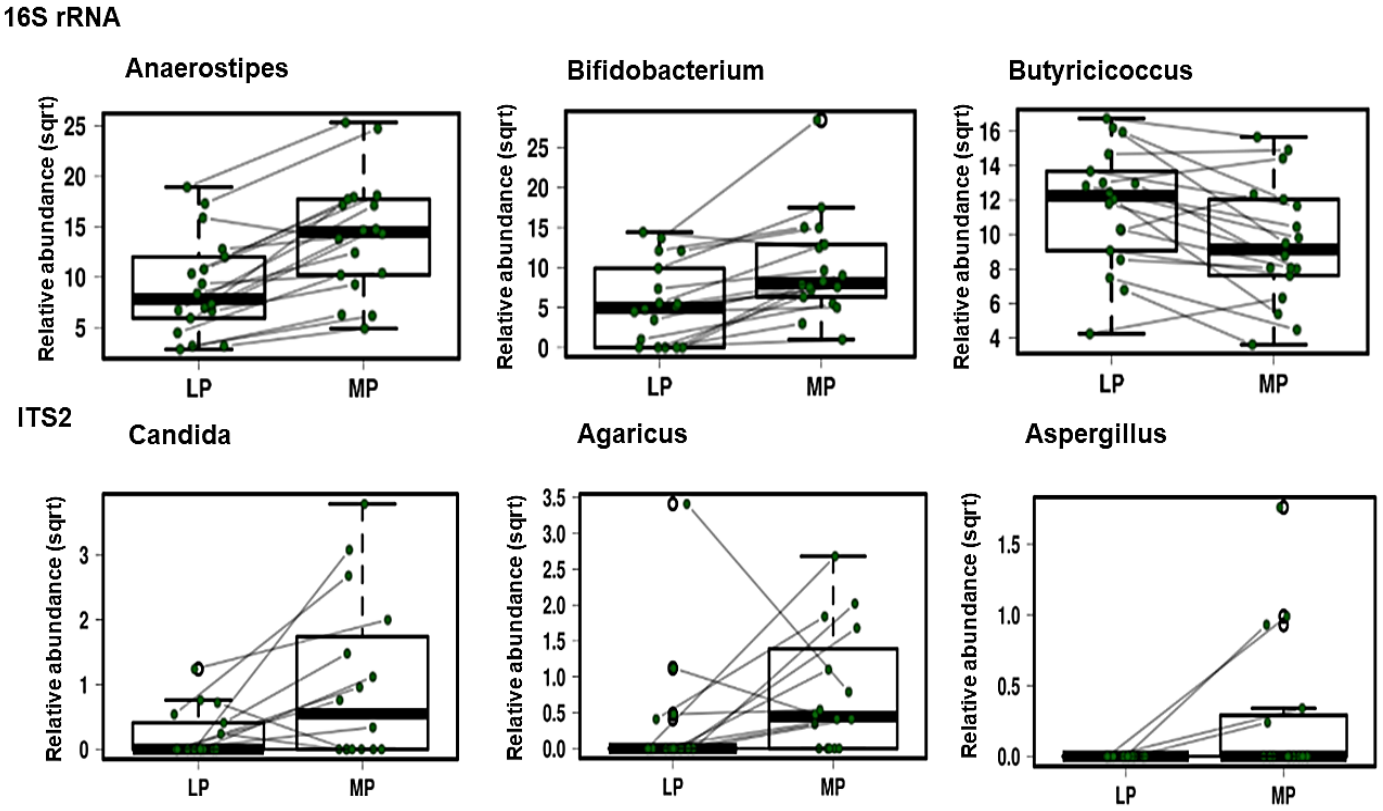

Figure 5.6 Mixed effect linear regression (MELR) analysis of 16S rRNA and ITS2 data identified significant increase in the relative abundance of bacteria *Anaerostipes* ($p < 0.001$, FDR < 0.001) *Bifidobacterium* ($p < 0.001$, FDR < 0.001) and significant decrease in the relative abundance of *Butyricicococcus* ($p = 0.001$, FDR= 0.022) after consumption of the Moderate Prebiotic (MP) diet. MELR also identified increase in the relative abundance of *Candida* ($p = 0.003$, FDR = 0.087) *Agaricus* ($p = 0.033$, FDR = 0.2) and *Aspergillus* ($p = 0.027$, FDR = 0.2) after consumption of the Moderate Prebiotic (MP) diet. Here, sampling time point was set as a fixed effect and individual subject as a random effect. Low Prebiotic (LP) and Moderate Prebiotic (MP) refer to the relative abundance of these taxa measured at LP and after consumption of the MP diet, respectively. Data collected from the same subject are connected by the lines.

LefSe and sPLS-DA analyses were then used in an attempt to identify any additional bacterial and/or fungal taxa that may be considered "discriminatory" for the communities observed following consumption of either the LP or MP diet. The LefSe analysis of the 16S rRNA and ITS2 profiles also showed that *Bifidobacterium* (LDA = 3.52) and *Anaerostipes* (LDA= 3.71) spp., as well as *Aspergillu*s (LDA = 3.79) and *Agaricus* (LDA= 3.77), are discriminatory and enriched in response to consumption of the MP diet (Appendix Figure 7.11). The sPLS-DA analyses suggested that, in addition to *Anaerostipes* and *Bifidobacterium*, *Prevotella 7* and members of *Lachnospiraceae* ND3007 group discriminate between the Bacteria/Archaea communities in response to the MP diet; and *Haemophilus* was discriminatory of the communities observed following consumption of the LP diet (Figure 5.7a).

The sPLS-DA of the ITS2-derived profiles also supported the finding that *Aspergillus* and *Agaricus* are expanded and discriminatory in response to the MP diet, whereas *Byssochlamys*, *Meira* and *Leucosporidium* were discriminatory of the communities present following consumption of the LP diet (Figure 5.7b). Collectively, these results suggest that the prebiotic content of the diets affect both the bacterial and fungal community members in healthy subjects.

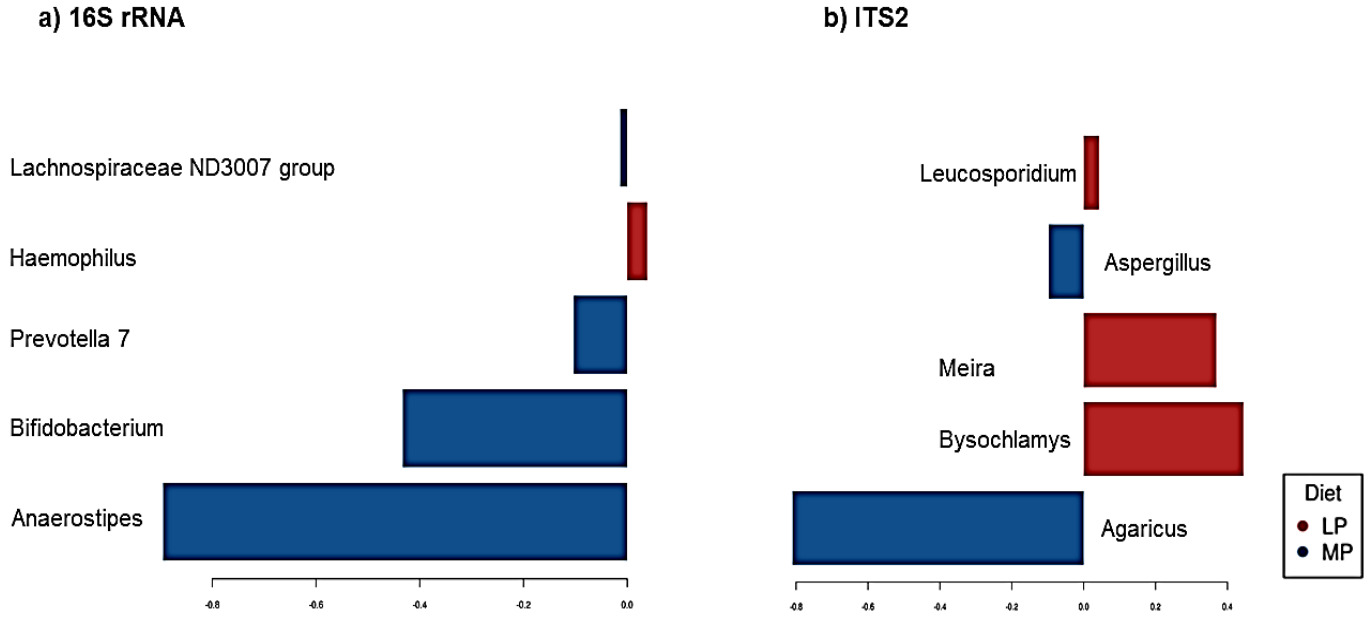

Figure 5.7 Bacterial (16S rRNA) and Fungal (ITS2) genera differentiating the stool microbiota profiles of subjects after consumption of Low Prebiotic (LP; red) and after consumption of the Moderate Prebiotic (MP; blue) diet identified by sPLS-DA.

5.4.5 Stool microbial communities inferred from MGS data

Table 5.5 shows the genus and species level assignments and prevalence rates for the stool bacteria predicted from the MGS data and the 20 most abundant bacterial genera and species from across the cohort, and their relative abundance range from the samples collected at baseline, and after the LP and MP diets, are illustrated in Figure 5.8 and Table 5.5. Briefly, the profiles were similar to those identified from the 16S rRNA data with 9/20 of the most abundant bacterial genera shared between the datasets (*Bacteroides*, *Faecalibacterium*, *Eubacterium*, *Subdoligranulum*, *Roseburia*, *Blautia*, *Coprococcus*, *Dorea* and *Parabacteroides). Bacteroides* spp., were the most abundant with 2-65% of the MGS data assigned to this genus. At the species level, a number of *Bacteroides (B. cellulosilyticus*, *B. dorea*, *B. massillensis*, *B. pectinophilus*, *B. plebeius*, *B. stercoris*, *B. uniformis* and *B. vulgatus* comprised the list of the top 20 species detected in the samples. Furthermore, the variations in relative abundances of *Bacteroides* and *Prevotella 9* observed from the 16S rRNA amplicon profiles was also retained within the MGS data; as those samples with a relatively small abundance of *Bacteroides* spp. (2-11%) possessed a greater abundance of *Prevotella copri* (18-43%), which according to the SILVA database, is a member of *Prevotella 9* grouping. Similar to the 16S rRNA amplicon data, *Faecalibacterium prausnitzii* was found to be present in all the samples and the second most abundant taxon, representing 2-24% of the MGS data from individual samples. Additionally, *Eubacterium* spp. including *E. eligens*, *E. rectale* and *E. halii* were also abundant in the samples; and *Subdoligranulum* spp. and *Ruminococcus obeum* were detected in all the samples (Figure 5.8 and Table 5.5).

In contrast to the 16S rRNA amplicon data, the representation and prevalence of methanogenic archaea within the MGS data was much less, but there was a detectable amount of viral DNA recovered from the samples. *Methanobrevibacter* spp. was detected in fewer samples (18/50 c.f. 22/50) and frequently at a lesser relative abundance (0.2-2.5% c.f. 0.04-7%). The contrast was starker for *Methanosphaera* spp. detected from the MGS data in only one sample (at 0.1% relative abundance) in comparison to 10/50 samples (at 0.2-2.0%) in 16S rRNA amplicon profiles. Viral DNA was detectable within the MGS data from 8/50 samples, the *C2-like virus* most prevalent and abundant (5/8 samples, 0.1-1.1%) with *Flavivirus* (*West Nile virus*) also found in 3 samples (0.2-1.1%).

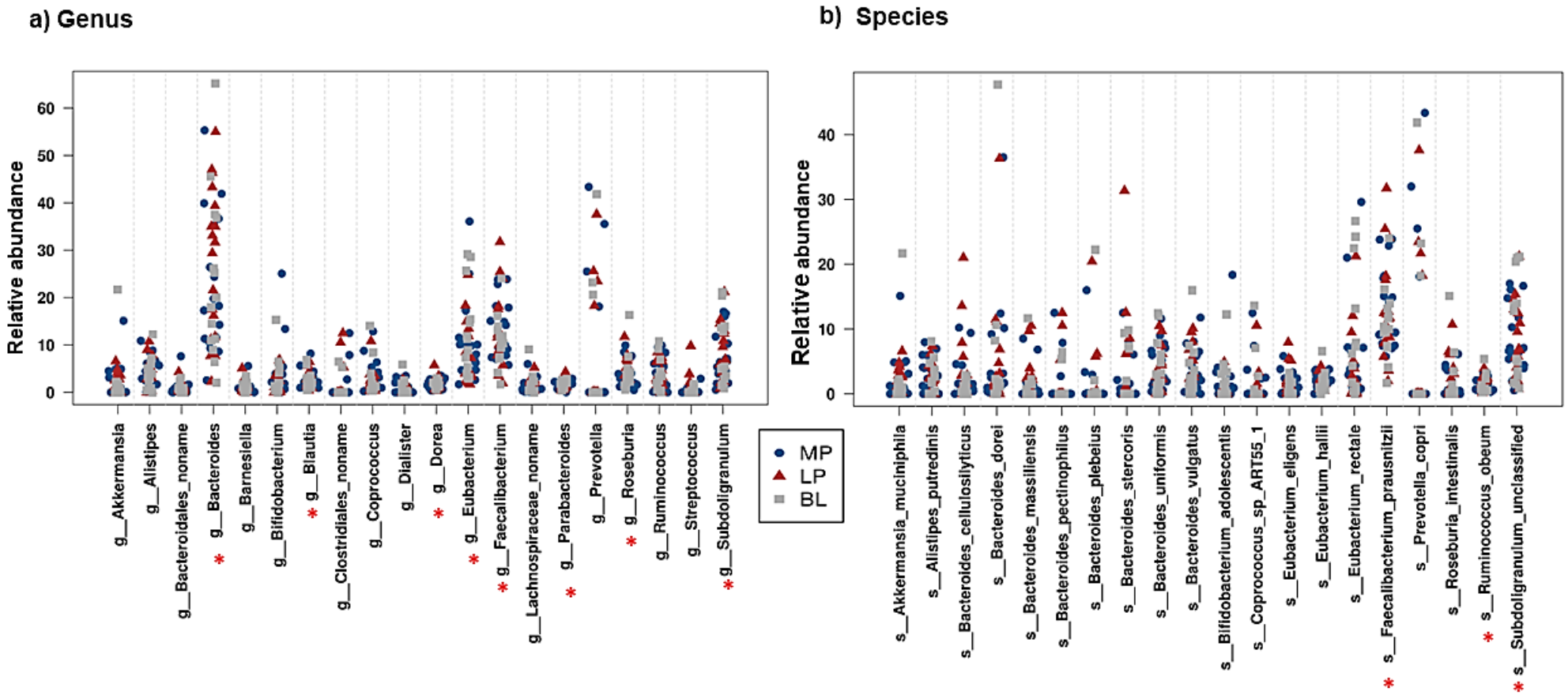

Figure 5.8 The relative abundance scores for the top 20 a) genera and b) species present in the stool samples collected from healthy adult subjects during consumption of their habitual diet (baseline, grey squares) and following their consumption of the LP (red triangles) and MP diets for three weeks (blue circles). These genera were ranked based on the mean relative abundance scores calculated from all the samples (i.e. n=50), based on the Metaphlan2 normalised read counts. Those genera with a prevalence rate of 100% are annotated by *.

Table 5.5 Abundance and prevalence of top 20 genera and species in the samples according to the MGS data obtained from all the samples collected at BL and post dietary interventions.

MGS at genus level			MGS at species level		
Taxonomy	**Range (%)**	**Prevalence (%)**	**Taxonomy**	**Range (%)**	**Prevalence (%)**
Bacteroides	2.03-65.15	100	*Faecalibacterium_prausnitzii*	1.69-31.73	100
Blautia	0.47-8.17	100	*Ruminococcus_obeum*	0.15-5.38	100
Coprococcus	0.23-14.04	100	*Subdoligranulum_unclassified*	0.55-21.22	100
Dorea	0.31-5.74	100	*Eubacterium_hallii*	0.30-6.60	96
Eubacterium	1.70-36.08	100	*Bacteroides_dorei*	0.001-47.71	94
Faecalibacterium	1.70- 31.72	100	*Bacteroides_uniformis*	0.18-12.43	92
Parabacteroides	0.02-4.31	100	*Eubacterium_rectale*	0.02-29.60	90
Roseburia	0.48-16.31	100	*Bacteroides_cellulosilyticus*	0.03-21.01	88
Subdoligranulum	0.55-21.21	100	*Bacteroides_vulgatus*	0.28-15.96	88
Lachnospiraceae noname	0.04-9.04	98	*Eubacterium_eligens*	0.01-7.94	84
Alistipes	0.08-12.19	94	*Bacteroides_caccae*	0.01-5.28	80
Ruminococcus	0.06-10.74	92	*Alistipes_putredinis*	0.13-8.10	70
Barnesiella	0.01-5.55	88	*Roseburia_intestinalis*	0.002-15.09	66
Bifidobacterium	0.10-25.06	84	*Akkermansia_muciniphila*	0.03-21.68	64
Bacteroidales noname	0.05-7.61	78	*Bifidobacterium_adolescentis*	0.02-18.35	64
Streptococcus	0.003-9.80	78	*Bacteroides_stercoris*	0.34-31.35	42
Akkermansia	0.03-21.67	64	*Bacteroides_massiliensis*	0.44-11.67	40
Dialister	0.08-5.87	36	*Coprococcus_sp_ART55_1*	0.65-13.62	26
Prevotella	0.01-43.37	26	*Bacteroides_plebeius*	0.02-22.23	24
Clostridiales noname	0.01-12.50	18	*Prevotella_copri*	18.09-43.36	22

Alpha diversity as measured by the MELR repeated measures test showed no significant difference in the Shannon index and evenness scores between the LP and the MP groups (Figure 5.9a and b); and a trend towards a decrease in bacterial richness in response to the MP diet (Figure 5.9c, p=0.08). However, the MGS data reflected the trends observed from the 16S rRNA data, of reduced bacterial richness in the MP group as compared to the LP group. In summation, based on the 16S rRNA and the MGS data these results suggest that increasing the prebiotic content of the diet has no significant effect on the alpha diversity shannon index and the evenness scores however, there appears to be a reduction in bacterial richness measure with the increased prebiotic content diet.

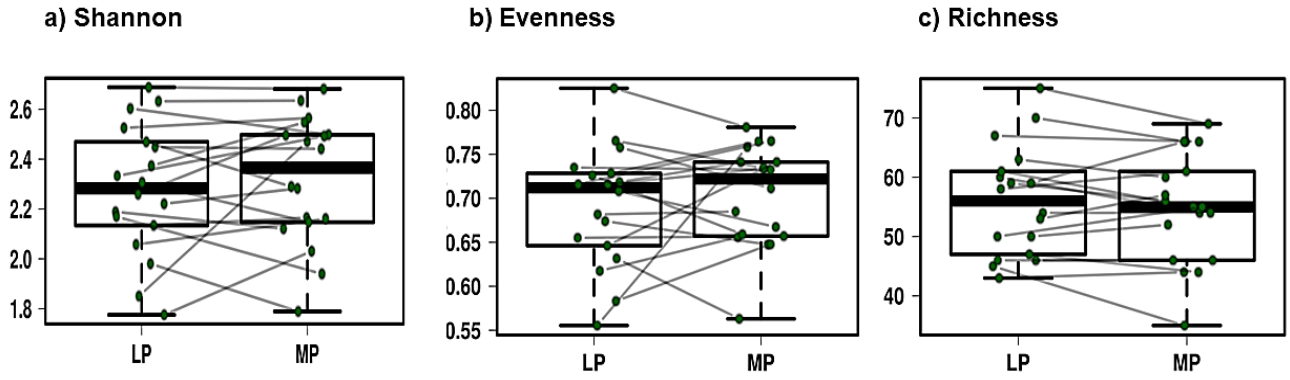

Figure 5.9 Alpha diversity measures calculated for prokaryotes from the MGS data. There is a reduction in prokaryotic richness (p=0.08) when subjects consumed a diet with moderate prebiotic content. Here, sampling time point was set as a fixed effect and individual subject as a random effect. Low Prebiotic (LP) and Moderate Prebiotic (MP) refer to the relative abundance of these taxa measured at LP and after consumption of the MP diet, respectively. Data collected from the same subject are connected by the lines.

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5.4.6 MGS data analysis reflected the trends in bacterial shifts between the LP and MP groups as observed from the 16S rRNA data

MELR analysis from the MGS data revealed significant increase in the relative abundance of genus *Bifidobacterium* ($p < 0.001$, FDR= 0.034) (Figure 5.10). Although analysis from the MGS data identified an increase in the relative abundance of *Anaerostipes* in response to the MP dietary intervention similar to the 16S rRNA profiles, this increase was not found to be statistically significant (p= 0.069, FDR= 1). At the species level, MELR analysis revealed an increase of *Bifidobacterium* species *B. adololescentis* (p= 0.003, FDR= 0.11), *B. longum* (p= 0.01, FDR= 0.17) and an increase of

Eubacterium rectale (p= 0.004, FDR= 0.11) following the consumption of MP diet (Figure 5.10). However, this increase was not statistically significant once FDR corrections were applied.

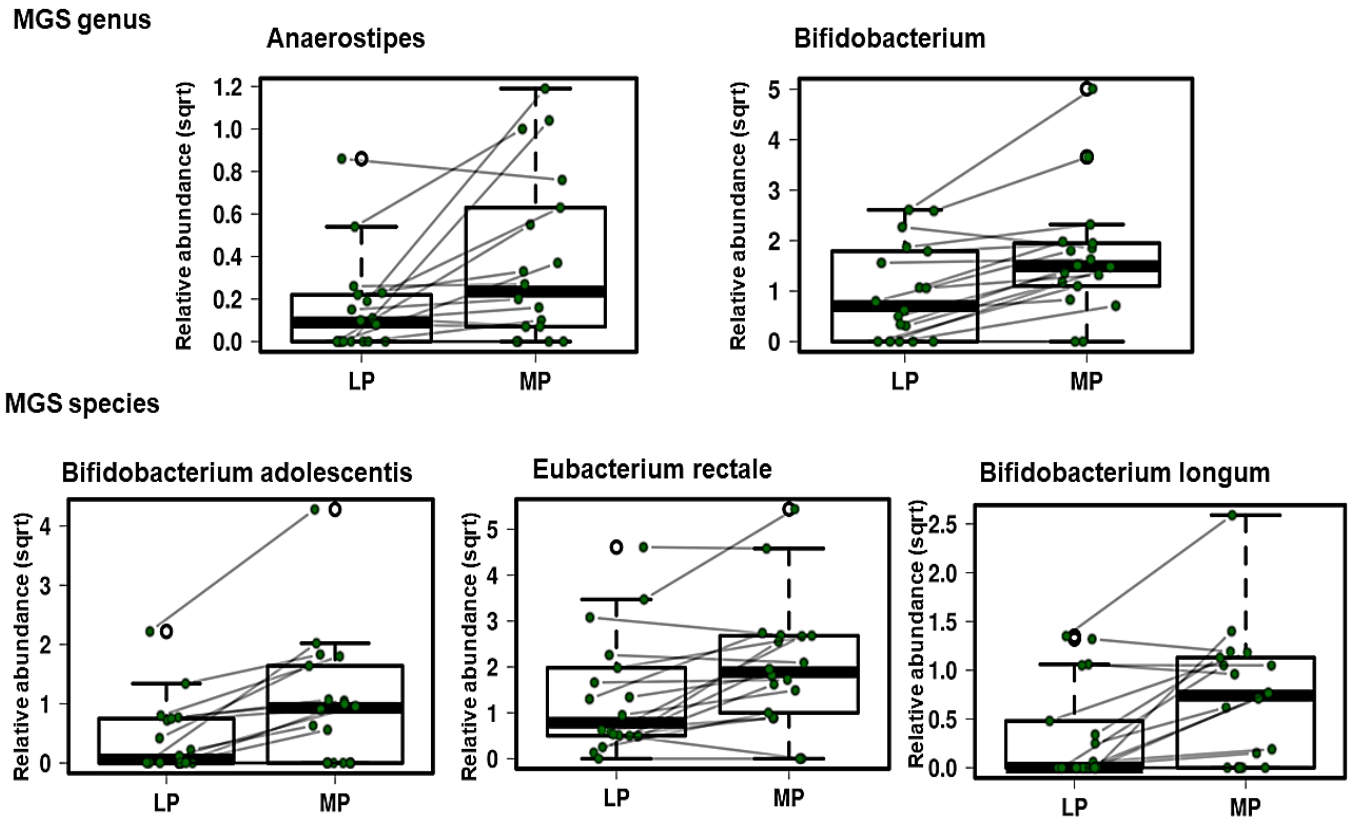


Figure 5.10 Mixed effect linear regression (MELR) of the metagenomic shotgun (MGS) data identified significant increase in the relative abundance of genera *Anaerostipes* (p = 0.069, FDR = 1), *Bifidobacterium* (p < 0.001, FDR=0.034) and species *B. adolescentis* (p=0.003, FDR= 0.11), *E. rectale* (p = 0.004, FDR= 0.11) and *B. longum* (p = 0.01, FDR=0.17) after consumption of the Moderate Prebiotic (MP) diet. Low Prebiotic (LP) and Moderate Prebiotic (MP) refer to the square root transformed normalised relative abundance of these taxa measured at LP and after consumption of the MP diet, respectively.

LefSe analysis for the MGS data identified species *Eubacterium hallii* (LDA=3.58) and *Lachnospiraceae_5_1_63FAA* (LDA = 3.51) to be discriminatory and enriched in response to the MP dietary intervention group as compared to the LP group (Appendix Figure 7.12). Analysis by the sPLS-DA of the same subject samples for MGS data further identified the relative abundance of *Anaerostipes* and *Bifidobacterium* to be enriched and increased in the MP samples and the relative abundance of *Adlercruetzia* to be higher following the LP dietary intervention (Figure 5.11a and b).

At the species level, *Lachnospiraceae bacterium 5_1_63 FAA*, *Eubacterium rectale*, *Eubacterium hallii* and *Anaerostipes hadrus* were identified to be discriminatory and enriched in subjects consuming a MP diet whereas *Adlercruetzia equolifaciens* was differentially affected and enriched when LP diet was consumed.

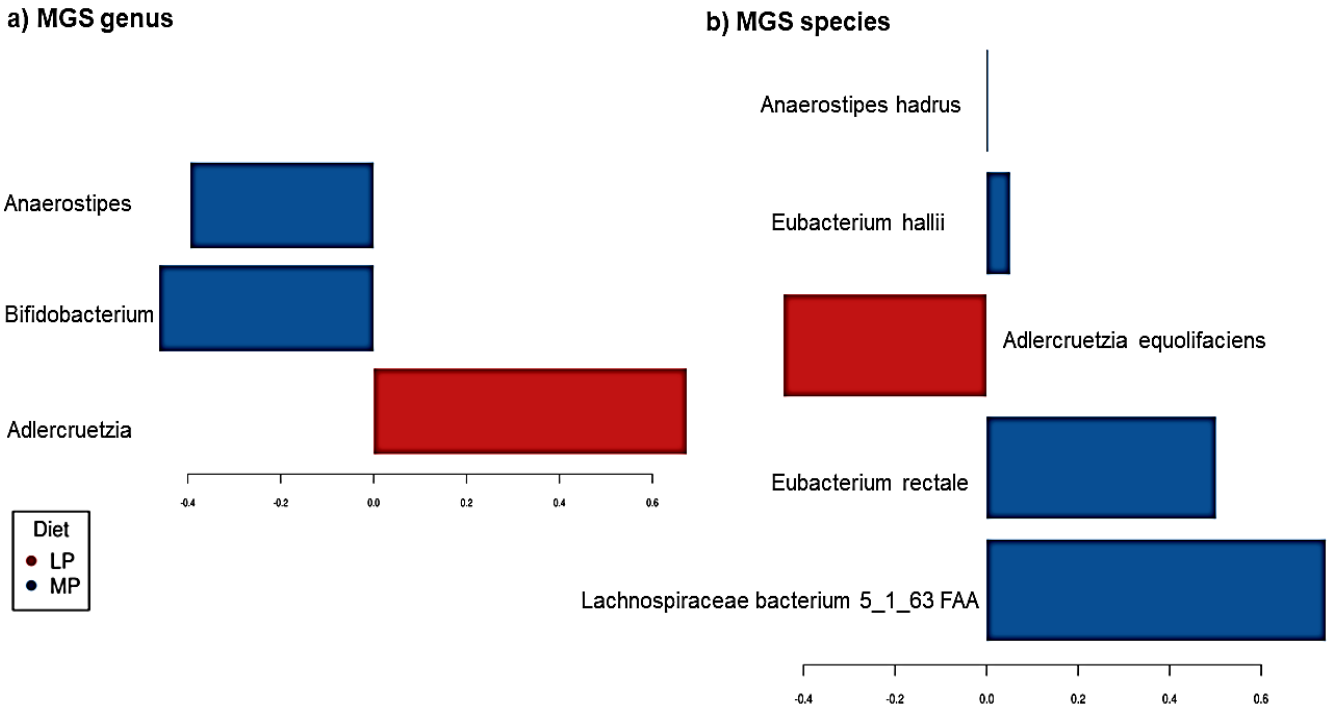


Figure 5.11 Bacterial **a)** genera and **b)** species differentiating the stool microbiota profiles of subjects after consumption of Low Prebiotic (LP; red) and after consumption of the Moderate Prebiotic (MP; blue) diet identified by sPLS-DA from the MGS data.

5.4.7 The taxonomic changes in gut bacteria in response to the MP diet are also apparent in functional characteristics predicted from the MGS data

Table 5.6 shows the MGS data metrics from the samples analysed in this study. The Bowtie2 alignment against the human hg19 database removed ~1% of the reads, and the range of paired-end reads remaining was similar for all the dietary groups. The coverage of the MGS data produced from the individual samples was also found to be similar across the three groups with BL having a mean % coverage of 73% ± 18 (n=14), LP: 71.4% ± 15.2 (n=18) and MP: 71% ± 15.42 (n=18).

Table 5.6 Table showing the range of number of raw reads, filtered reads and % coverage of the MGS data

Diet group	Range (number of reads) before bowtie2 (x 10^6)	Range (number of reads) after bowtie2 (x 10^6)	Range % coverage
BL (n=14)	1.2746- 21.6883	1.2742-21.6830	36-92
LP (n=18)	1.7650-21.8615	1.7646-21.8513	51-91
MP (n=18)	1.48865-21.6915	1.48828-21.6743	46-94

The resultant gene family abundance output files were normalised to copies per million (cpm) and reclassified into Gene Ontology (GO) and Protein Family (Pfam) categories, which resulted in the highest regrouping of the gene family output files (56% and 49%, respectively) compared to KEGG Orthology (KO) and Enzyme Category (EC) categories, which resulted in only 12% and 8% of data being regrouped, respectively. Hence, subsequent functional data analysis has been performed on the GO and Pfam categories. I then used the regrouped GO functional data to compute alpha diversity metric scores and found that while there are no differences in the Shannon diversity and evenness scores, there was a significant decrease in the richness of the functional gene counts following consumption of the MP diet (Figure 5.12). This suggests that the MP diet results in a smaller repertoire of gut microbial metabolic functions as compared to the LP group.

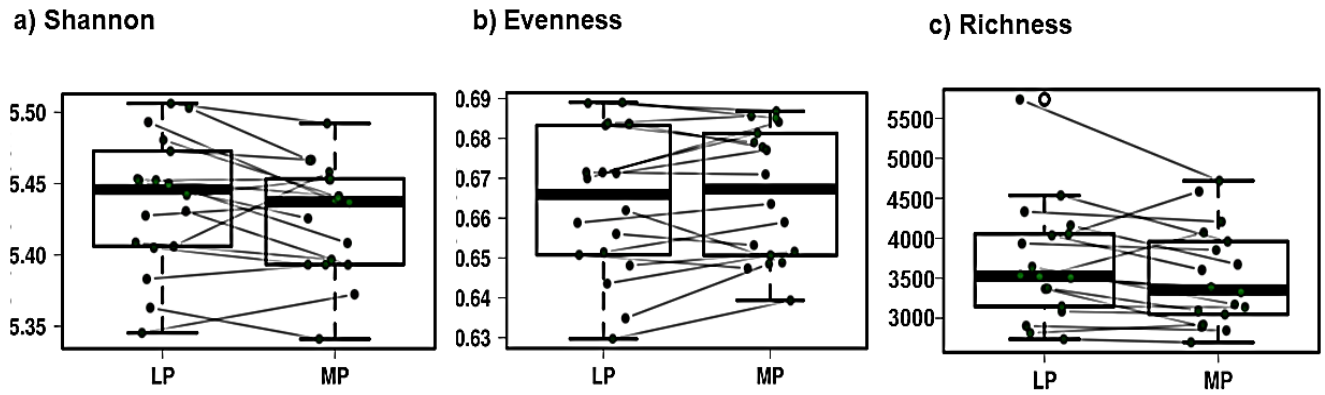


Figure 5.12 Alpha diversity measures calculated for functional Gene Ontology (GO) data produced from HUMAnN2 pipeline. There is a significant reduction in functional richness ($p<0.001$) when subjects consumed a diet with moderate prebiotic content. No changes in Shannon and evenness scores were observed between the two groups ($p=1$).

5.4.8 Distinct differences in functional microbial pathways between the LP and MP groups

In total 7040 GO categories were revealed from the functional assignment by HUMAnN2. These categories are further divided into three sub-ontologies as GO provides description of the biological process (molecular events suitable for the functioning of living units), molecular function (activities such as catalysis and binding describing the action of a gene product) and cellular components (part of the cell or extracellular environment where the gene product is located) of gene products. Mixed effect linear regression analysis was performed on all the GO categories and the pathways exhibiting statistically significant differences ($p<0.05$) between the LP and the MP groups were organised according to the three broader annotations of GO terms: biological process, molecular function and cellular components (Figure 5.13a, b and c). However, the statistical significance was lost once FDR correction of <0.05 was applied.

The MP group had higher abundance across 38 of the 74 biological processes categories (eg, translation, response towards cytokines, folic acid, vitamin A; alpha glucan biosynthetic process, sorbitol catabolism, mannitol metabolism, L ascorbic acid metabolism, antibiotic biosynthesis, fucose metabolism, cardiolipin biosynthesis, DNA restriction-modification systems and sulfur compound metabolism) (Figure 5.13a). A total of 20 molecular functions were significantly different ($p<0.05$) between the LP and the MP dietary interventions according to the MELR analysis. Of these 12/20 were found to be greater in the MP group compared to the LP group (eg: nucleic acid, rRNA, mRNA, ATP, cation and DNA binding, transposase activity, etc.) (Figure 5.13b). Nine cellular components were found to be significantly different ($p<0.05$) between the LP and the MP groups according to the repeated measures MELR analysis; with the ribosome and large ribosomal subunit categories greater in the MP group as compared to the LP group. In contrast, components associated with outer membrane, periplasmic space, cell wall, and cell outer membrane were greater in the LP group (Figure 5.13c).

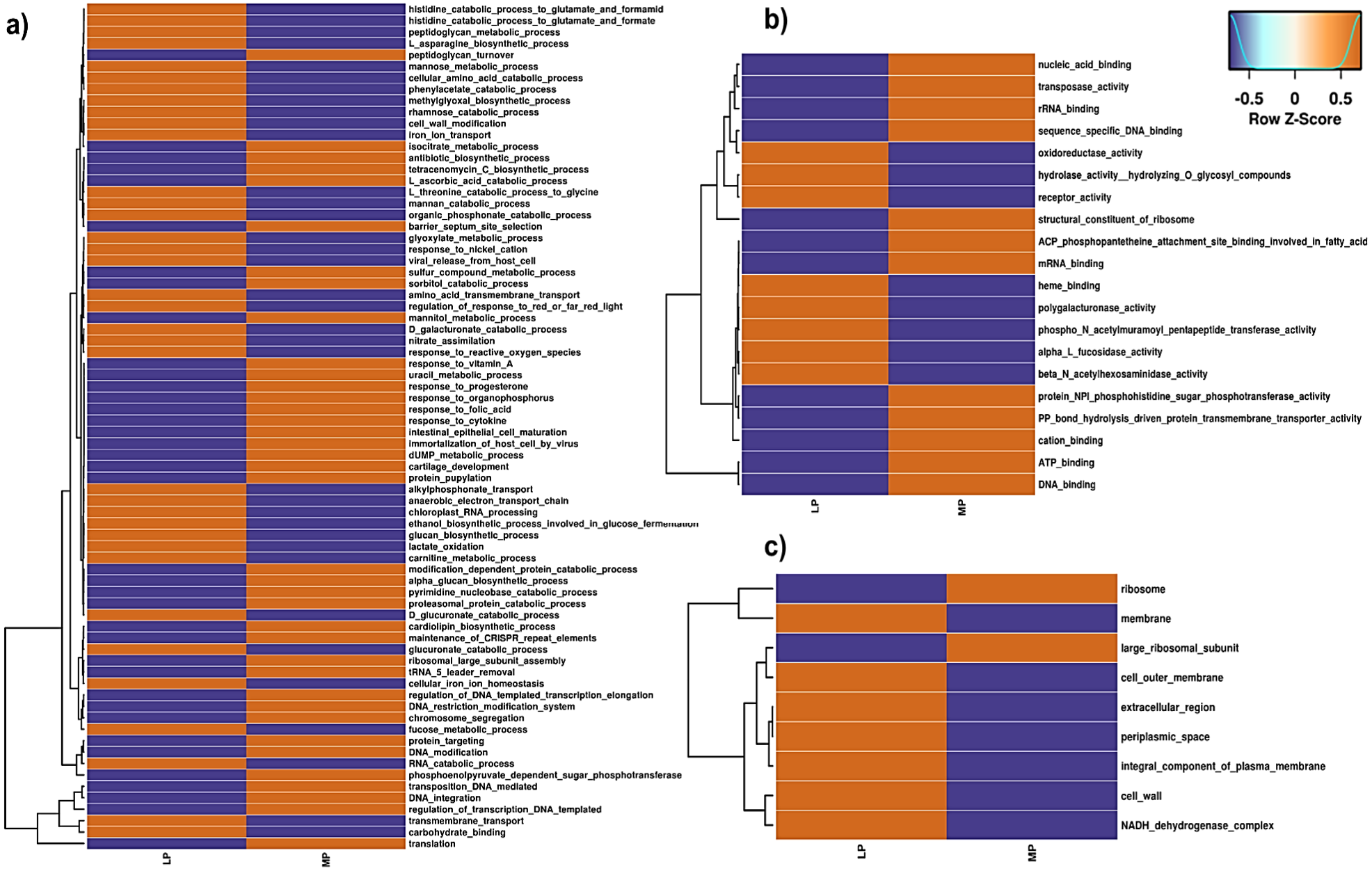

a)
histidine_catabolic_process_to_glutamate_and_formamid
histidine_catabolic_process_to_glutamate_and_formate
peptidoglycan_metabolic_process
L_asparagine_biosynthetic_process
peptidoglycan_turnover
mannose_metabolic_process
cellular_amino_acid_catabolic_process
phenylacetate_catabolic_process
methylglyoxal_biosynthetic_process
rhamnose_catabolic_process
cell_wall_modification
iron_ion_transport
isocitrate_metabolic_process
antibiotic_biosynthetic_process
tetracenomycin_C_biosynthetic_process
L_ascorbic_acid_catabolic_process
L_threonine_catabolic_process_to_glycine
mannan_catabolic_process
organic_phosphonate_catabolic_process
barrier_septum_site_selection
glyoxylate_metabolic_process
response_to_nickel_cation
viral_release_from_host_cell
sulfur_compound_metabolic_process
sorbitol_catabolic_process
amino_acid_transmembrane_transport
regulation_of_response_to_red_or_far_red_light
mannitol_metabolic_process
D_galacturonate_catabolic_process
nitrate_assimilation
response_to_reactive_oxygen_species
response_to_vitamin_A
uracil_metabolic_process
response_to_progesterone
response_to_organophosphorus
response_to_folic_acid
response_to_cytokine
intestinal_epithelial_cell_maturation
immortalization_of_host_cell_by_virus
dUMP_metabolic_process
cartilage_development
protein_pupylation
alkylphosphonate_transport
anaerobic_electron_transport_chain
chloroplast_RNA_processing
ethanol_biosynthetic_process_involved_in_glucose_fermentation
glucan_biosynthetic_process
lactate_oxidation
carnitine_metabolic_process
modification_dependent_protein_catabolic_process
alpha_glucan_biosynthetic_process
pyrimidine_nucleobase_catabolic_process
proteasomal_protein_catabolic_process
D_glucuronate_catabolic_process
cardiolipin_biosynthetic_process
maintenance_of_CRISPR_repeat_elements
glucuronate_catabolic_process
ribosomal_large_subunit_assembly
tRNA_5_leader_removal
cellular_iron_ion_homeostasis
regulation_of_DNA_templated_transcription_elongation
DNA_restriction_modification_system
chromosome_segregation
fucose_metabolic_process
protein_targeting
DNA_modification
RNA_catabolic_process
phosphoenolpyruvate_dependent_sugar_phosphotransferase
transposition_DNA_mediated
DNA_integration
regulation_of_transcription_DNA_templated
transmembrane_transport
carbohydrate_binding
translation
LP
MP
b)
-0.5
0
0.5
Row Z-Score
nucleic_acid_binding
transposase_activity
rRNA_binding
sequence_specific_DNA_binding
oxidoreductase_activity
hydrolase_activity__hydrolyzing_O_glycosyl_compounds
receptor_activity
structural_constituent_of_ribosome
ACP_phosphopantetheine_attachment_site_binding_involved_in_fatty_acid
mRNA_binding
heme_binding
polygalacturonase_activity
phospho_N_acetylmuramoyl_pentapeptide_transferase_activity
alpha_L_fucosidase_activity
beta_N_acetylhexosaminidase_activity
protein_NPI_phosphohistidine_sugar_phosphotransferase_activity
PP_bond_hydrolysis_driven_protein_transmembrane_transporter_activity
cation_binding
ATP_binding
DNA_binding
LP
MP
c)
ribosome
membrane
large_ribosomal_subunit
cell_outer_membrane
extracellular_region
periplasmic_space
integral_component_of_plasma_membrane
cell_wall
NADH_dehydrogenase_complex
LP
MP

Figure 5.13 Heatmaps of the significantly different (mixed-effect linear regression $p < 0.05$) **a)** Biological processes **b)** Molecular functions and **c)** Cellular compartments between the LP and the MP groups generated by the HUMAnN2 pipeline. Displayed values represent Z score for the normalised, square root transformed data in copies per million.

Less stringent sPLS-DA further revealed pathways associated with sorbitol metabolism, hydrolysis-driven protein transmembrane transporter activity, translation, ribosome structure and assembly, pyrimidine base metabolism, cardiolipin biosynthesis, and peptidoglycan turnover were all discriminatory of the microbiota changes in response to the MP diet (Figure 5.14). In contrast, pathways involved in peptidoglycan metabolism, glucuronate and mannan catabolism, methylglyoxal anabolism, and extracellular region were enriched and discriminatory of the microbiota in response to consumption of the LP diet (Figure 5.14). Based on these findings, the known differences in LP and MP content, and the taxonomic alterations in the gut microbiota outlined above, I chose to focus primarily on those pathways involved with carbohydrate metabolism.

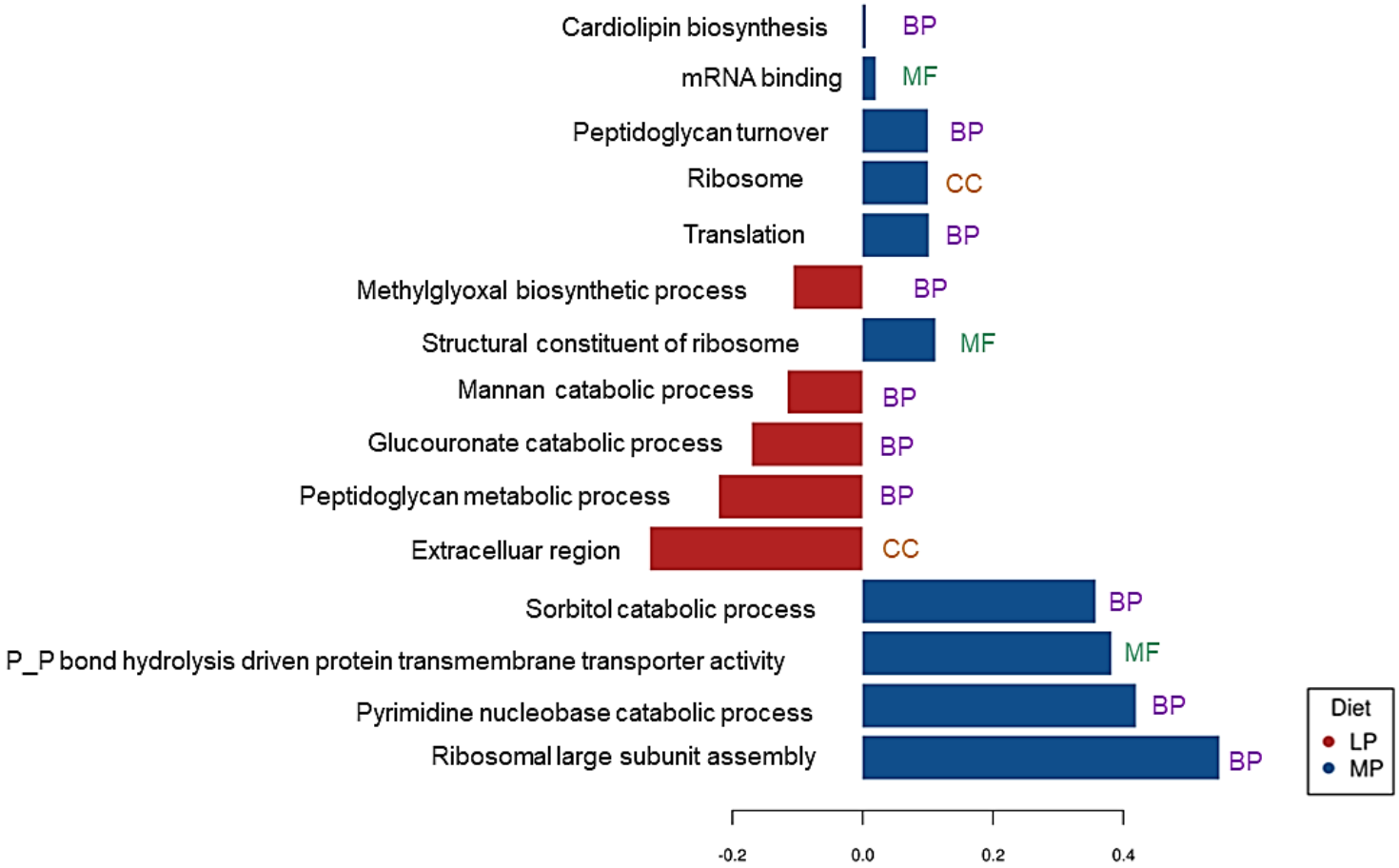


Figure 5.14 Gene Ontology (GO) groups shown by sPLS-DA analysis to be discriminatory of the microbiota changes associated with consumption of the LP (red) and MP (blue) diets. The BP, MF, and CC annotations for each GO, represent functions assigned to the Biological Process, Molecular Function and Cellular Compartment categories, respectively.

5.4.9 Functional metagenome analysis of the Pfam data revealed higher abundance of PTS systems associated with the MP dietary intervention group

sPLS-DA analysis was then performed on the regrouped Pfam functional data, which revealed that the relative abundances of Polysaccharide Lyase family 8, Glycoside Hydrolase families 2, 20 and 30, alpha-fucosidase and Glycosyl Transferase family 4 are discriminatory of the microbiota following consumption of the LP diet. In contrast, protein families of PTS systems predicted to be involved with lactose/cellobiose, sorbitol, sorbose/mannose/fructose, as well as starch binding module 26 were found to be discriminatory of the microbiota changes observed following consumption of the MP diet (Figure 5.15) .

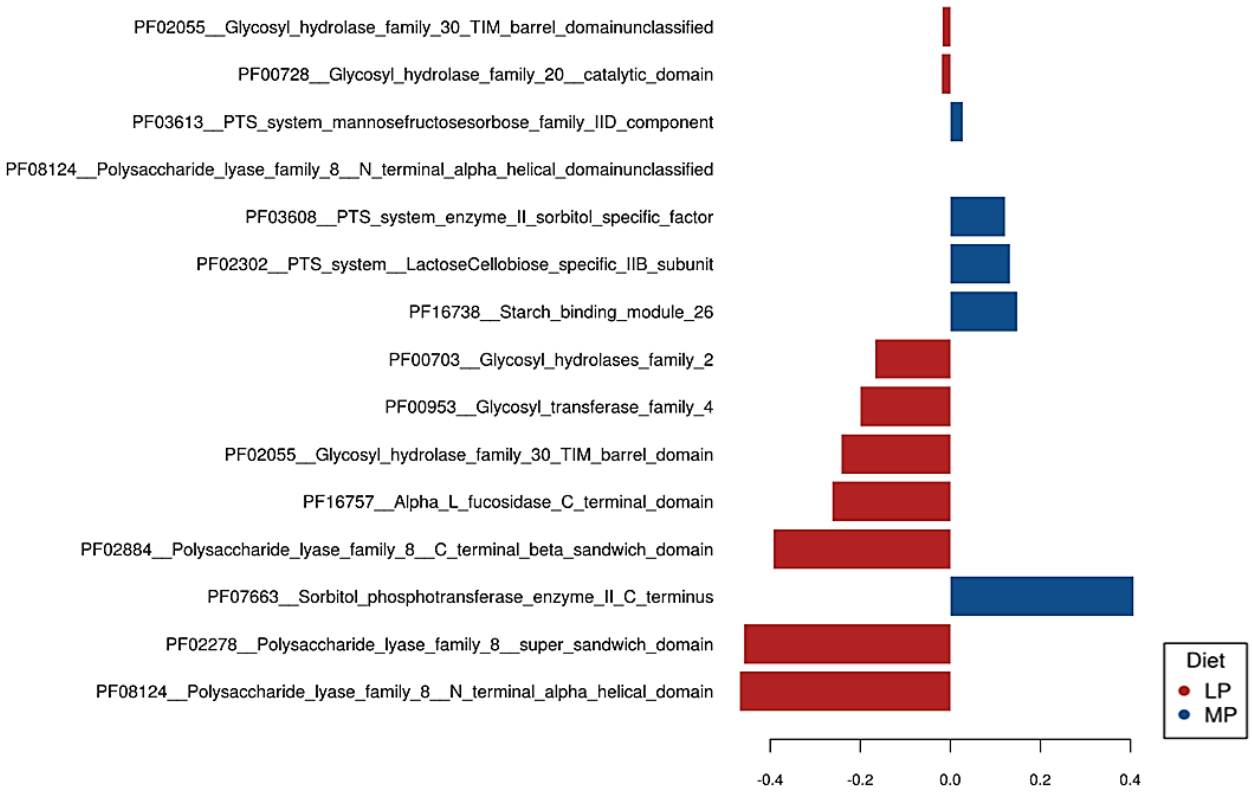


Figure 5.15 Protein families (Pfam) differentiating the stool microbiota profiles of subjects after consumption of Low Prebiotic (LP; red) and after consumption of the Moderate Prebiotic (MP; blue) diet identified by sPLS-DA.

MELR analysis on these PTS pathways further confirmed the microbiota changes with the MP diet possess significantly greater gene counts of sorbose subfamily II component (p=0.0026, FDR=0.026), Glucitol sorbitol specific IIA component (p=0.0026, FDR=0.026), Lactose cellobiose specific IIB component (p=0.014, FDR=0.11) and Fructose IIA component (p=0.014, FDR=0.11) PTS pathways as compared to the LP group (Figure 5.16).

In summation, these results suggest that in response to the compositional changes of the diet from LP to MP, the primary drivers of the microbiota changes appear to be an enrichment of microbes capable of utilizing the sugar alcohols provided by the MP diet.

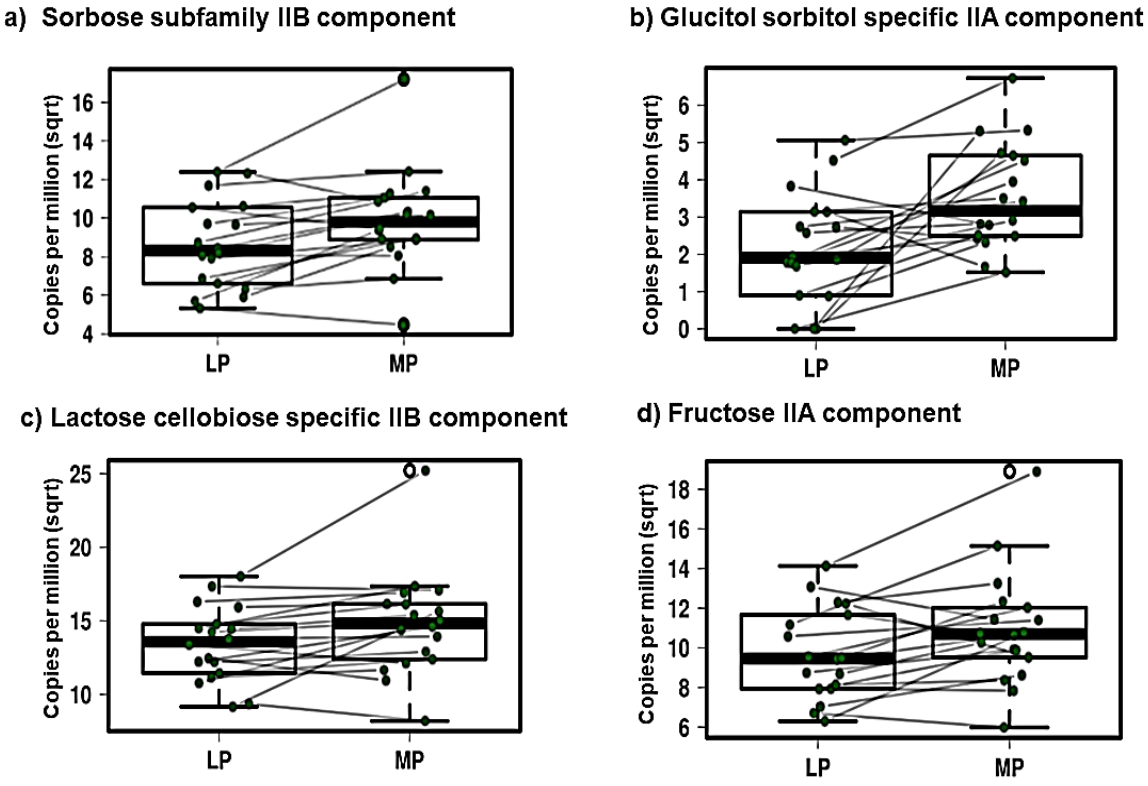


Figure 5.16 Mixed effect linear regression (MELR) analysis of the PTS pathways identified significant increase in the gene counts of of Sorbitol subfamily II component ((**a**), p = 0.0026, FDR= 0.026), Glucitol sorbitol specific IIA component ((**b**), p = 0.0026, FDR= 0.026), Lactose Cellobiose specific II B subunit ((**c**), p = 0.014, FDR= 0.11) and Fructose IIA component ((**d**), p = 0.014, FDR= 0.11) after consumption of the Moderate Prebiotic (MP) diet. Here, sampling time point was set as a fixed effect and individual subject as a random effect. Low Prebiotic (LP) and Moderate Prebiotic (MP) refer to the square root transformed TSS normalised gene counts of these pathways measured at LP and after consumption of the MP diet, respectively. Data collected from the same subject are connected by the lines.

5.4.10 MAGs recovered from the LP and MP groups show the differential capability to metabolise sugar alcohols

The 3Gbp sequencing depth used enabled the recovery of 27 and 18 high quality (>80% complete and <10% contaminated) MAGs from the LP and MP datasets, respectively; and their taxonomy determined via PATRIC are consistent with the predominant bacterial taxa inferred from the MGS using MetaPhlan2 and the 16S rRNA gene amplicon data (Table 5.7).

Table 5.7 List of high-quality Metagenome Assembled Genomes (MAGs; >80% complete and <10% contaminated) extracted from the LP and the MP groups, taxonomically identified through hierarchical clustering using Mash in PATRIC.

MAGs recovered from LP group			
BinId	**%completeness**	**%contamination**	**PATRIC taxonomy**
bin0.15	98.1	10.94	*Coprococcus sp*
bin0.22	88.87	2.46	*Roseburia CAG100*
bin0.23	96.43	7.84	*Bacteroides spp. CAG 545*
bin0.26	93.89	8.9	*Eubacterium spp. CAG252*
bin0.31	94.34	9.89	*Catenibacterium sp. AM22-6LB*
bin0.32	88.81	3.86	*Barnesiella spp*
bin0.35	95.59	7.75	*Alistipes CAG 268*
bin0.37	80.96	5.06	*Clostridium spp.*
bin0.4	89.85	2.84	*Bacteroides sp.*
bin0.42	81.24	6.7	*undefined*
bin0.43	83.42	6.97	*Eubacterium spp.*
bin0.45	88.59	6.96	*Roseburia spp. UBA11770*
bin0.47	97.99	4.77	*Bacteroides spp. CAG144*
bin0.5	94.85	5.65	*Ruminococcaceae st. UBA9126*
bin0.57	97.99	6.02	*Butyrivibrio crossotus strain*
bin0.62	88.44	3.66	*Clostridium spp.*
bin0.67	93.96	3.12	*Eubacterium spp. CAG180*
bin0.71	95.39	5.35	*Bifidobacterium animalis*
bin0.75	84.36	0.8	*Ruminococcus spp.*
bin0.81	85.4	5.93	*Ruminococcus*
bin0.86	87.14	10.17	*Ruminococcaceae st. UBA2089*
bin0.87	83.06	2.2	*Phascolarctobacterium faecium*
bin0.9	93.91	10.41	*Bacteroides sp..CAG98*

bin0.90	86.52	2.09	*Alistipes putredinis*
bin0.92	80.41	1.76	*Butyricicoccus*
bin0.94	82.82	6.63	*Ruminococcus*
MAGs recovered from MP group			
BinId	**%completeness**	**%contamination**	**PATRIC taxonomy**
bin0.11	91.41	7.16	*Bacteroides spp CAG 98*
bin0.23	95.84	10.08	*Undefined*
bin0.27	96.27	4.26	*Eubacterium spp.*
bin0.29	91.99	5.79	*Barnesiella sp. Strain UBA11816*
bin0.3	97.97	10.03	*Eubacterium spp.*
bin0.32	95.11	6.93	*Coprococcus*
bin0.4	92.07	6.98	*Alistipes CAG 268*
bin0.46	86.13	8	*Undefined*
bin0.53	94.42	7.63	*Bifidobacterium adolescentis*
bin0.54	80.89	0.4	*Ruminococcaceae bacterium*
bin0.59	80.84	3.38	*Clostridium spp CAG 75*
bin0.63	85.21	3.89	*Phascolarctobacterium faecium*
bin0.68	86.67	5.44	*Clostridium spp. CAG 138*
bin0.69	85.81	4.79	*Roseburia CAG18*
bin0.77	98.67	4.35	*Undefined/Acholeplasmatales*
bin0.81	86.54	2.92	*Alistipes putredinis strain UBA9494*
bin0.9	87.87	5.26	*Clostridiales strain/Ruinococcaecae*
bin0.96	80.12	1.8	*Bifidobacterium longum*

Three *Bifidobacterium* MAGS were produced from the data, one from LP (bin .71) and two from the MP (bin .53 and .96) datasets. Given the MGS data showed that the MP diet not only supports an increased relative abundance of Bifidobacteria, but also drives a shift in their population structure, the *Bifidobacterium* MAGs recovered from the two dietary groups were compared. Re-ordering and aligning the MAGs (bin .71, bin .53 and bin .96) was first performed and *B.animalis* subsp. *lactis* DSM 10140 was used as the reference genome for the Mauve alignments. As expected, the alignments confirmed there was a large amount of genetic synteny between the MAGs, although the three MAGs were readily differentiated by their xenologous regions (unique to each MAG, Figure 5.17).

The Average Nucleotide Identity (ANI) scores calculated for the Bifidobacteria MAGs are shown in Figure 5.18 and confirms the accuracy of the PATRIC-based assignments for MAGs Bin.53 and Bin.96 to *B. longum* and *B. adolescentis,* respectively and further confirms the abundance of different Bifidobacterial species in response to the LP and MP diets. These findings further substantiate the results of the MELR analyses shown in Figure 5.10 above, where the significant increase in the relative abundance of *Bifidobacterium* spp. following consumption of the MP diet is attributable to increases in both *B. adolescentis* and *B. longum*. Furthermore, the CAZyme profiles of the three MAGs were also found to be consistent with the GO and Pfam analysis of the paired-end MGS data, in that the *B. adolescentis* and *B. longum* MAGs recovered from the MP datasets also possess a greater gene count for sorbitol/mannitol metabolism than the *B. animalis* MAG (Table 5.8).

In summation, these findings further confirm that the composition of the MP diet has expanded the size of Bifidobacterial population via the selective enrichment of *B. adolescentis* and *B. longum* strains, and their ability to metabolise polyols such as sorbitol and mannitol.

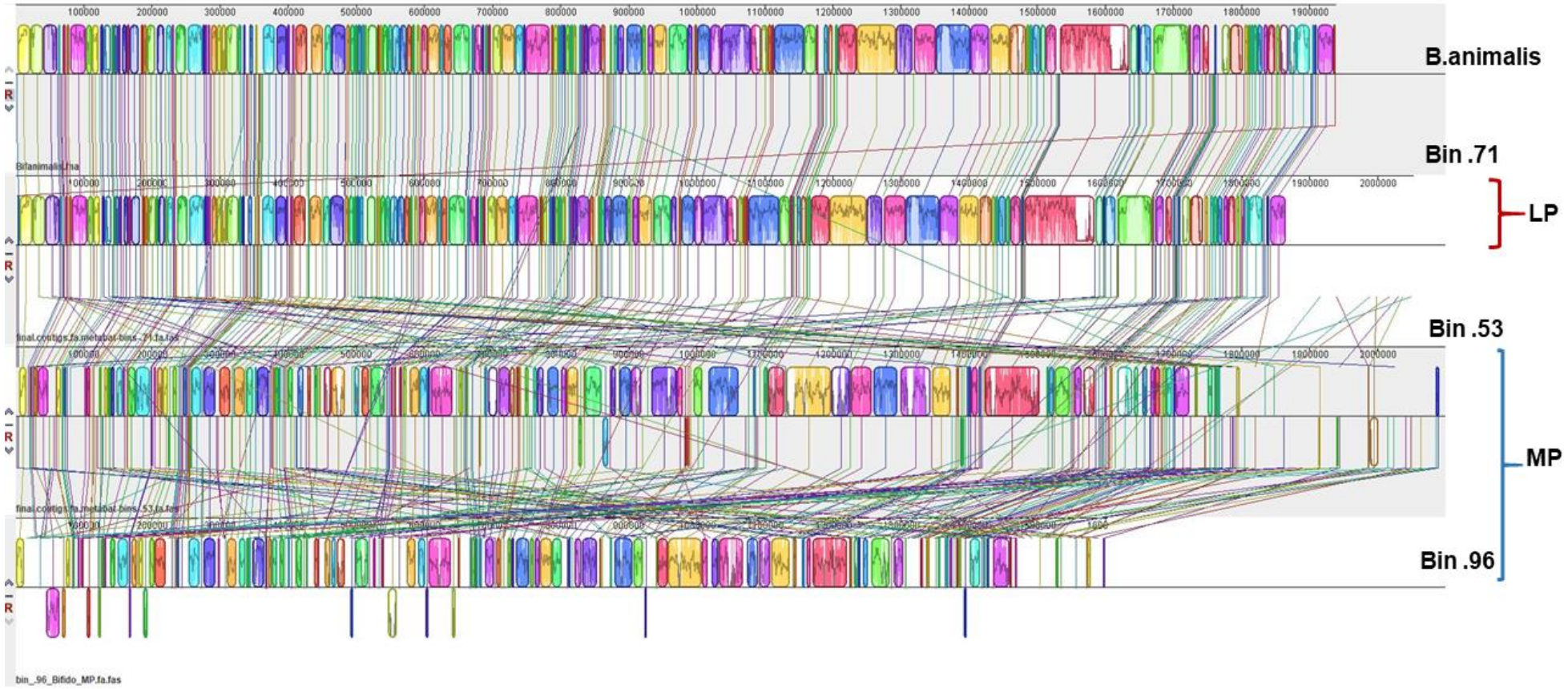


Figure 5.17 Mauve alignment of the *Bifidobacterium* MAGs produced from the LP (Bin.71) and MP (Bin.53 and Bin.96) MGS datasets and using the closed genome from *B.animalis* subsp. *lactis* DSM 10140 as the reference genome. All four genomes possess a large amount of genetic synteny as depicted by the individual coloured blocks, and particularly, between the *B. animalis* and *Bin .71* genomes. The MAGs Bin .53 and Bin .96 possess some xenologous regions depicted as the blank spaces, and a greater degree of genome rearrangements, as depicted by the connecting lines.

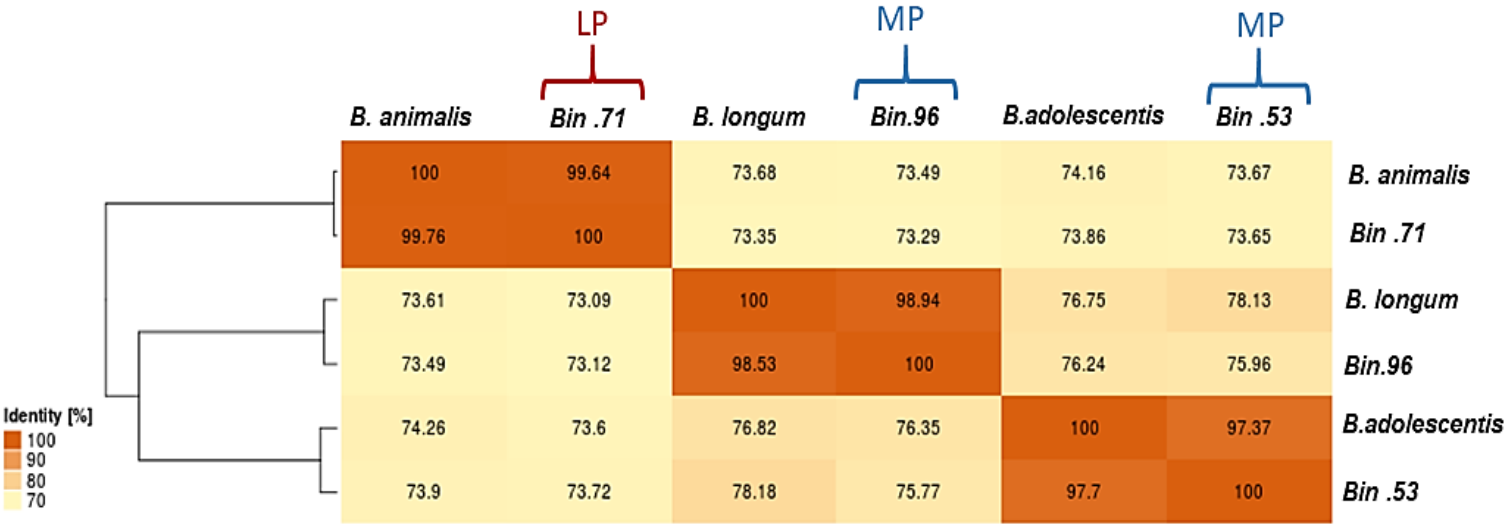


Figure 5.18 The average nucleotide identity (ANI) matrix of the Bifidobacterial MAGs calculated from BLAST hits between orthologous genes of the core genome in EDGAR. The Average Nucleotide Identity (ANI) scores calculated for the Bifidobacterial MAGs revealed 99.64% identity between bin .71 and the reference genome *B. animalis* subsp. *lactis* DSM 10140 suggesting bin .71 to be a *B. animalis* strain. Reference genomes of *B. longum* NCC2705 and *B. adolescentis* ATCC 15703 were also included to build the ANI matrix. It revealed that bin .53 showed 97.7% identity to the reference genome of *B. adolescentis* ATCC 15703 and bin .96 had 98.53% identity to the reference genome of *B.longum NCC2705*.

Table 5.8 The gene counts of the enzymes known for carbohydrates and sugar alcohol degradation in the three recovered Bifidobacteria Metagenome Assembled Genomes (MAGs).

Carbohydrates degrading enzymes (oligosaccharides & sorbitol)	Bin.71	Bin.53	Bin.96
Alpha mannosidase (EC 3.2.1.24)	0	3	2
Alpha-xylosidase (EC 3.2.1.177)	0	2	0
Beta galactosidase (E.C. 3.2.1.23)	8	7	5
Beta glucosidase (EC 3.2.1.21)	2	5	2
Beta xylosidase	0	2	0
Periplasmic beta-glucosidase (EC 3.2.1.21)	0	2	0
Sorbitol dehydrogenase (EC 1.1.1.140)	0	1	1
Xylan 1,4-beta-xylosidase (EC 3.2.1.37)	2	3	1

5.4.11 Correlation analysis shows significant specific bacterial-fungi associations in response to the prebiotic content of the diet

In order to better understand the intra- and/or inter-Domain interactions following consumption of the LP and MP diets, I performed three different forms of correlation analyses, using the difference in the relative abundance values between the MP and LP diets of specific bacterial/archaeal and fungal taxa (i.e. Δ=MP-LP). First, I performed a correlation analysis using the 16S rRNA amplicon and ITS2 datasets, to expand the analyses to lesser abundant taxa, such as the Archaea. Next, I combined the bacterial species profiles predicted from the MGS data using MetaPhlan2, with the ITS2 (fungal) profiles. Thirdly, I investigated whether different microbial networks were apparent following consumption of the LP and MP diets. The findings of these analyses are illustrated in Figures 5.18-5.20, respectively.

The correlation matrix using only the 16S rRNA and ITS2 datasets are shown in Figure 5.18. In particular, the Δ*Bifidobacterium* values were positively correlated with Δ*Anaerostipes,* but negatively correlated with Δvalues for taxa assigned to *Ruminococcaceae.* Δ*Saccharomyces* values were positively correlated with the Δvalues calculated for *Anaerostipes* and *E. hallii;* and the ΔArchaea *(Methanobrevibacter*) values were positively correlated with the Δvalues calculated for the *Ruminococcaceae* family and *Akkermansia*. Furthermore, these results also showed a strong negative correlation between *Methanobrevibacter* and *Akkermansia* with both *Faecalibacterium* and *Roseburia*.

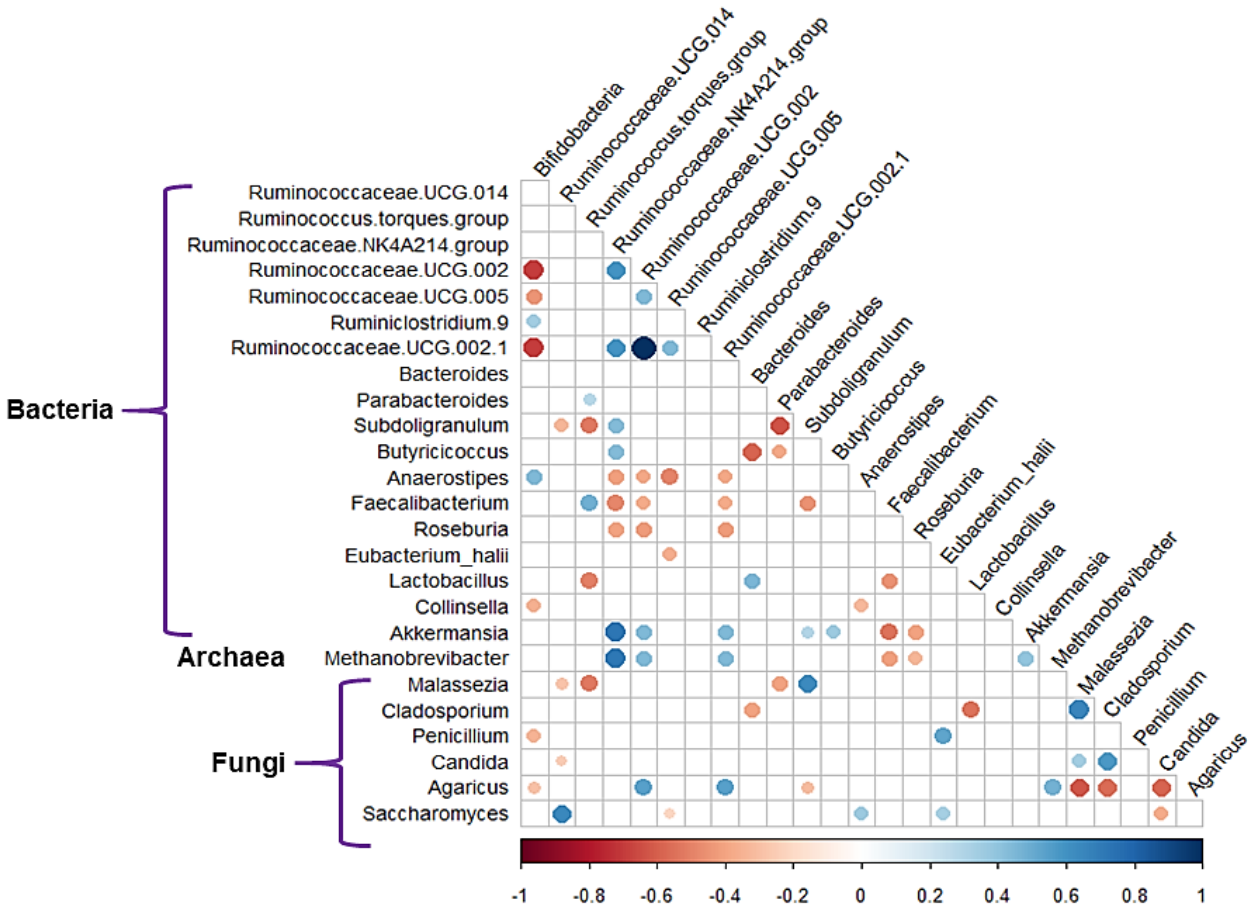


Figure 5.19 Spearman correlation analyses of the changes in relative abundance between the MP and LP diets (i.e. Δvalues) for the key bacterial, archaea and fungal taxa identified by from the 16S rRNA and ITS2 profiles. Only those correlations with adjusted P-values < 0.05 following a False Discovery Rate correction are shown. Red circles denote negative correlations and blue circles denote positive correlations, with both color intensity and the size of the circle proportional to the strength of the correlation.

I then categorised the bacterial species profiles predicted from the MGS data into either primary carbohydrate degraders or secondary carbohydrate fermenters, because I wanted to investigate whether the fungal profiles were more readily correlated with either or both of these "metabolic guilds" of bacteria. Interestingly, *Saccharomyces* amongst all the other fungi had highest number of significant associations with the bacterial species (Figure 5.20) with significant positive correlations observed between Δ*Saccharomyces* and Δvalues for *B. longum*, *A. hadrus* and *E. hallii.* Positive correlations were also found between Δ*Agaricus* with the Δvalues calculated for *B. adolescentis*, *B.*

longum and *A. hadrus*. In addition to these bacterial-fungal interactions, the positive correlations predicted between *Anaerostipes* and *E. rectale* and *E. hallii* suggest mutualistic interactions between these bacterial taxa. Furthermore, positive associations were seen between Δ*E.hallii* with Δvalues calculated for *B. adolescentis* and *B.longum*. In contrast, a negative correlation was identified between the Δ*Saccharomyces* and Δ*R. torques* values. Negative correlations were also estimated between Δ*R. obeum* and Δvalues calculated for *Agaricus* and *E. hallii.* A negative correlation between *Bifidobacterium animalis* and *E. rectale* was found (Figure 5.20).

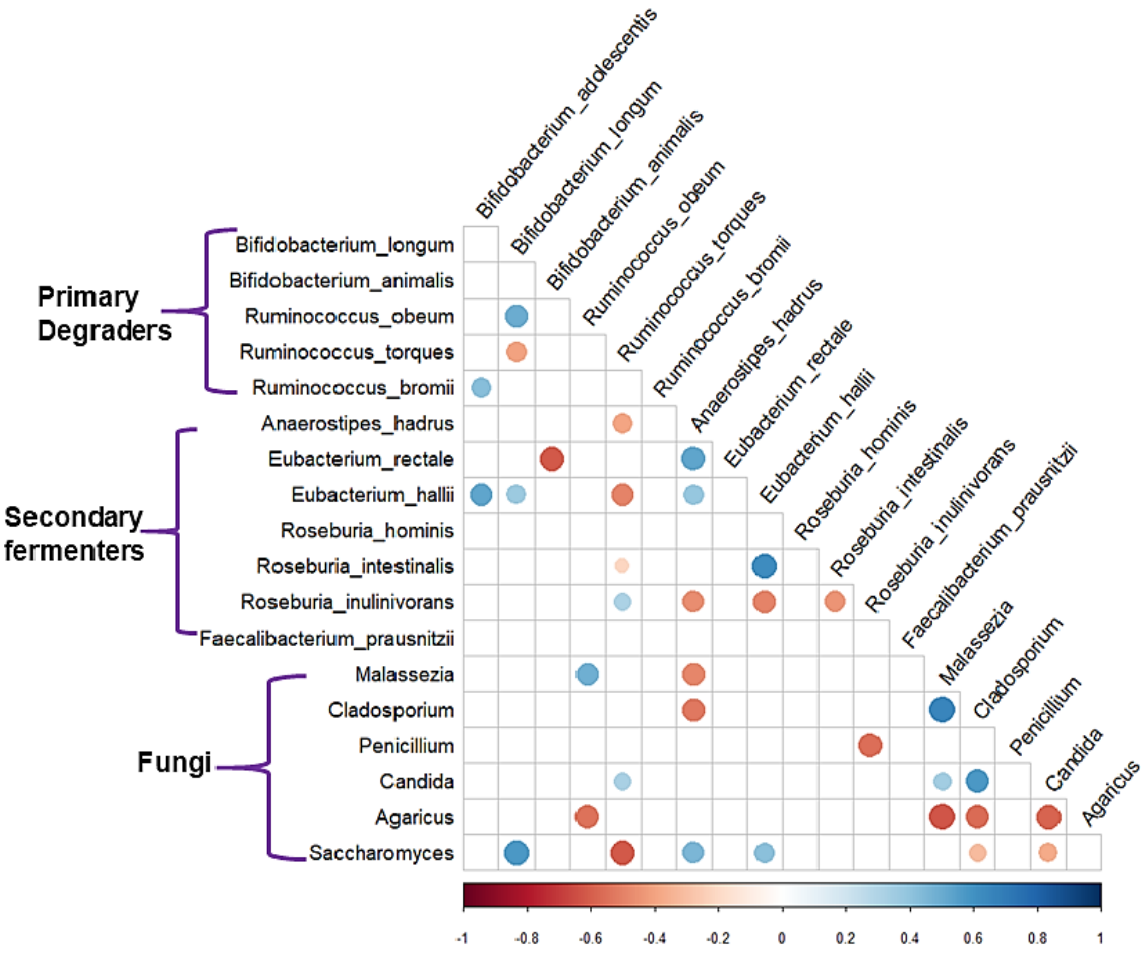
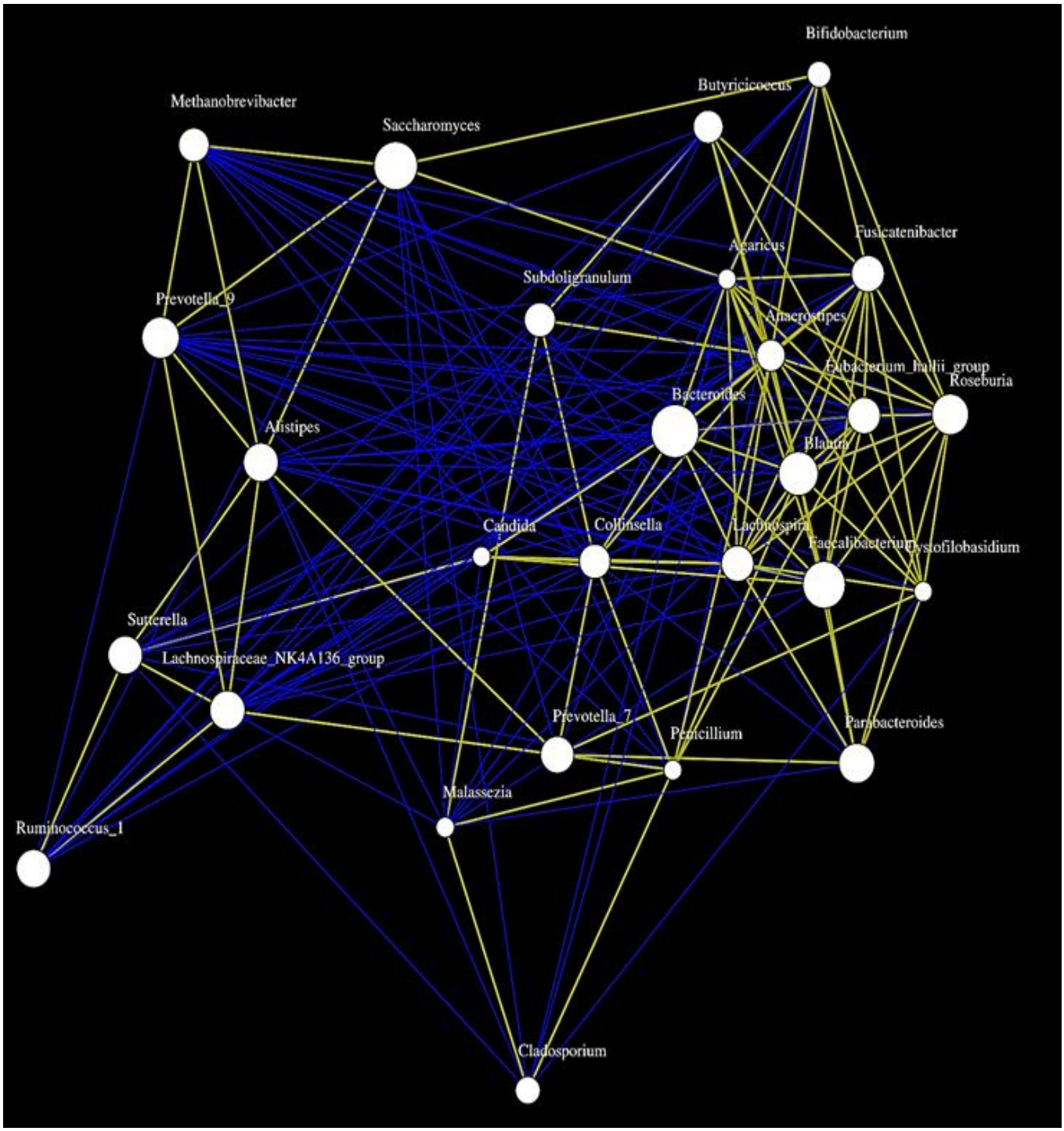


Figure 5.20 Spearman correlation analyses of the changes in relative abundance between the MP and LP diets (i.e. Δvalues) for the key bacterial, archaea and fungal taxa identified by from the MGS and ITS2 profiles. Only those correlations with adjusted P-values < 0.05 following a False Discovery Rate correction are shown. Red circles denote negative correlations and blue circles denote positive correlations, with both color intensity and the size of the circle proportional to the strength of the correlation.

The correlation networks produced using the abundance values (rather than the Δvalues) from the 16S rRNA and ITS2 datasets for the LP and MP diets are shown in Figure 5.21 a and b, respectively. Both diets appear to sustain two separable networks, a smaller network comprised primarily of *Saccharomyces, Methanobrevibacter, Prevotella 9* and *Ruminococcus 1;* and a second larger network including *Bifidobacterium, Bacteroides, Faecalibacterium, Roseburia* and *Anaerostipes.* Interestingly, the bacterial and the fungal taxa that were increased following the consumption of MP diet were found to have positive associations within the same network. For example; *Anaerostipes* was found to be positively associated with Bifidobacteria and the fungi *Agaricus.* Notably, *Candida* appears to have positive correlations with the *Eubacterium hallii* group and have greater number of positive associations with other taxa in the MP group compared to the LP group. Taken together, these results suggest a complex relationship between bacterial and fungal communities in the gut environment and that specific alterations are present in LP and MP groups. The microbial networks predicted following consumption of the LP and MP diets include both prokaryote and fungal taxa, with a dense correlation network with the MP diet.

a) Network plots: LP



b) Network plots: MP

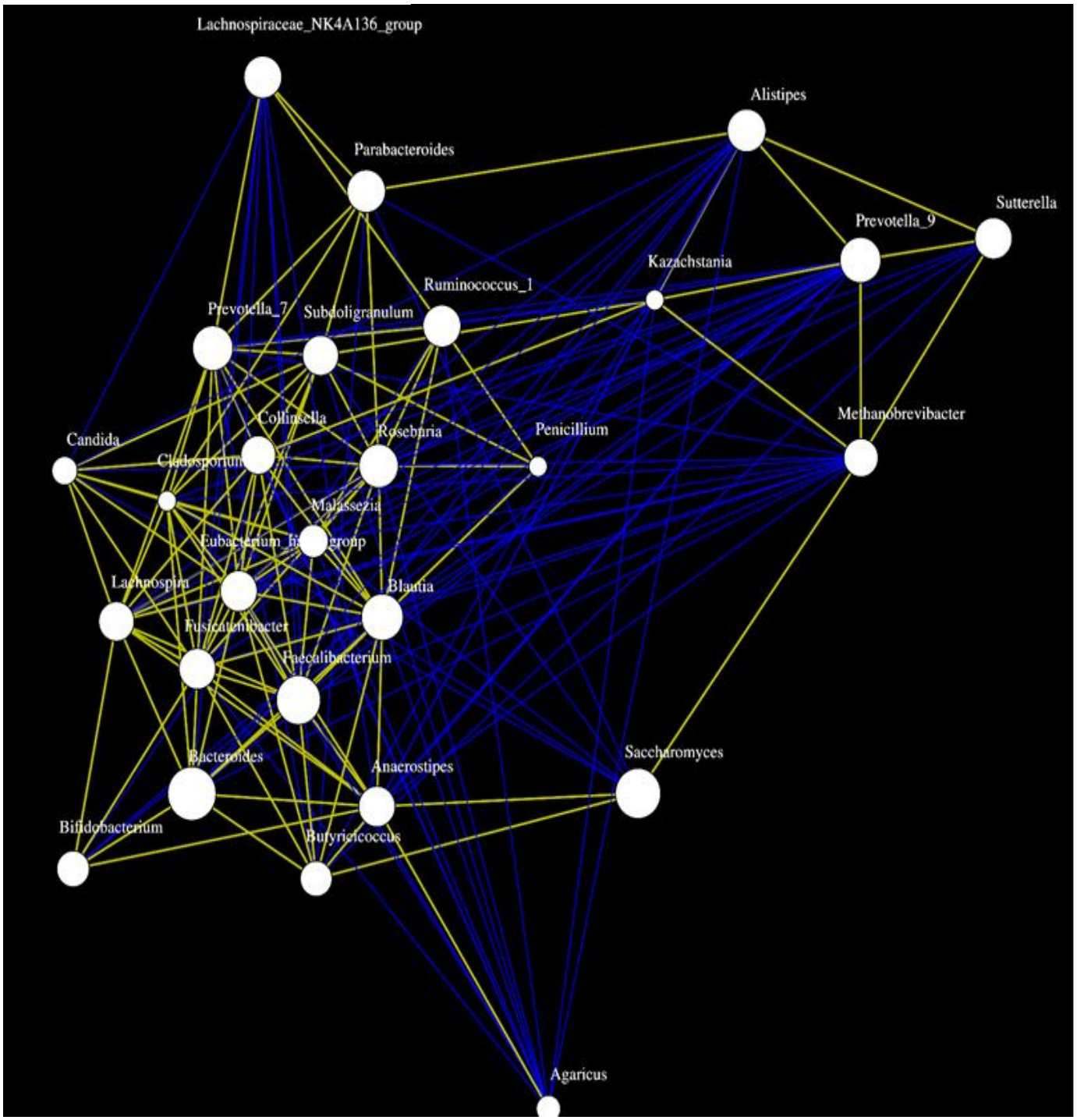


Figure 5.21 Network plots from the 16S rRNA and ITS2 data, showing positive (yellow, r>0.25) and negative (blue, r<-0.25) Spearman correlations among the top 20 bacterial and top 10 fungal taxa along with inclusion of *Methanobrevibacter* (Archaea), Bifidobacteria and *Anaerostipes* in the network plots following consumption of the **a)** LP diet **b)** MP diet.

5.5 Discussion

This Chapter presents a combination and integration of 16S rRNA and ITS2 profiles with MGS data to provide the first multi-Domain characterization of the effects from diets providing either a low or a moderate daily intake of prebiotic-containing foods in healthy human subjects.-The differences in prebiotic intake specifically relate to the quantity of oligosaccharides and sugar alcohols (mannitol and sorbitol) present in these foods (Table 5.1 above) and as such, could also be described as diets providing a low or moderate daily intake of FODMAPS. This study was conducted in "healthy" subjects and did not report any abnormal or persistent gastrointestinal symptoms prior to the study. Care was taken to monitor participant compliance to the dietary interventions throughout the study and no marked changes were reported in terms of abdominal discomfort and/or pain with the LP and MP diets. These findings are consistent with those from other studies where the "healthy" subject groups show no marked changes in GI symptoms in response to the FODMAP content of their diet (81,188).

5.5.1 Taxonomic variations in stool microbiome communities in response to diet.

I have integrated the findings of the gut microbiota profiles predicted from the 16S rRNA (or ITS2) amplicon and MGS datasets in Figure 5.22. In summation, the results using both are both confirmatory and complementary and provide a more holistic insight of the stool microbiome than either method alone. The majority (11/20) of the top 20 taxa identified in the stool samples were present in both the PCR based and MGS datasets (i.e. *Bacteroides, Faecalibacterium, Eubacterium, Subdoligranulum, Roseburia, Blautia, Coprococcus, Dorea* and *Parabacteroides).* Furthermore, the statistical analyses of the community profiles derived from the 16S rRNA amplicon and MGS datasets both show that the MP diet is associated with increases in the relative abundances of *Bifidobacterium, Anaerostipes,* and *Eubacterium* spp. with a commensurate reduction in bacterial richness score. However, at the sequencing depth used here (3 Gbp) both the Archaea and Fungi were underrepresented in the MGS datasets, and therefore, the ITS2 amplicon profiles were the only way to reveal the significant increase in fungal richness scores and the relative abundances of *Candida, Agaricus* and *Aspergillus* that appear to occur in response to consumption of the MP diet. Overall, a combination of PCR based, and MGS based methods have provided insights on the effects of "in-situ" prebiotic diets on the gut bacterial, fungal and archaeal communities.

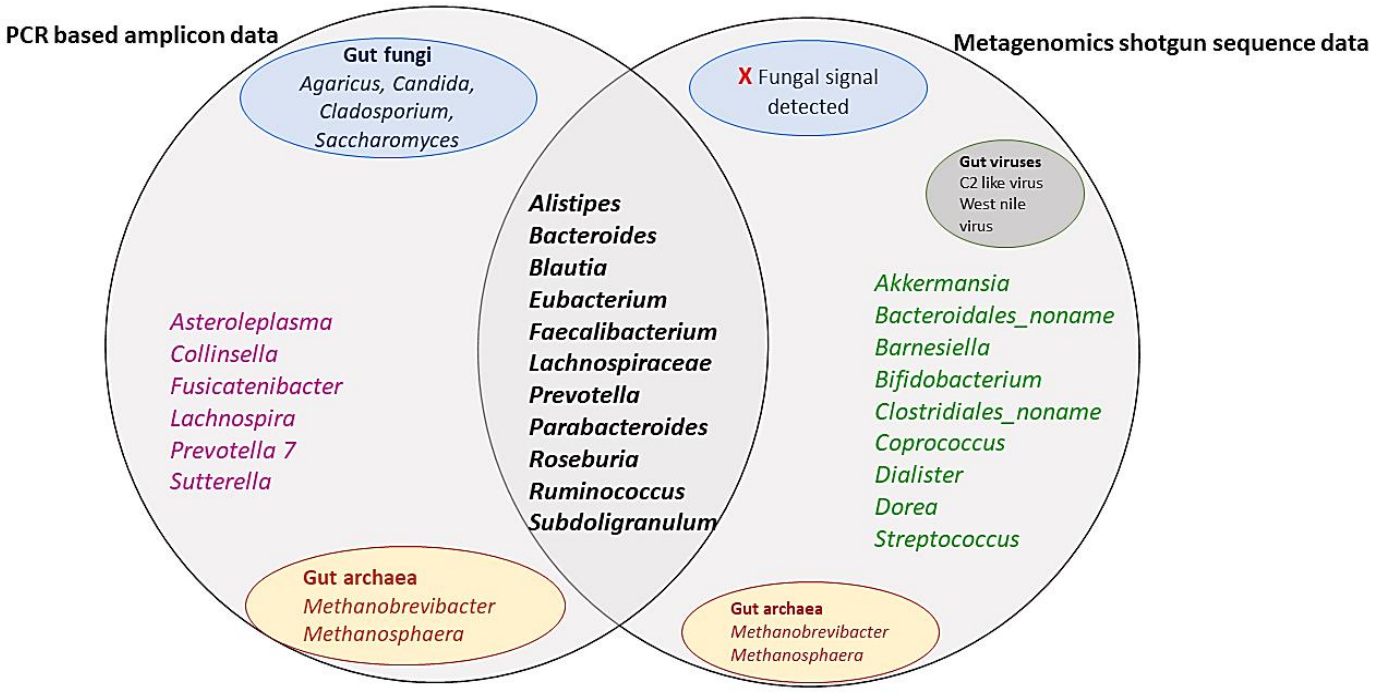


Figure 5.22 Overview of the convergence and differences between Amplicon (16S rRNA and ITS-2) and 3 Gbp MGS data, limited to the top 20 bacterial groups as identified by both the approaches and the top 5 fungal genera as identified by the ITS2 PCR based method have been shown here.

The 16S rRNA amplicon data contained sequences assigned to *Prevotella 7* and *Prevotella 9* and both were in the top 20 genera. *Prevotella* was also identified to be one of the top 20 genera from the MGS data, with *Prevotella copri* found to be a major species. My examination of the SILVA database shows that *Prevotella copri* is assigned to the *Prevotella 9* lineage. In the current study, the amplicon-based 16S method effectively captured the gut archaeal signals as compared to the MGS method. While both *Methanobrevibacter* and *Methanosphaera* were detected by both these methods, they were detected in a higher number of samples from the amplicon-based method. *Methanosphaera* was detected in 10 samples from the amplicon-based method as compared to MGS method where it was detected in only 1 sample. Furthermore, *Methanobrevibacter* was detected in 22 samples from the amplicon-based method as compared to MGS method where it was detected in 18 samples. This difference between the MGS and amplicon-based methods in detecting the gut archaea could be due to the choice of the PCR primers (V6-V8 region of 16S rRNA gene) used in the amplicon method. These primers seem to better capture the archaeal signals compared to the MGS method not deep enough (3 Gbp) for complete coverage of these low abundant gut members. For example, Pasolli et al. (189) recovered 150,000 MAGS from a total of 9,428 MGS datasets produced from different human body sites, ages, lifestyle and countries. However, most of the datasets used by Pasolli et al. (189) had limited to no coverage of the archaea, and only 675 good quality archaeal MAGS were

recovered and of these, 487 represent *Methanobrevibacter smithii* and related spp. Furthermore, only four good quality *Methanosphaera* MAGs were recovered which suggests the low abundance of this archaea in the gut environment. Additionally, our MGS datasets appeared to possess no reads from fungi, despite the ITS2 sequencing validating the presence of a stool mycobiome responsive to the prebiotic content of these diets. Again, this may be more of a reflection of the low abundance of fungi in these microbiomes. A study by Nash et al. (190) mined the HMP faecal healthy cohort metagenomic sequence data produced at a depth of 10 gigabases per sample to recover the fungal genomes. In their study, out of the >27 billion metagenomic sequences produced, only 0.01% aligned to the fungal genomes. Overall, my findings suggest that while MGS can provide increased taxonomic resolution (to the species level of classification), these data come with a caveat of lower sensitivity and detection of important but less abundant taxa. Until the cost effectiveness of MGS sequencing allows greater sequencing depth, the amplicon-based methods (16S rRNA and ITS2) offer a cost-effective and highly complementary approach to holistically examine the bacteria, archaeal and fungal communities resident within the human gut.

Both the amplicon and MGS datasets show that bacterial richness is reduced by consumption of the MP diet, but no significant changes in either the Shannon alpha or evenness scores. Such findings suggest that the MP diet supports the expansion of numerically predominant members of the gut community such as *Bifidobacterium*, *Anaerostipes* and *Eubacterium* spp. which were all observed to increase following the MP dietary intervention and with limited impacts on bacterial evenness, but at the expense of lesser abundant bacterial species which will reduce bacterial richness. In that context, the mixed effect linear regression analyses of both the amplicon- and MGS-derived profiles showed that the relative abundance of *Bifidobacterium* spp. increased on consumption of the MP diet, which is consistent with the effects reported when prebiotic supplements such as fructans, galacto-oligosaccharides and inulin have been added to the diets of healthy adult subjects (87,89,139,191–194). The less stringent statistical tests such as sPLS-DA and LefSe also provided similar findings, but the MGS data provided a more detailed characterization of these changes. No less than 6 *Bifidobacterium* spp. were identified in the samples analysed by MGS: *B. adolescentis* was the most abundant (and with 64% prevalence) with *B. longum*, *B. animalis*, *B. bifidum*. *B. pseudocatenulatum* and *B. catenulatum* present at lesser abundances. The increases in *B. adolescentis* and *B. longum* following consumption of the MP diet are consistent with the findings reported by Baxter et al. (194) from healthy young adults in response to adding inulin (20 g/day) to their diets. The 16S rRNA data also revealed significant increase in the relative abundance of genus *Anaerostipes* following the MP dietary intervention when compared with the LP group. I observed a similar trend from the MGS data

analysis, although this increase in relative abundance of *Anaerostipes* populations was not statistically significant.

5.5.2 Functional variations in response to diet determined from MGS datasets.

The MGS data also revealed an increase in the relative abundance of *Eubacterium spp. (Eubacterium rectale* and *Eubacterium hallii*) in response to consumption of the MP diet. A plausible explanation for the increase of *Eubacterium* and *Anaerostipes* spp. could be their utilization of *Bifidobacterium*-derived fermentation products such as lactate and acetate as well as prebiotic degradation products (144,195,196). In that context, culture-based studies with *Anaerostipes* spp. have shown it is not able to degrade oligofructose when it is provided as the sole carbon and energy source in mono-cultures, but will grow on the fructose released by oligofructose degradation when co-cultured with *Bifidobacterium longum* suggesting that the growth of *Anaerostipes* might be rate limited by the size of Bifidobacterial population and its provision of the depolymerizing enzymes (197). Furthermore, the bacterial and fungal correlation analysis showed that change in the relative abundance of *B. adolescentis* and *B. longum* are positively related to the change in the relative abundance of secondary fermenters *E. hallii* which is then positively related to *Anaerostipes hadrus*. This suggests another plausible distinct mechanism of metabolic cross-feeding of the partial breakdown products from complex substrates (Figure 5.20 above in results section).

Interestingly, neither the amplicon nor the MGS data show any significant changes in the relative abundance of *Faecalibacterium prausnitzii* in response to the LP or MP diets. This bacterium is well known to metabolize and ferment prebiotics such as inulin (87,198). These findings are similar to what have been reported by Baxter et al. (17) where inulin increased the abundance of different species of *Bifidobacterium, Anaerostipes hadrus* and *Eubacterium rectale* but no other bacterial taxa. In addition, prebiotic supplementation in mice has also been associated with an increased abundance of *Akkermansia muciniphila* – a mucin degrader and a healthy gut commensal (64). However, in the current study I did not observe significant shifts in *A. muciniphila* populations in response to the LP or MP diets, although the MGS data shows it to be among the top 20 genera.

In summary, these findings suggest the MP diet supported an expansion of numerically predominant members of the gut community such as *Bifidobacterium, Anaerostipes* and *Eubacterium* spp. which were all observed to increase following the MP dietary intervention, and at the expense of lesser abundance bacterial species such as *Prevotella, A. muciniphila* etc. These findings corroborate and expand those determined from the systematic review and meta-analysis by So et al. (146), who reported that only *Bifidobacterium* spp. showed a consistent, significant increase in its abundance in response to dietary fibre supplements in healthy individuals. Their analyses also suggest there are no

changes in Shannon alpha diversity in response to dietary fibre supplements, but importantly, my results do reveal there is a reduction in bacterial richness in response to consuming a diet with a moderate prebiotic content.

In contrast to the bacterial taxa, my findings show that the MP diets increases fungal richness within the gut microbiome. Other studies have shown that the Ascomycota (genera *Saccharomyces, Candida, Penicillium, Aspergillus, Cladosporium*) and Basidiomycota (genera *Malassezia*) are the primary members of the fungal communities in the human gut (49,53,61,190,199). Here *Saccharomyces* spp. were the most dominant and prevalent genus detected in the entire cohort. Both *S. cerevisiae* and *S. boulardii* have been reported to reduce colitis in mice, exhibit anti-inflammatory properties in several colitis models, and have beneficial effects in the prevention of diarrhoea and *C. difficile* infection in human subjects (200–202). Furthermore, the relative abundance of *S. cerevisiae* was found to reduce in IBD subjects compared to the healthy subjects (53). Polysaccharide rich food supplement (3 g/d) obtained from the chemical hydrolysis of *Saccharomyces cerevisiae* is shown to reduce waist circumference and body fat in overweight and obese subjects (203). Collectively these findings suggest the beneficial role of *Saccharomyces* towards human health and its high abundance and prevalence in the human gut might be a characteristic of a healthy gut mycobiome.

Here, *Candida, Agaricus* and *Aspergillus* spp. were detectable following the consumption of the MP diet, and currently, there is very little information relating their prevalence and/or abundance with diet. Hoffman et al. (61) reported the abundance of *Candida* to be positively associated with the consumption of carbohydrate-based diets by healthy human subjects suggesting the potential impact of dietary carbohydrates on the abundance of *Candida*. Despite having low abundance reads and low prevalence rate of these fungal taxa in the samples, the increase in fungal richness scores following MP dietary intervention suggests a possible influence of diet on these taxa and future studies with more number of subjects needs to be conducted with stronger focus on these fungal communities.

Interestingly, my correlation analyses revealed positive relationships between *Saccharomyces* and the change in the abundance and *B. longum*, *A. hadrus* and *E. hallii* (Figure 5.20 above in results section). Sokol et al. (53) also reported positive correlations between abundance of *Saccharomyces* and bacteria such as *Bifidobacterium*, *Roseburia*, *Ruminococcus* and *Blautia* that were reduced in IBD patients. Such findings suggest complex associations between the gut mycobiome and microbiome which can be further affected by factors such as nutrient availability (diet) and/or health status of subjects. In contrast, a study in mice reported an expansion of the gut mycobiome upon antibiotic treatment, and its reduction again upon antibiotic cessation, suggesting that the two kingdoms compete for the nutrients in the gut environment, and bacteria seems to outcompete fungi for the

shared nutrient niches (204). As such, it seems plausible to suggest that a consequence of the reduced bacterial richness in response to the MP diet is an increase in fungal richness. More detailed studies of the nutritional ecology of the gut mycobiome is warranted.

The MGS data showed there is a significant increase in gene counts of sorbitol and mannitol metabolic pathways in response to the MP diet. As mentioned previously, in addition to the oligosaccharides, the polyol content was a key difference between the LP and the MP diets. Sugar alcohols are a type of low digestible carbohydrate and are found in certain fruits, vegetables and artificial sweeteners (205). As such, polyols are key component of the dietary FODMAPs, and a moderate amounts of polyols have been shown to increase Bifidobacteria abundance in healthy individuals (205). A study by Gostner et al. (206) evaluated the effects of consumption of controlled basal diet enriched with either 30 g isomalt or 30 g sucrose daily in 19 healthy volunteers over a double-blind, placebo-controlled, cross over design study. The study reported an increase in Bifidobacteria in response to the isomalt diet compared with the sucrose diet showing the bifidogenic property of isomalt and suggesting its potential use as a prebiotic. Another randomised, longitudinal, double-blind study in 75 healthy adults by Finney et al. (207) investigated the effects of low-doses of lactitol on the human fecal microbiome. In this study, 25-g tablets of milk chocolate containing 10 g sucrose and lactitol in ratios of 10:0, 5:5, or 0:10 were given to the subjects daily for 7 days. Significant increase in Bifidobacteria were reported in the group that consumed the 0:10 ratio suggesting that the low doses of lactitol might be bifidogenic and may function as a prebiotic (207). In another study, the effects of L-sorbose and xylitol were reported to promote the growth and metabolic activity of a butyrate producer *Anaerostipes* in healthy human fecal culture (208). Moderate amounts (10 g/day) of sorbitol or mannitol have been shown to induce gastrointestinal symptoms in IBS patients but did not have any effect on the healthy subjects (209). Given the evidence of other polyols on healthy human gut microbiome and my results from the current study, it is plausible that the higher polyol content of the MP diet might have a role in the increased Bifidobacterial and *Anaerostipes* abundance. Collectively these findings suggest that polyol sorbitol and mannitol consumption in moderate amounts by healthy human subjects induces a healthy colonic microbiome and can therefore be beneficial as potential prebiotics. Furthermore, I also observed an increase in the gene counts of the polyol associated Phosphoenolpyruvate-carbohydrate phosphotransferase systems (PTS) in response to the MP diet. The PTS pathways are associated with the transport of carbohydrates such as glucose, mannose, fructose and cellobiose into the bacterial cell and consists of cytoplasmic proteins- (Enzyme I and HPr) and different carbohydrate-specific Enzymes IIA, IIB and IIC to catalyze carbohydrate translocation, uptake and phosphorylation (210). These findings suggest that MP dietary intervention results in an increase of increases the expression of microbial pathways responsible for optimal

utilization of prebiotics in a more complex carbohydrate environment as compared to the LP group. Indeed, the recovered Bifidobacteria MAGs from the MP group were different from that recovered from the LP group, particularly in terms of their ability for sorbitol/mannitol metabolism.

In conclusion, here I have reported for the first time that the "in-situ" prebiotic diet increases the relative abundance of Bifidobacteria *(*with increase in *B.adolescentis, B. longum* species) whereas increases fungal and decreases bacterial richness in healthy human subjects. MP diet was found to invoke significant increase in gene counts for polyol metabolism and associated PTS pathways. Inter-Domain associations were found to be impacted by the prebiotic content of the diet. Overall, the findings show plausible links with the prebiotic content of the diet, gut bacterial, fungal and archaeal populations and improves our understanding of the consequences of prebiotic diets on the gut structure and function.

Chapter 6

Chapter 6 General Discussion and Future Directions

It is widely recognised that the commensal microbiota can impart positive impacts on the structural, nutritional and protective functions of the host gastrointestinal tract, contributing to homeostasis (211) and diet is one of the main contributors affecting the diversity and composition of these commensals in the gastrointestinal tract. The overall aim of this thesis was to investigate diet x microbiota interactions in two key groups of subjects: elite race walkers and healthy adults. Prior to my Ph.D., I had not worked with human clinical samples and microbiome data and joining the Morrison lab allowed me to develop new skills in working with these types of samples and analysing large microbiome datasets. To that end, I was able to learn and successfully use methods to work safely with human saliva and stool samples, extract genomic DNA from these, prepare 16S rRNA libraries and perform bioinformatics analysis of 16S rRNA, fungal ITS2 and MGS data analysis.

My initial efforts are outlined in Chapter 2 and highlight the importance of appropriate handling and preservation of the stool samples for microbiome profiling studies. One of the initial steps that could potentially affect the microbiome analysis is proper sample storage conditions which are an integral part of any microbiome profiling study design. The results show that inappropriately stored stool samples are subject to degradation in Gram-negative bacterial biomass and reflected in high abundances of Firmicutes and lesser abundances of Gram-negative Bacteroidetes and Proteobacteria. As a healthy human gut would consist of somewhat equal abundances of Firmicutes and Bacteroidetes, this observed trend was quite abnormal (123). Several other studies have reported how the storage of samples at different temperatures and for varying times affects bacterial integrity, including one study that reported a significant decrease in the relative abundance of Bacteroidetes and an increase in Firmicutes phyla after 30 minutes compared to 15 minutes of fecal sample storage at room temperature, as measured by qPCR (212). In this Chapter, the results also show that stool samples collected, then thawed and pooled, despite being stored at -80ºC, also return profiles with a greater relative abundance of Firmicutes as compared to Bacteroidetes, at least when compared to a single sample transferred and stored in OMNIgene gut tubes. Other studies with rumen digesta samples have also reported a significant loss in Bacteroidetes when the samples were frozen without a cryoprotectant (i.e., glycerol/phosphate-buffered saline) and thereby thought to negatively impact the profiling of the archaeal and bacterial communities as measured by qPCR analysis of the 16S rRNA gene (213). During the course of my Ph.D. program, studies have also reported that the OMNIgene gut (OMR-200) collection tubes as a reliable way to collect high-quality fecal samples for gut microbiome studies (214,215). Overall, my results show that it is

imperative to store the samples appropriately to avoid any post-collection biases in microbiome profiles that could lead to misleading interpretations. It is also important to ensure that all the samples are stored in the same manner to avoid any bias. For these reasons, all the samples collected as part of the work with AIS and Monash University were appropriately stored in OMNIgene tubes.

The results in Chapters 3 and 4 were enabled by the Supernova 1 study via AIS, which aimed to investigate the effect of different approaches to dietary carbohydrate intake on metabolism, economy and performance of world-class endurance race walkers. The saliva and stool samples were collected from a cohort of 21 male athletes prior to the 2016 Brazil Olympics. While several studies have been published examining diet x microbiome interactions in elite athletes (63,68) these studies were conducted with "power sports" and to my knowledge, these results presented in Chapters 3 and 4 (and now published) are the first with elite "endurance sports" athletes.

The human oral cavity harbours billions of bacteria and is one of the most investigated microbiomes due to its association with several oral infectious diseases, periodontal diseases, and caries. The oral microbiota is also known to contribute to significant non-communicable diseases such as diabetes, cardiovascular diseases and cancers (73). However, little is known about the oral microbiome of athletes, and any possible links with athlete performance. The analyses from Chapter 3 showed that following the consumption of the LCHF diet, the relative abundances of *Haemophilus, Neisseria* and *Prevotella* spp. were decreased, while the relative abundance of *Streptococcus* spp. was increased. Interestingly, the bacterial taxa found to decrease following the consumption of the LCHF diet are known to be the key governors of the enterosalivary nitrate-nitrite-nitric oxide (NO) axis within the oral cavity. Previous studies have demonstrated associations between the LCHF diet and impaired plasma nitrate/nitrite conversion following supplementation with potassium nitrate, compared with the response to a HCHO diet in healthy active men (115) suggesting that a LCHF diet might alter the nitrate-nitrite-NO pathway to NO-related health and performance benefits in athletes. One of the major outcomes of the parent Supernova 1 study was a reduction in the exercise economy (i.e. an increased oxygen cost of exercise) across a range of walking speeds in the LCHF group (99). So, it seems plausible that this reduced exercise efficiency might be attributed in part to an altered oral microbiome. In particular, the results from this work highlights interest in further investigation of the effect of ketogenic LCHF diets on the functional potential of oral microbiome, its relationship with NO homeostasis, and downstream impacts on cardiovascular, circulatory and metabolic function.

Results from Chapter 4 showed that the stool microbiota profiles of elite race walkers could be separated into "*Bacteroides*" and "*Prevotella*" dominated enterotypes similar to what has been reported on healthy mainstream subjects and other endurance athletes (68,123,124) and these enterotypes appeared to be stable after the 3-week dietary interventions. Highly digestible carbohydrates are often the preferred source of energy for endurance sports athletes, somewhat in contrast to the diets favoured by power athletes, where protein can account for considerably more of the total energy intake. As such, the somewhat subtle impacts from the HCHO and PCHO dietary interventions on the stool microbiota could be that these diets were similar to the typical diets consumed by the endurance athletes and/or the carbohydrate content of the diets might be digestible and with little resistant carbohydrates available to the colonic microbiota. The ketogenic LCHF diet did invoke more profound changes on the stool microbiota profiles of the athletes as compared to the HCHO and PCHO dietary interventions, with a significant increase in the relative abundances of *Bacteroides* and *Dorea* and decrease in the relative abundance of *Faecalibacterium*. Wu et al. (124) have shown that *Bacteroides* spp. and a *Bacteroides-dominant* enterotype is found in healthy mainstream subjects who report long-term dietary pattern rich in animal fats and protein, whereas the *Prevotella-dominant* enterotype was found in those subjects with a long-term dietary pattern of high fibre containing foods. Shankar et al. (136) have reported similar findings from their comparative study of US and Egyptian children, considered to consume diets rich in animal products and processed foods (US) or a Mediterranean-type diet of whole grains, complex carbohydrates and lesser amounts of animal proteins and fats. Furthermore, the relative abundance of *Bacteroides* and *Dorea* particularly in response to the LCHF diet were also found to be significantly negatively associated with fat oxidation and economy measures, respectively.

Although the findings are largely enabled by the repeated sampling from each athlete (at entry and after the 3-week dietary intervention period), I feel the study would have enhanced and with greater impact if the Supernova 1 study had, like the studies of Clarke et al. (63) also recruited a non-athlete cohorts of subjects with similar or "normal" BMI, and consumed the same types of diets. Unfortunately, it was also not possible for the AIS team to compile a detailed food diary for each athlete during the study periods. Furthermore, the small sample size and the lack of comprehensive data on the athletes habitual baseline diet are some of the limitations of this work. However, and despite these limitations the results arising from these two Chapters do justify the additional studies of how diet x microbiome interactions could affect athletic performance, and in particular, the impacts of dietary pattern on the oral and stool microbiome and its effects on physiology and athlete performance.

The final Chapter of the thesis provided me more opportunities to expand my skills in microbiota data analysis via the combined use of 16S rRNA and ITS2 amplicon data as well as MGS data. It has expanded my knowledge from the bacterial world to fungal communities, and the potential impacts of inter-Kingdom associations on the function of the gut microbiome in healthy adults. I believe the study is the first of its kind to produce a combination of 16S rRNA, ITS2 and MGS data to investigate how a diet designed to provide a low and moderate prebiotic content affects the gut microbiota of healthy human subjects. The analysis showed that although MGS provides increased taxonomic resolution of the Bacterial Domain, the data produced at the intended sequencing depth (3 Gbp), which currently is a cost-effective compromise, comes with a caveat of limited or no detection of important but less abundant taxa such as gut fungi and archaea. Overall, the findings are similar to those reported by Douglas et al. (216) where 16S amplicon-based profiling performed better than MGS for detecting less abundant taxa. The sequencing was performed at ACE in two batches approximately one year apart (i.e. BL, LP and MP samples for subjects 1-9; and then BL, LP and MP samples for subjects 10-18). and the first batch of sequencing was found to produce less paired end reads, and the coverage estimates indicated differences in coverage between the first and second datasets. However, the alpha and beta diversity metrics for the 16S rRNA gene amplicon data provided clear evidence that the comparisons between diets were consistent, and while the MGS sequencing might have a bearing on the statistical significance of the differences observed, it has not impacted the biological relevance of the findings presented here.

Even though the principal aim of the study presented in Chapter 5 was to compare the effects of the prebiotic content of two diets on gut microbiome of the subjects, it still was worthwhile to study just the baseline profiles of the subjects, which represent the microbiota resident within these subjects in response to their habitual dietary pattern. Baseline samples were collected from 14 subjects. The unsupervised beta diversity PCoA plots of the 16S rRNA gene amplicon data using the Bray-Curtis distance measure revealed that the subjects at baseline could be separated into no less than 3 distinct clusters (Figure 6.1a). The genus-level profiles of these three clusters are shown in Figure 6.1c, with 5/14 subjects found to possess a "*Bacteroides-Faecalibacterium* dominant" communities (cluster 1) and 6/14 subjects possessing remarkable abundance of *Methanobrevibacter* (cluster 2). The remaining 2 subjects clustering together and having higher abundance of "*Prevotella 9*" (cluster 3) and 1 subject having genus level profiles similar to that of cluster 1. Adonis further confirmed significant difference between clusters 1 and 2. I was unable to perform any statistical test on the remaining 3 points on the PCoA plot due to low number of samples in each group. Furthermore, these clusters were retained following the dietary-intervention periods, as shown when these profiles were included in the beta diversity analysis (Figure 6.1b).

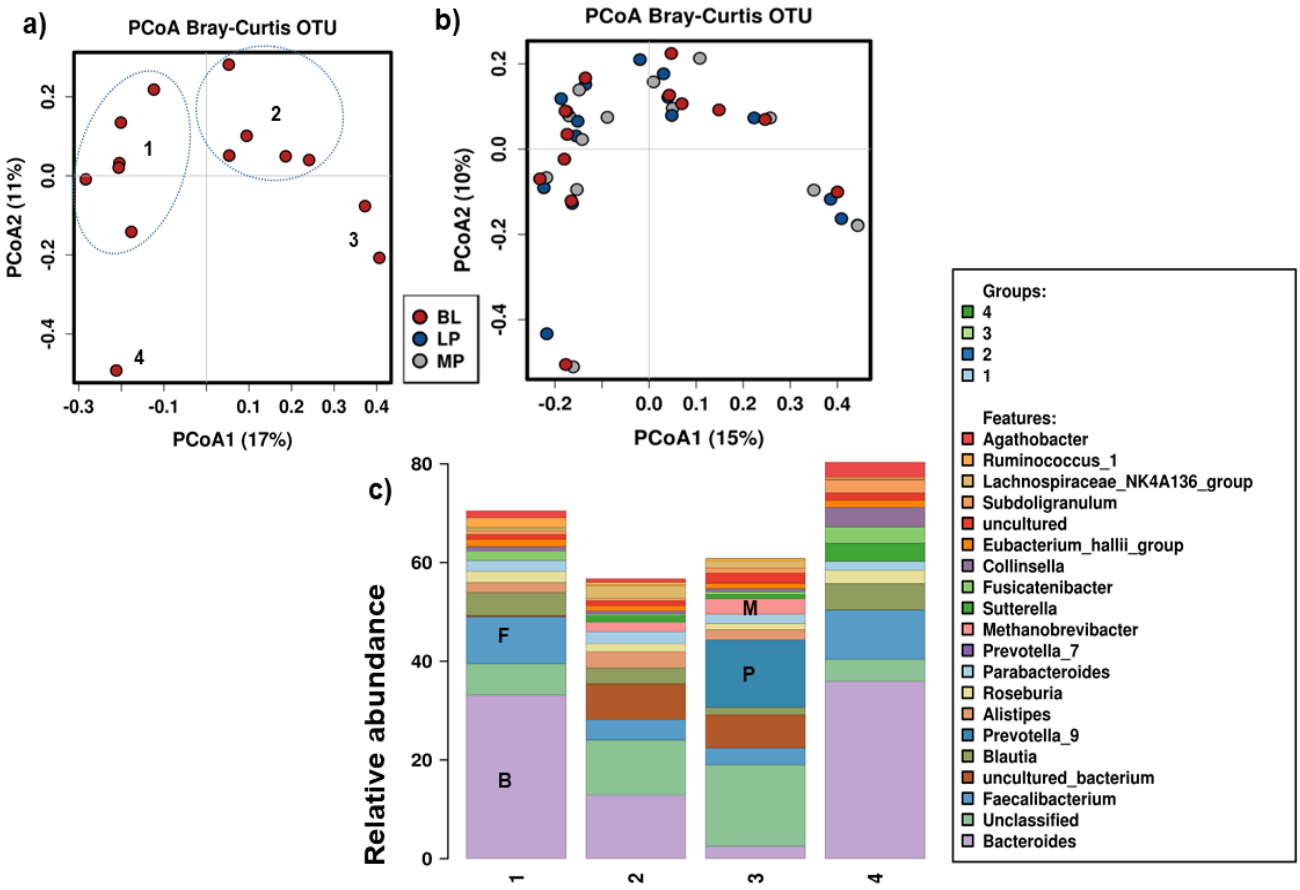


Figure 6.1 Principal coordinates analysis (PCoA) of the Bray-curtis distances produced from 16S rRNA data of the stool samples of subjects collected at baseline (BL; red) only (**a**); and combined with their matching stool samples collected after the Low Prebiotic (LP; blue) and Moderate Prebiotic (MP; grey) dietary-interventions period (**b**). The subjects are separated into three distinct clusters and importantly, this clustering did not appear to be disrupted in response to the dietary intervention. The first two Principal Coordinates and the amount of variation each explains are shown (PCoA1, PCoA2). (**c**) The profiles of the predominant taxa present in the baseline stool samples of with either a *Bacteroides* (B) and *Faecalibacterium* (F)-dominant (cluster 1; n= 6), a *Prevotella* 9 (P) and *Methanobrevibacter* (M) dominant (cluster 3; n = 2) or a mixed cluster (cluster 2; n = 5) "enterotype".

I then wanted to investigate whether there was a prebiotic diet x cluster interaction evident in these subjects similar to what was found with LCHF x enterotype interaction in Chapter 3. However, paired t-test revealed no significant differences between the BL vs. LP, BL vs. MP and LP vs. MP groups in either of the two clusters (Appendix Figure 7.13 and Figure 7.14). This suggests that even though there appears to be some clustering of the subjects at BL, there were no diet x cluster interactions and the clustering observed from BL data had no significant effect on the response of these subjects

towards the prebiotic dietary interventions. As there was no statistical difference between the groups at BL, further analysis was performed using the group samples as an LP vs. MP comparison.

The results presented in Chapter 5 show that a specific impact of the MP diet was to increase the gene counts involved with sugar alcohol metabolism and their associated transport (via PEP-PTS systems). Polyols such as lactitol and isomalt are well recognised to selectively enrich *Bifidobacterium* spp. abundance (206,207) and I believe the moderate amounts of both sorbitol and mannitol provided with the MP diet are the key factor behind the increase of select *Bifidobacterium* spp. with this diet. In that context, the Bifidobacterial MAGs that were produced from the MGS data from subjects consuming the MP diet favoured strains with the distinct ability to metabolise sugar alcohols, which further validates the veracity of the taxonomy and the functional profiling results. In addition to *Bifidobacterium*, an increase in relative abundances of *Anaerostipes* and *Eubacterium* following the consumption of the MP diet was also observed. Additionally, from the correlation analysis, significant positive associations of these taxa with found with *Bifidobacterium,* suggesting mutualistic cross-feeding interactions between these bacterial communities.

The results from the ITS2 amplicon profiles identified increase in the relative abundances of *Candida*, *Agaricus* and *Aspergillus* in response to the MP diet. Though not much is known about the effect of diets on gut fungi, one study has reported a positive association of carbohydrate-based diets and *Candida* in healthy subjects (61). However, the biological importance of these gut fungi in host health warrants further consideration. Scant reports are available on the bacterial-fungal-archaeal associations in the healthy human gut. Disease specific trans-kingdom alterations between IBD and healthy subjects have been shown by Sokol et al (53) and in this regard, the results have also shown distinct associations between the bacterial and fungal community members of the gut. Amongst the fungi, *Saccharomyces* was found to have more associations with bacterial taxa, and interestingly, strong positive correlations with *B. longum*, *A. hadrus* and *E. hallii;* all of which are increased following the consumption of the MP diet.

Fermentable fibres stimulate the growth of colonic microbes and the production of microbial metabolites including short-chain fatty acids (SCFA). The systematic review and meta-analysis by So et al. (146) reported that while fecal butyrate concentrations are increased in response to dietary fibre supplements in healthy individuals, no significant changes in other SCFA concentrations are observed. I have calculated the molar proportions of acetate, propionate and butyrate in stool samples and found there were no significant differences in the molar ratios of these SCFAs between the LP and the MP diets (Figure 6.2). These results suggest that the magnitude of the microbiota shifts induced by the MP diet are not sufficiently large to drive a shift in the SCFA profiles in stool. These

findings corroborate those reported by Baxter et al. (194) that showed microbiomes with relatively larger populations of Bifidobacteria do not result in an increase in butyrate production in response to inulin or resistant potato starch even though increase in the relative abundances of butyrate producers such as *A. hadrus* and *E. rectale* were observed.

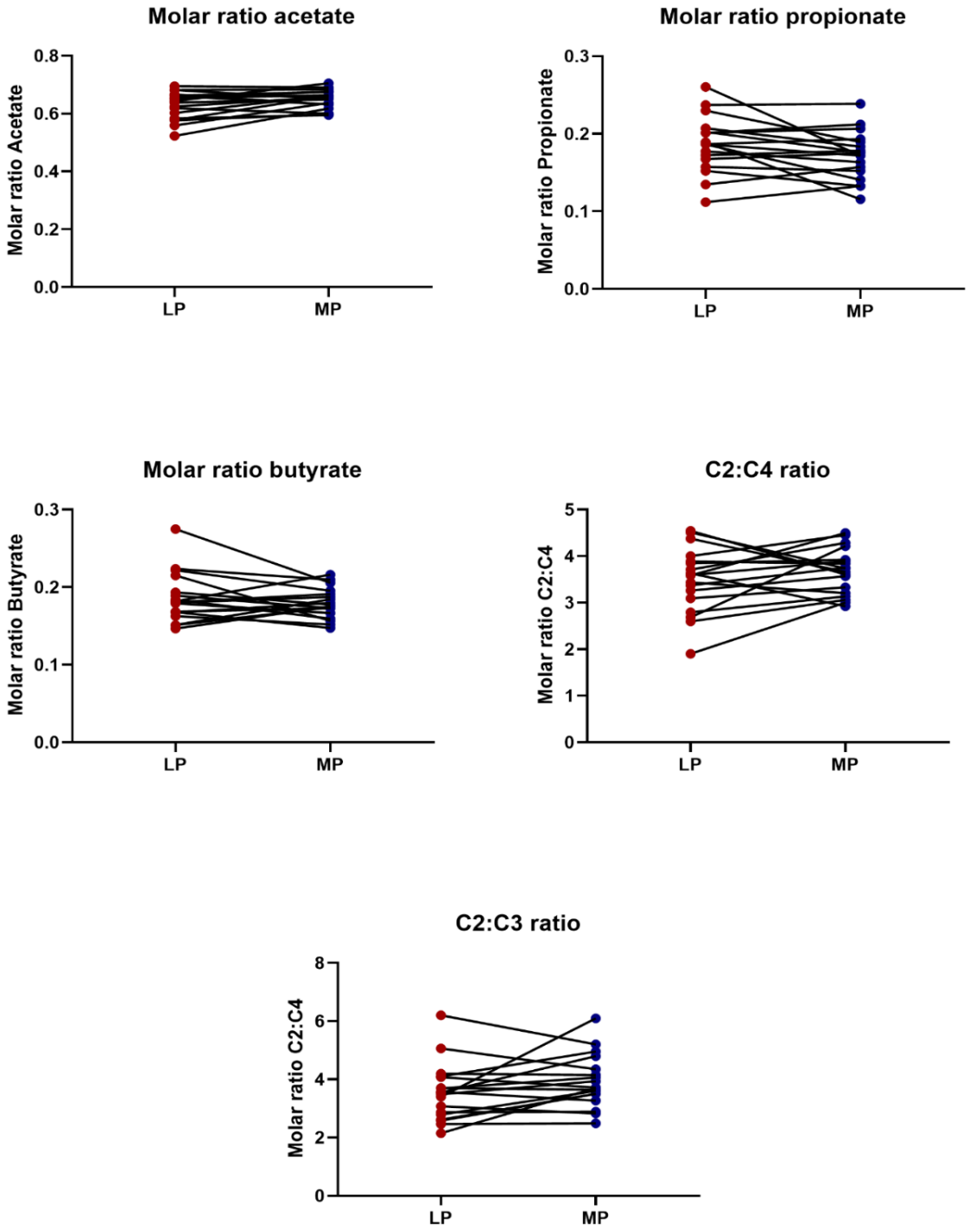


Figure 6.2 Molar ratios of short chain fatty acids (SCFAs) acetate, propionate, butyrate and acetate:propionate (C2:C3) and acetate:butyrate (C2:C4) ratios in subjects following the consumption of Low Prebiotic (LP; red) and Moderate Prebiotic (MP; blue) diets.

In relation to the studies presented in Chapter 5 there are several possible directions for future research. Our collaborators at Monash University have collected data measuring gut symptoms, stool frequency and output, as well as measures of ammonia, p-cresol and SCFA. It will be interesting to

determine whether and how these data might be associated with the gut microbiota profiles that were observed following consumption of the LP and MP diets. The production of more detailed metabolomics data would also advance our understanding of the interactions between the microbes, their metabolites, dietary factors and the host. Such a multi-omics approach was beyond the scope of my Ph.D. research. The DNA samples could also be used to determine the absolute abundances of select bacterial, fungal and archaeal taxa. For instance, qPCR analyses of select Bifidobacteria (*B. longum*, *B. adolescentis* and *B. animalis*) would further confirm the enrichment of these species in response to either the LP or MP diet. Similarly, qPCR analyses of other bacterial taxa such as *Anaerostipes* and *Eubacterium* spp. could also be performed. However, the adaptation of qPCR approaches to fungal taxa such as *Saccharomyces*, *Candida*, *Agaricus*, and *Aspergillus* would further confirm the actual population size of the microbes in gut communities and how shifts in their absolute abundance relative to bacteria and archaea affect health. The cross-over study design used for the Monash study does raise the possibility that "carry over" effects might have influenced the changes in microbiota profiles. Although I feel my results are not significantly impacted by any carryover effects, I would recommend sampling at the end of the washout period (as well as increasing the time for the washout period) to ensure that the microbiota profiles at the end of the washout period are similar to the subject's habitual or baseline microbial profiles, so any effect(s) arising from intervention can be assessed.

In conclusion, this thesis presents novel insights of diet x microbiome interactions from samples collected as part of two longitudinal nutritional trials of either healthy mainstream adults, or elite endurance athletes. I believe more of these types of studies are needed, to better understand the nutritional ecology of the gut, the characteristics of a "healthy microbiome", and how elastic the healthy gut microbiome may be to dietary interventions. Such understanding is needed to complement the relative extensive examination of the microbial dysbiosis observed with chronic, non-communicable diseases such as IBD, obesity and other diet-related disorders. Only when the "healthy microbiome" is better characterised can the development of diets with either preventative or corrective impacts on the gut microbiota be developed and used with greater confidence for meaningful impacts on health or well-being. To that end, I believe my efforts are both meaningful and insightful to our achievement of these goals for improved health and nutrition.

Chapter 7

Chapter 7 Appendix

7.1 Sequence clean up protocol

Reagents needed:

AMPure XP beads (bring to room temp)	20µL per sample
80% ethanol (prepare fresh)	400µL per sample
10mM Tris pH 8.5	52.5µL per sample
Magnetic bead stand	
Dispensing trough (for multichannel pipette)	

1. Use 25µL of the amplicon PCR product, in PCR tubes/96-well PCR plate for the clean-up.
2. Centrifuge the amplicon PCR plate at 1000 x g for 1 min (can use little green benchtop spinner; or large benchtop centrifuge if using plate).
3. Vortex AMPure beads for 30 sec. Add to dispensing trough.
4. Add 20µL of beads to each PCR sample (use multichannel). Gently pipette up and down 10 times to mix.
5. Incubate at room temp. for 5 mins.
6. Place in magnetic stand for 2 mins, or until supernatant has cleared.
7. While still in the magnetic stand, remove and discard supernatant (use multichannel)
8. While still in the stand, wash the beads with 80% ethanol.
    a. Add 200µL of ethanol to reaction PCR tube (use multichannel)
    b. Incubate for 30 seconds
    c. Carefully remove and discard supernatant.
9. Repeat a second ethanol wash as above. After this one, use a P20 pipette to remove any residual ethanol.
10. While still in the magnetic stand, let the beads air dry for 10 mins.
11. Remove amplicon PCR tubes from stand. Add 52.5 µL of 10mM Tris to each PCR tube. Gently pipette up and down 10 times to mix. Make sure beads are fully resuspended.
12. Incubate at room temp for 2 mins.
13. Place in magnetic stand for 2 mins, or until supernatant has cleared.
14. Carefully transfer 50µl of the supernatant into a clean labelled tube.

7.2 Supplementary figures Chapter 3

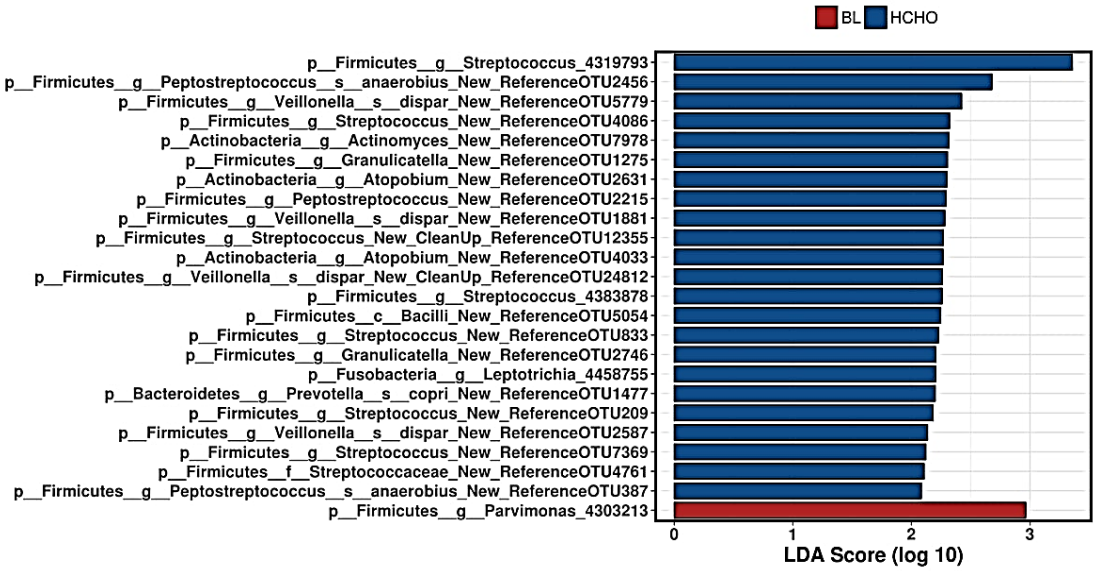


Figure 7.1 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the High Carbohydrate diet (HCHO, blue) identified by LefSe.

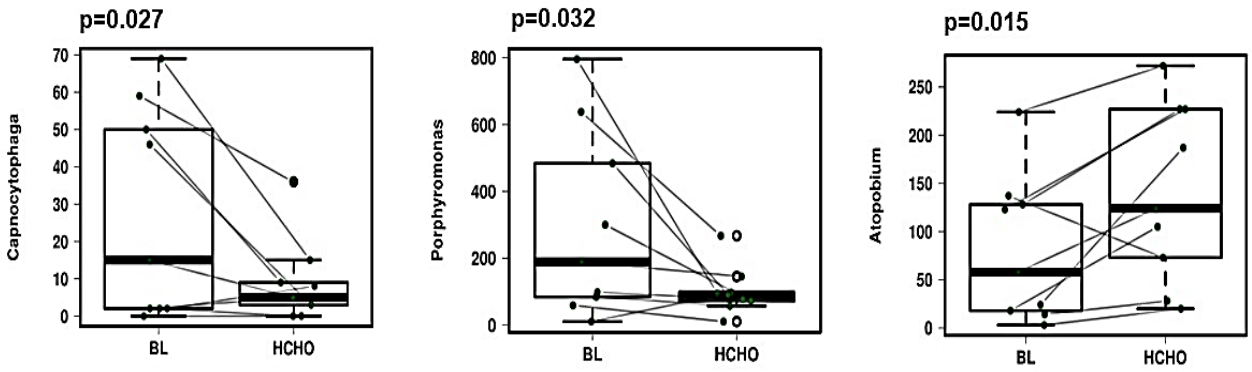


Figure 7.2 Mixed effect linear regression analysis of the oral microbiota profiles at BL and following High Carbohydrate (HCHO) diet training intervention identified significant reductions in the relative abundances of *Capnocytophaga* (p=0.027) and *Porphyromonas* (p=0.032) whereas significant increase in the relative abundance of *Atopobium* (p=0.015) following the HCHO diet training intervention. Relative abundance was compared by mixed effect linear regression, including sampling

time point as fixed effect and athlete as random effect. BL: baseline. Samples collected from the same individual are connected by lines.

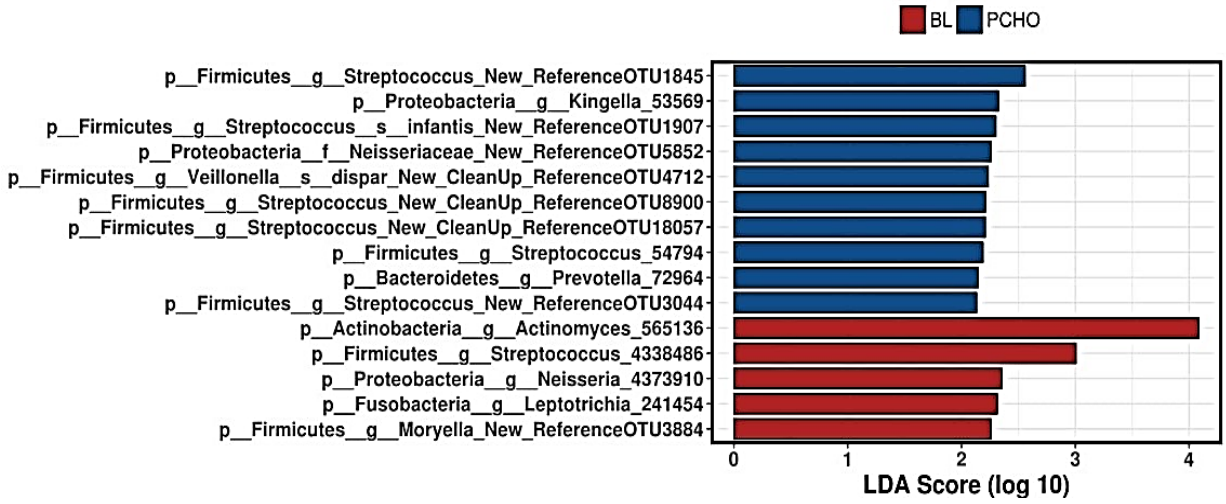


Figure 7.3 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Periodised Carbohydrate diet (PCHO, blue) identified by LefSe.

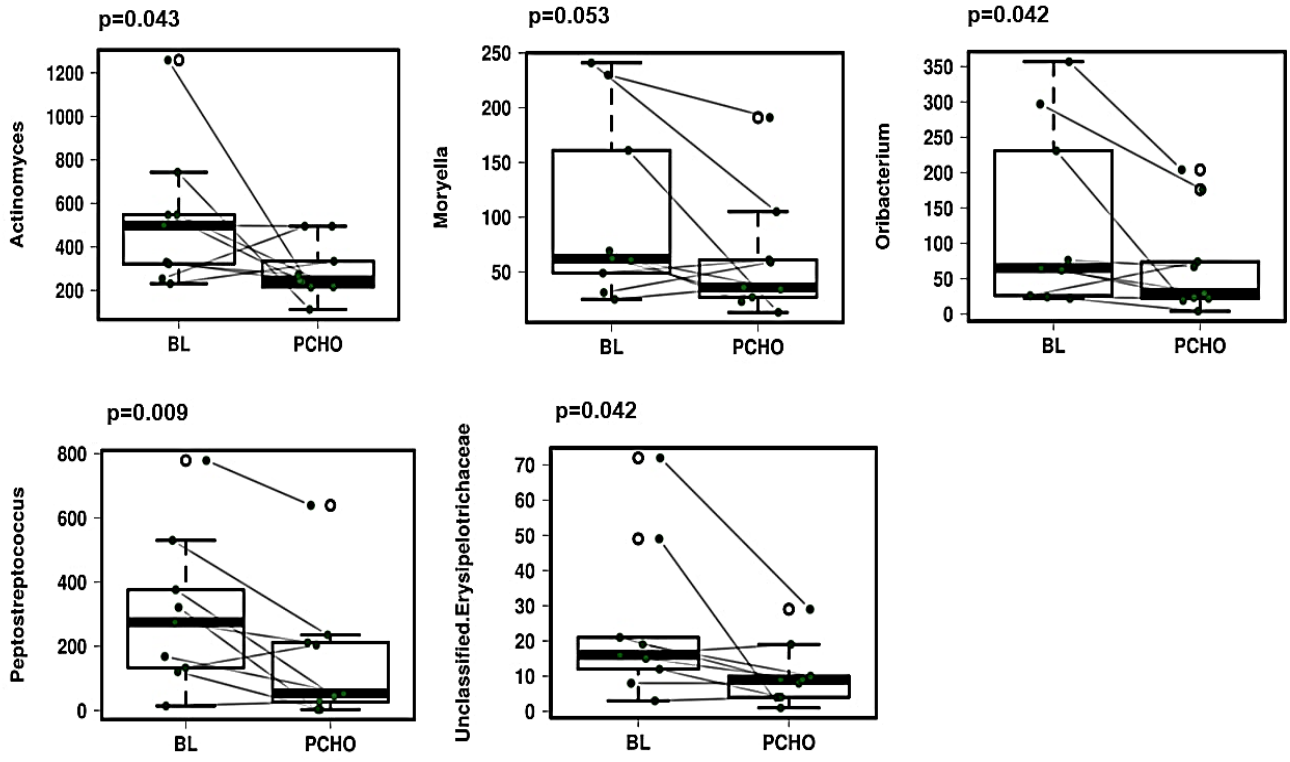


Figure 7.4 Mixed effect linear regression analysis of the oral microbiota profiles at BL and following Periodised Carbohydrate diet (PCHO) training intervention identified significant reductions in the relative abundances of *Actinomyces* (p=0.043), *Moryella* (p=0.053), *Oribacterium* (p=0.042), *Peptostreptococcus* (p=0.009) and *Unc. Erysipelotrichaceae* (p=0.042) after consumption of the PCHO diet. Relative abundance was compared by mixed effect linear regression, including sampling time point as fixed effect and athlete as random effect. BL: baseline. Samples collected from the same individual are connected by lines.

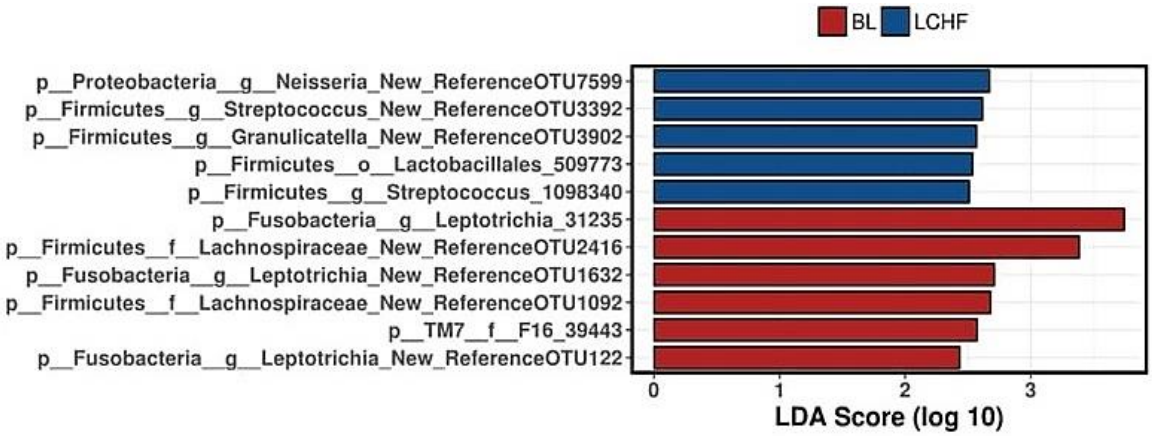


Figure 7.5 Genera differentiating between the oral microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Low Carbohydrate High Fat diet (LCHF, blue) identified by LefSe.

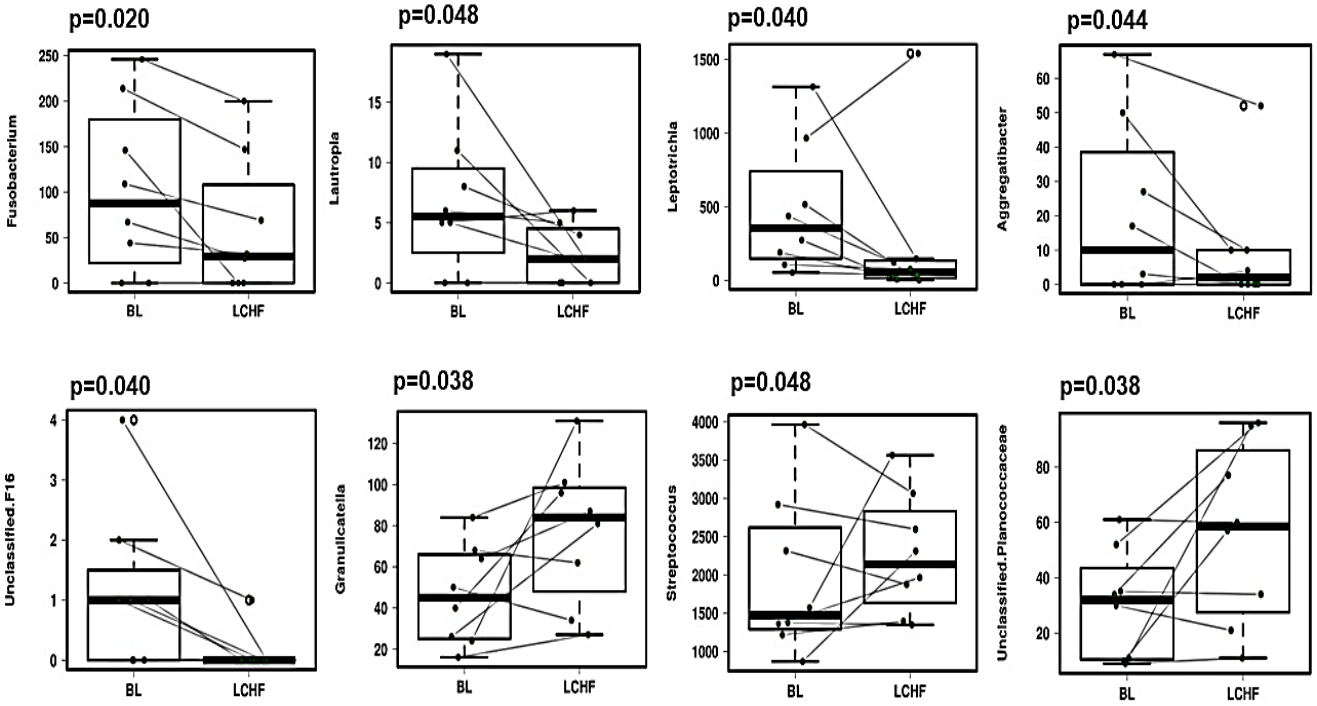


Figure 7.6 Mixed effect linear regression analysis of the oral microbiota profiles at BL and following Low Carbohydrate High Fat (LCHF) diet training intervention identified significant reductions in the relative abundances of *Fusobacterium* (p=0.020), *Lautropia* (p=0.048), *Leptotrichia* (p=0.040), *Aggregatibacter* (p=0.044) and Unc. F16 (p=0.040) whereas significant increase in the relative abundances of *Granulicatella* (p=0.038), *Streptococcus* (p=0.048) and *Planococcaceae* (p=0.038) after consumption of the LCHF diet. Relative abundance was compared by mixed effect linear regression, including sampling time point as fixed effect and athlete as random effect. BL: baseline. Samples collected from the same individual are connected by lines.

7.3 Supplementary figures Chapter 4

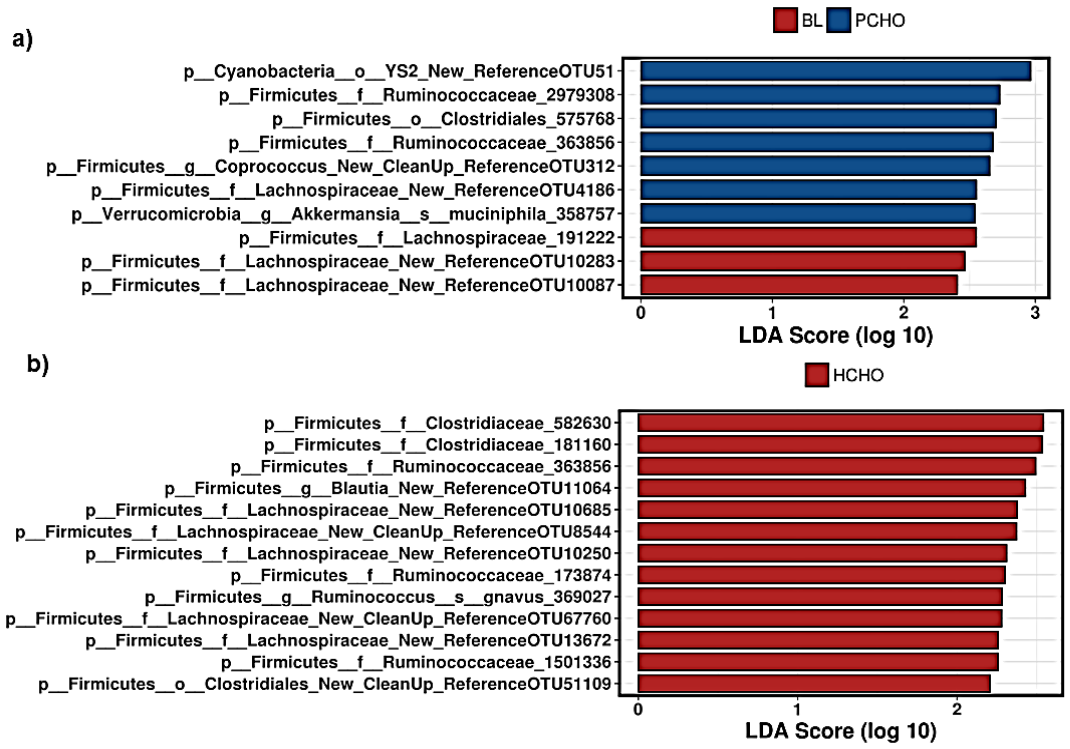


Figure 7.7 Genera identified by LefSe analyses that differentiate between the stool microbiota profiles of athletes at baseline (BL, red) or after their consumption of the Periodised Carbohydrate diet (PCHO, blue) **(b)** Genera identified by LefSe analysis that differentiate between the stool microbiota profiles of athletes at baseline (BL, blue) and after their consumption of the High Carbohydrate diet (HCHO, red).

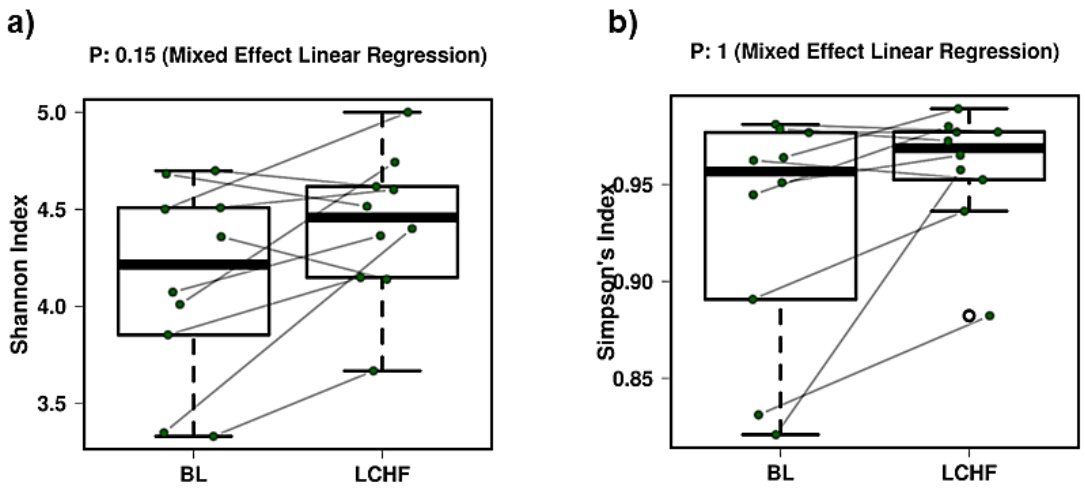


Figure 7.8 Shannon and Simpson alpha diversity measures of the stool microbiota profiles for athletes at baseline (BL) and after their consumption of the Low Carbohydrate High Fat diet (LCHF). The diversity measures at the two sampling times were compared by mixed effect linear regression, including sampling time point as fixed effect and athlete as a random effect, and were not statistically different.

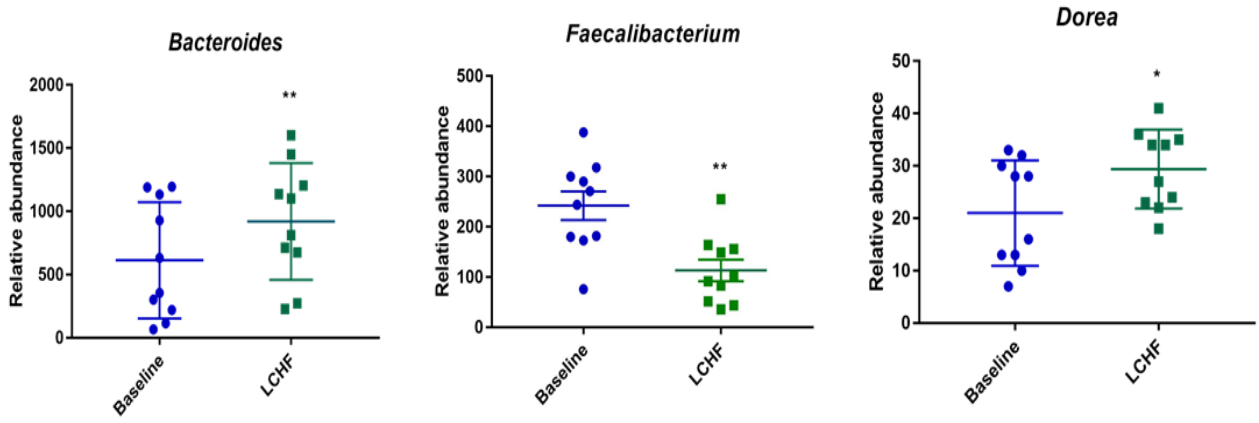


Figure 7.9 Wilcoxon rank t-test of the stool microbiota profiles for athletes at baseline (BL) and after their consumption of the Low Carbohydrate High Fat diet (LCHF) identified significant reductions in the relative abundances of *Faecalibacterium* (p=0.0003) and significant increases in the relative abundance of both *Bacteroides* (p=0.0022) and *Dorea* (p=0.0068) after consumption of the LCHF diet.

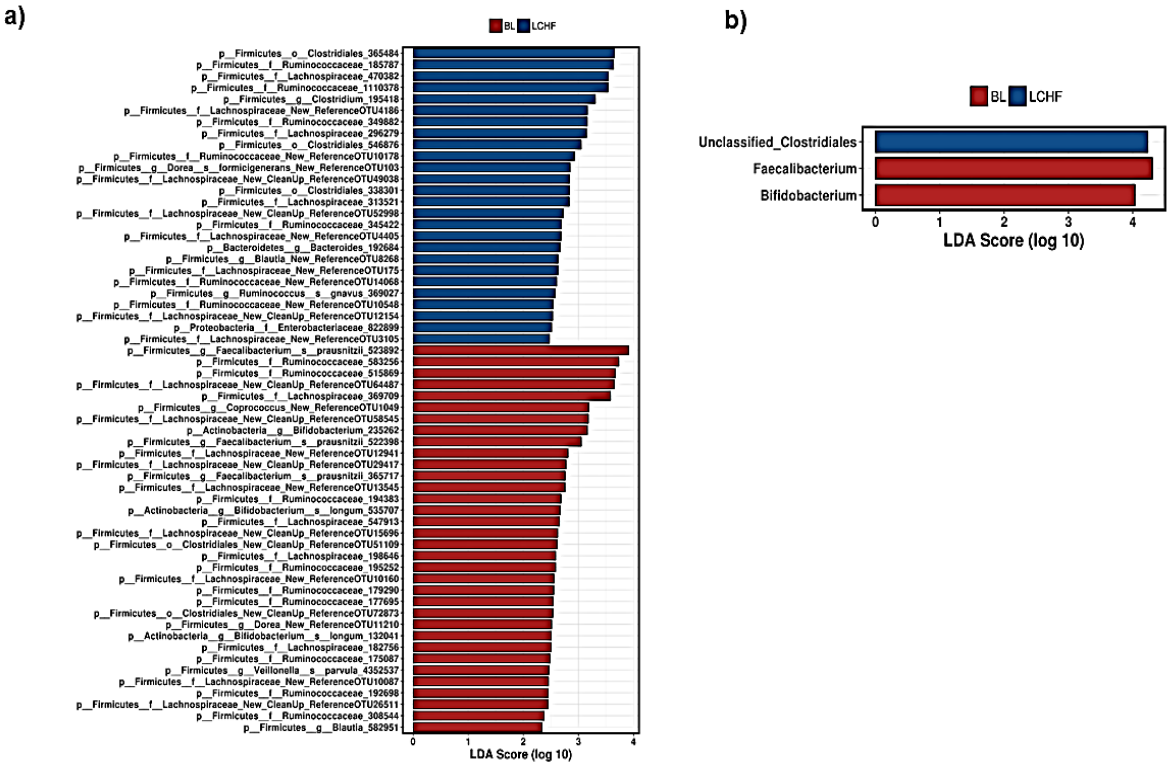


Figure 7.10 Genera identified by LefSe analysis that differentiate between the stool microbiota profiles of athletes at baseline (BL, red) and after their consumption of the Low Carbohydrate High Fat diet (LCHF, blue) at either the OTU level **(a)**, or at the genus level **(b)**.

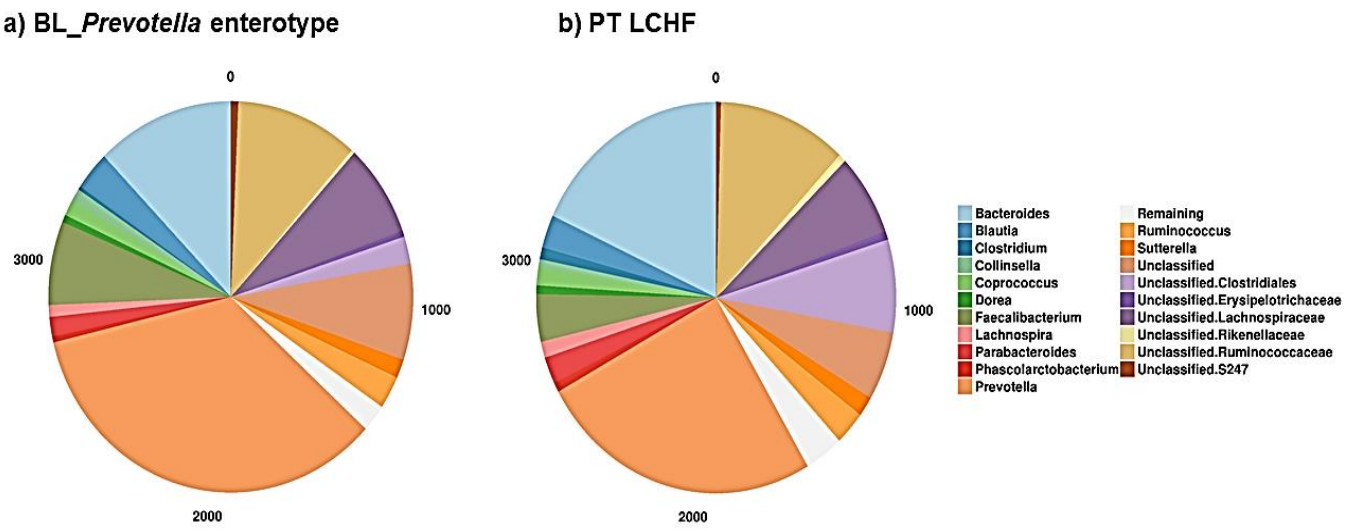


Chart 7.1 Pie charts showing the genus level profiles of those athletes with the *Prevotella*-dominant enterotype at: **(a)** Baseline (BL) and **(b)** Post treatment (PT), i.e. after consumption of the Low Carbohydrate High Fat (LCHF) diet. The relative abundance of *Bacteroides* and *Unc. Clostridiales* were increased, whereas the relative abundance of *Faecalibacterium* was decreased in these athletes, PT.

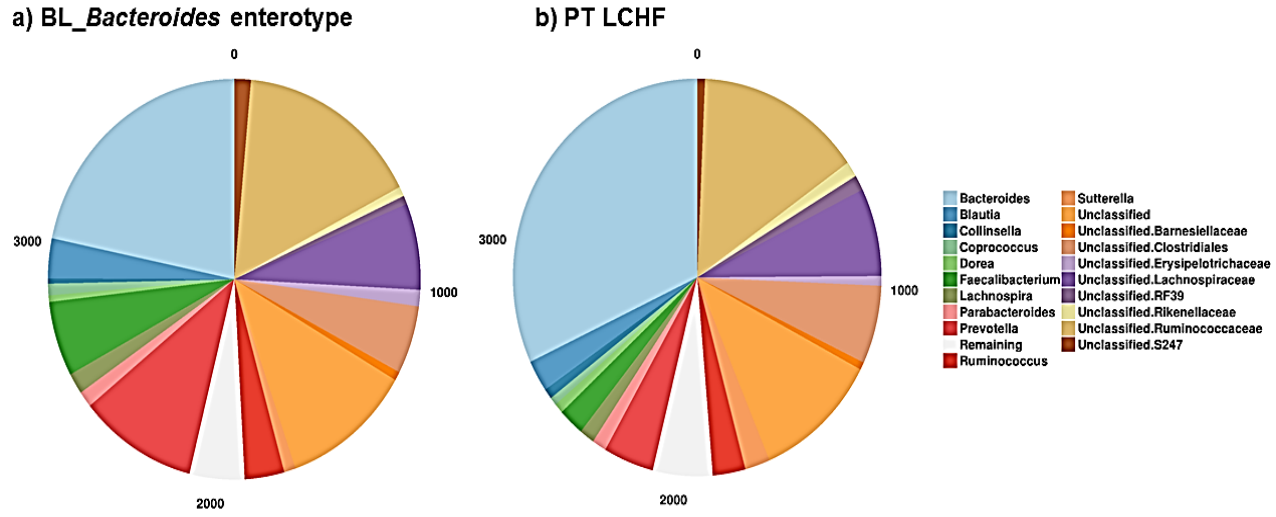


Chart 7.2 Pie charts showing the genus level profiles of those athletes with the *Bacteroides*-dominant enterotype at: **(a)** Baseline (BL) and **(b)** Post treatment (PT), i.e. after consumption of the Low Carbohydrate High Fat (LCHF) diet. The relative abundance of *Bacteroides* and *Sutterella* were increased, whereas the relative abundance of *Faecalibacterium* was decreased in these athletes, PT.

7.4 Supplementary figures Chapter 5

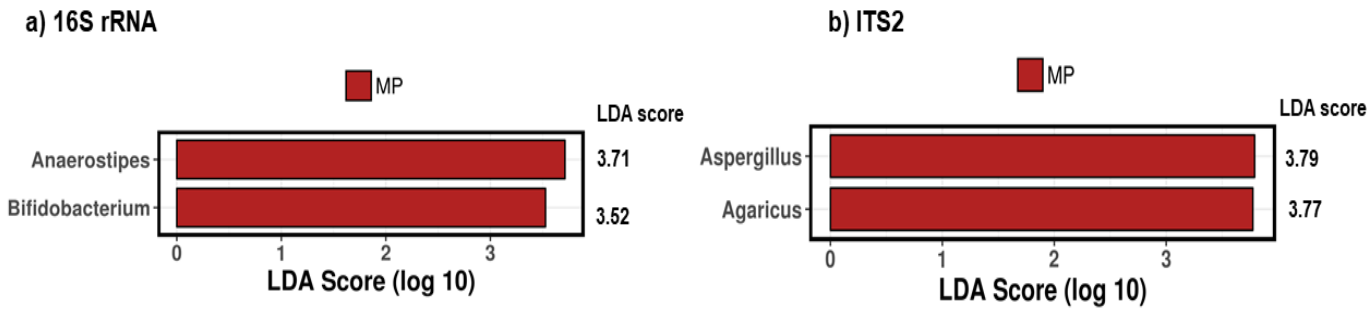


Figure 7.11 Bacterial (16S rRNA) and Fungal (ITS2) genera differentiating the stool microbiota profiles of subjects after consumption of the Moderate Prebiotic (MP; red) diet identified by LefSe.

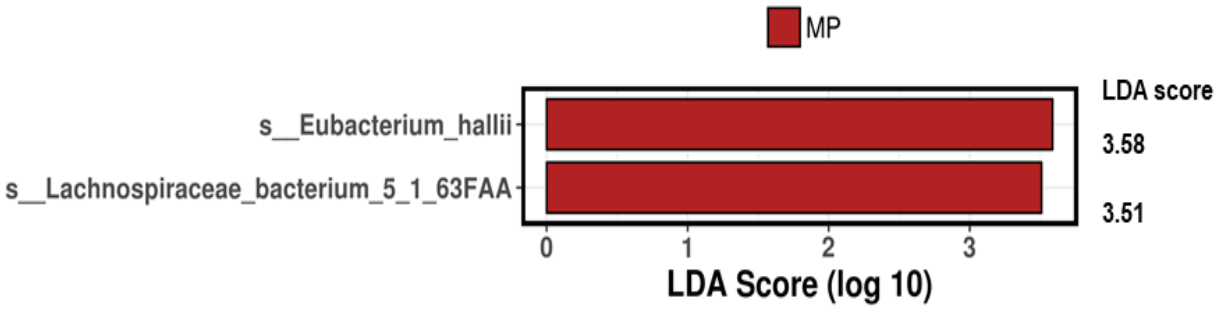


Figure 7.12 Bacterial species (from the MGS data) differentiating the stool microbiota profiles of subjects after consumption of the Moderate Prebiotic (MP; red) diet identified by LefSe.

7.5 Supplementary figures Chapter 6

CLUSTER 1: Paired t test LP vs MP (N=6)

Taxa	Group	P	P.adj	FDR qValue	MeanLP	MeanMP
Eubacterium_hallii_group	All groups	0.0310	1.0000	0.7750	275.1667	335.0000
Bifidobacterium	All groups	0.0310	1.0000	0.7750	60.6667	219.8333
Subdoligranulum	All groups	0.0590	1.0000	0.7750	77.0000	191.0000

CLUSTER 1: Paired t test BL vs MP (N=6)

Taxa	Group	P	P.adj	FDR qValue	MeanBL	MeanMP
Lachnospiraceae_NK4A136_group	All groups	0.0310	1.0000	0.5714	103.3333	270.8333
Ruminococcaceae_UCG003	All groups	0.0310	1.0000	0.5714	46.0000	111.1667
Lachnospiraceae_ND3007_group	All groups	0.0630	1.0000	0.5714	79.6667	153.5000

CLUSTER 1: Paired t test BL vs LP (N=6)

Taxa	Group	P	P.adj	FDR qValue	MeanBL	MeanLP
Subdoligranulum	All groups	0.0590	1.0000	0.6111	191.8333	77.0000
Butyricicoccus	All groups	0.0630	1.0000	0.6111	70.3333	161.8333
Ruminococcaceae_UCG003	All groups	0.0630	1.0000	0.6111	46.0000	86.3333

Figure 7.13 Wilcoxon rank paired t-test of stool microbiota profiles of healthy subjects from Cluster 1 (high *Bacteroides* and high *Faecalibacterium* cluster, n=6) identified no significant changes in the relative abundances of bacteria when comparisons were made between Low Prebiotic (LP) vs Moderate Prebiotic (MP); Baseline (BL) vs. LP; BL vs. MP groups.

CLUSTER 2: Paired t test LP vs MP (N=5)

Taxa	Group	P	P.adj	FDR qValue	MeanLP	MeanMP
Ruminococcaceae_UCG014	All groups	0.0580	1.0000	0.8267	173.0000	51.0000
Subdoligranulum	All groups	0.0620	1.0000	0.8267	124.2000	159.2000
Dorea	All groups	0.0620	1.0000	0.8267	87.0000	116.4000
Anaerostipes	All groups	0.0620	1.0000	0.8267	40.8000	157.6000
Lachnospiraceae_UCG004	All groups	0.0620	1.0000	0.8267	63.0000	36.6000
Bifidobacterium	All groups	0.0620	1.0000	0.8267	22.0000	57.2000

CLUSTER 2: Paired t test BL vs MP (N=5)

Taxa	Group	P	P.adj	FDR qValue	MeanBL	MeanMP
Ruminococcaceae_UCG005	All groups	0.0620	1.0000	1.0000	201.2000	130.8000
Fusicatenibacter	All groups	0.0620	1.0000	1.0000	106.4000	155.0000
Anaerostipes	All groups	0.0620	1.0000	1.0000	68.6000	157.6000
Parasutterella	All groups	0.0620	1.0000	1.0000	49.8000	26.2000

CLUSTER 2: Paired t test BL vs LP (N=5)

Taxa	Group	P	P.adj	FDR qValue	MeanBL	MeanLP
Erysipelotrichaceae_UCG003	All groups	0.0580	1.0000	0.6857	58.8000	91.0000
Faecalibacterium	All groups	0.0620	1.0000	0.6857	733.8000	1056.4000
Ruminococcus_1	All groups	0.0620	1.0000	0.6857	155.0000	334.8000
Ruminococcaceae_UCG005	All groups	0.0620	1.0000	0.6857	201.2000	127.2000
Lachnospira	All groups	0.0620	1.0000	0.6857	50.0000	105.4000
Ruminococcaceae_UCG013	All groups	0.0620	1.0000	0.6857	23.6000	46.8000
Parasutterella	All groups	0.0620	1.0000	0.6857	49.8000	28.4000

Figure 7.14 Wilcoxon rank paired t-test of stool microbiota profiles of healthy subjects from Cluster 2 (mixed cluster, n=5) identified no significant changes in the relative abundances of bacteria when comparisons were made between Low Prebiotic (LP) vs Moderate Prebiotic (MP); Baseline (BL) vs. LP; BL vs. MP groups.

7.6 Bioinformatics scripts

16S rRNA sequence analysis: QIIME 1

- Unzipping all the .gz files and joining the paired end reads (To combine the forward and the reverse reads)

```
gzip . -rd *fastq.gz
multiple_join_paired_ends.py -i fastq_files -o joined_output
```

- Split Libraries (Quality control and demultiplexing step)

- To make a list of all the sequence file names and write to a file called output_names.

```
(cd joined_output && ls -d */) > output_names
```

- To make a comma-separated list of the sequence names and write to a file called step1.

```
sed 's_/_/fastqjoin.join.fastq_g' joined_output/output_names | tr "\n" , | sed 's/,$//' | sed 's/^/split_libraries_fastq.py -i /' > step1
```

- To make a barcode comma list and write to a file called step2.

```
tr "\n" , < sample_ids.txt | sed 's/,$/ --barcode_type 'not-barcoded' -q 19 -o split -m dummy_map.txt/' | sed 's/^/ --sample_id /' > step2
```

- To combine the files step1 and step2 together into a file called step3.

```
cat step1 step2 > step3
```

- Putting and running the script together.

```
sed -i "1 i\shellscript" step3
sed 's/shellscript/#!\/bin\/sh\n/' step3 > run_split_script.sh
chmod 700 run_split_script.sh
```

```
./run_split_script.sh
```

This result in a seqs.fna file which is then used for chimera filtering step.

- Identifying and filtering chimeras using USEARCH

```
identify_chimeric_seqs.py -i seqs.fna -m usearch61 -o usearch_checked_chimeras/ -r gg_97_otus_4feb2011.fasta

filter_fasta.py -f seqs.fna -o seqs_chimeras_filtered.fna -s chimeras.txt -n
```

- Assigning OTU's by open reference picking

```
pick_open_reference_otus.py -i seqs.fna -o workflow1/ -r 97_otus.fasta -s 0.1 -a
```

- Downstream filtering steps (Filtering low abundance OTU's (<0.01%), contaminated samples, blanks and samples with less than 1000 reads)

```
filter_otus_from_otu_table.py -i otu_table_micro.biom -o otu_table_micro_filtered.biom --min_count_fraction 0.0001

filter_samples_from_otu_table.py -i otu_table_filtered_excluded_below_1000reads.biom -o otu_table_filtered_excluded_below_1000reads_final.biom --sample_id_fp discard_controls.txt --negate_sample_id_fp
```

- Rarefaction of the OTU table

```
single_rarefaction.py -i otu_table_filtered_excluded_below_1000reads_final.biom -o otu_table_even1000.biom -d 1000
```

- Core diversity analysis

```
core_diversity_analyses.py -i otu_table_even1000.biom -o core_output -m map.txt -c Diet -t rep_set.tre -e 1000
```

Running Trimmomatic to remove adaptor sequences

[tnmurtaz@uqdi-login Nida]$ module load java
[tnmurtaz@uqdi-login Nida]$ java -jar /dmf/uqdi/HPC/PBSHOME/shared/applications/Trimmomatic-0.36/trimmomatic-0.36.jar PE -threads 24 SC7710_S1_R1_001.fastq.gz SC7710_S1_R2_001.fastq.gz S1_trimmed_1.fastq.gz S1_unpaired_1.fastq.gz S1_trimmed_2.fastq.gz S1_unpaired_2.fastq.gz ILLUMINACLIP:/dmf/uqdi/HPC/PBSHOME/shared/applications/Trimmomatic-0.36/adapters/NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 CROP:10000 HEADCROP:0 MINLEN:50

Merging the trim reads (R1 and R2)

[tnmurtaz@uqdi-login Nida]$ cat S1_trimmed_1.fastq.gz S1_trimmed_2.fastq.gz > S1.fastq.gz

Mapping against hg19 database to remove the human reads from the sample

[tnmurtaz@uqdi-login Nida]$ module load bowtie2_2.2.1
Bowtie2 (2.2.1) module loaded.
[tnmurtaz@uqdi-login Nida]$ bowtie2 -x hg19 -U S1.fastq.gz -S S1_mapped_and_unmapped.sam

Converting sam to bam format

[tnmurtaz@uqdi-login Nida]$ export MODULEPATH=/dmf/uqdi/Immunology/Morrison_Group/modules:$MODULEPATH
[tnmurtaz@uqdi-login Nida]$ export MODULEPATH=/dmf/uqdi/HPC/PBSHOME/shared/modules:$MODULEPATH
[tnmurtaz@uqdi-login Nida]$ module load samtools-1.3.1
[tnmurtaz@uqdi-login Nida]$ samtools view -bS S1_mapped_and_unmapped.sam > S1_mapped_and_unmapped.bam

Extracting unmapped reads from bam file

[tnmurtaz@uqdi-login Nida]$ samtools view -b -f 4 S1_mapped_and_unmapped.bam > S1_unmapped.bam

Converting unmapped bam to fastq

[tnmurtaz@uqdi-login Nida]$ samtools bam2fq S1_unmapped.bam > S1_unmapped.fastq

Script to run HUMAnN2 on unmapped fastq file

#!/bin/bash
#PBS -l select=1:ncpus=48:mem=248gb

```
#PBS -l walltime=100:00:00
pwd
cd /share/uqdi/Immunology/Morrison_Group/Nida
module load humann2/0.11.1
module load biopython/1.66
module load metaphlan/2.0
humann2 --input S1_unmapped.fastq --output S1_hummann2_50 --nucleotide-database
/dmf/uqdi/Immunology/Morrison_Group/Nida/ACE/humann2/chocophlan --diamond
/dmf/uqdi/Immunology/Morrison_Group/JingJie/POCER_MGS/diamond --protein-database
/dmf/uqdi/Immunology/Morrison_Group/Nida/ACE/trial_humann2/uniref50/ --metaphlan-
options="--mpa_pkl /dmf/uqdi/HPC/sw/MetaPhlAn/2.0/databases/mpa_v20_m200.pkl --bowtie2db
/dmf/uqdi/HPC/sw/MetaPhlAn/2.0/databases/" --threads 30
```

Joining multiple output tables

```
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_join_tables –i genefamilies_all -o
merged_genefamilies.tsv --file_name genefamilies
```

Normalising the merged output table (unit as copies per million)

```
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_renorm_table --input merged_genefamilies.tsv
--output merged_genefamilies_cpm.tsv --units cpm
```

Regroup and rename to GO categories

```
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_regroup_table -i merged_genefamilies_cpm.tsv
-c ../utility_mapping/map_level4GO_uniref50.txt -o regroup_genefamilies_GO.tsv
```

```
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_rename_table -i
regroup_genefamilies_cpm_GO.tsv -c ../utility_mapping/map_level4GO_name.txt -o
rename_genefamilies_GO.tsv
```

Regroup and rename to Pfam categories

```
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_regroup_table -i merged_genefamilies_cpm.tsv
-c ../utility_mapping/map_level4ec_uniref50.txt -o regroup_genefamilies_cpm_Pfam.tsv
[tnmurtaz@uqdi-login genefamilies_all]$ humann2_rename_table -i
regroup_genefamilies_cpm_Pfam.tsv -c ../utility_mapping/map_level4ec_name.txt -o
rename_genefamilies_Pfam.tsv
```

R corrplot script for correlation analysis

```
MM = read.csv("change.csv")
MM_M = cor(MM,method="spearman")
library(corrplot)
MM.P = cor.mtest(MM_M)
M.adp = c()
as.data.frame(M.adp)
for (i in 1:28)
{
  MM.adp = p.adjust(MM.P$p[,i],method = "bonferroni")
  M.adp = cbind(M.adp,MM.adp)
}
col <- colorRampPalette(c("#BB4444", "#EE9988", "#FFFFFF", "#77AADD", "#4477AA"))
corrplot(MM_M, #col=col(200),
         type="lower",
         addCoef.col = "null",  #Add coefficient of correlation
         tl.col="black", tl.srt = 50, tl.cex = 1, number.cex = 1, #Text label color and rotation
         # Combine with significance
         p.mat = M.adp, sig.level = 0.05, insig = "blank",
         # hide correlation coefficient on the principal diagonal
         diag=FALSE
)
```

Script to run FastQC to estimate the number of metagenomic reads

```
#!/bin/bash
#PBS -l select=1:ncpus=24:mem=128gb
#PBS -l walltime=24:00:00
module load fastqc
module load java/1.8.0
pwd
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/ACE/shotgun/J228/repeat
fastqc S1.fastq.gz
```

Script to run Nonpareil to estimate metagenomic coverage

```
#!/bin/bash
#PBS -l select=1:ncpus=24:mem=200gb
#PBS -l walltime=200:00:00
module load fastqc
module load java/1.8.0
pwd
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/ACE/shotgun
export MODULEPATH=$MODULEPATH:/dmf/uqdi/HPC/PBSHOME/shared/modules
```

```
module load Miniconda-2.7
source activate nonpareil
nonpareil -s sample.fastq -T kmer -f fastq -b sample
```

Script to run qcIllumina

```
#!/bin/bash
#PBS -l select=1:ncpus=48:mem=248gb
#PBS -l walltime=24:00:00
pwd
cd uqdi/Immunology/Morrison_Group/Nida/qc
module load Miniconda-2.7
module load samtools-1.3.1
module load bbmap
python /dmf/uqdi/Immunology/Morrison_Group/Useful_software/qcIllumina/qcIllumina.py nextera
SC3298_S1_R1_001.fastq.gz SC3298_S1_R2_001.fastq.gz S1
```

Script to run MEGAHIT

```
#!/bin/bash
#PBS -l select=1:ncpus=38:mem=148gb
#PBS -l walltime=400:00:00
pwd
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/Assembly
/dmf/uqdi/HPC/PBSHOME/shared/applications/megahit/megahit-v1.1.1-2-g02102e1/megahit --
memory 0.7 --num-cpu-threads 30 --tmp-dir /scratch/nmurtaza/ -1 S1.qc.1.fq.gz,S2.qc.1.fq.gz -2
S1.qc.2.fq.gz,S2.qc.2.fq.gz -r S1.qc.singletons.fq.gz,S2.qc.singletons.fq.gz -o Megahit
```

Script to run BamM

```
#!/bin/bash
#PBS -l select=1:ncpus=38:mem=200gb
#PBS -l walltime=672:00:00
export PATH=/dmf/uqdi/HPC/PBSHOME/shared/applications/bwa-0.7.3a/:$PATH
export PATH=/dmf/uqdi/HPC/PBSHOME/shared/applications/samtools-1.3.1/bin/:$PATH
export PATH=/dmf/uqdi/HPC/PBSHOME/shared/applications/BamM-
test/bamm/:/dmf/uqdi/HPC/PBSHOME/shared/applications/BamM-
test/bin/:/dmf/uqdi/HPC/PBSHOME/shared/applications/Miniconda/bin/:$PATH
pwd
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/Assembly
bamm make -d /dmf/uqdi/Immunology/Morrison_Group/Nida/Assembly/final.contigs.fa -c
S1.qc.1.fq.gz S1.qc.2.fq.gz S2.qc.1.fq.gz S2.qc.2.fq.gz -t 30 -o
/dmf/uqdi/Immunology/Morrison_Group/Nida/all_bamm
pwd
```

Script to run MetaBat

```
#!/bin/bash
#PBS -l select=1:ncpus=38:mem=148gb
#PBS -l walltime=400:00:00
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/metabat
export PATH=/dmf/uqdi/HPC/PBSHOME/shared/applications/BamM-
test/bamm/:/dmf/uqdi/HPC/PBSHOME/shared/applications/BamM-
test/bin/:/dmf/uqdi/HPC/PBSHOME/shared/applications/Miniconda/bin:/dmf/uqdi/HPC/PBSHOM
E/shared/applications/metabat_v0.32.4_static_binary/:$PATH
runMetaBat.sh /dmf/uqdi/Immunology/Morrison_Group/Nida/Assembly/final.contigs.fa
/dmf/uqdi/Immunology/Morrison_Group/Nida/all_bamm/final.contigs.S*.qc.1.bam
```

Script to run CheckM

```
#!/bin/bash
#PBS -l select=1:ncpus=38:mem=148gb
#PBS -l walltime=400:00:00
cd /dmf/uqdi/Immunology/Morrison_Group/Nida/checkm
export MODULEPATH=/dmf/uqdi/Immunology/Morrison_Group/modules:$MODULEPATH
export MODULEPATH=/dmf/uqdi/HPC/PBSHOME/shared/modules:$MODULEPATH
module load pplacer-1.1
module load prodigal-2.6.3
module load hmmer-3.1b2
module load Miniconda-2.7
module load anaconda2
module load CheckM-1.0.7
checkm lineage_wf -t 25 -x fa /dmf/uqdi/Immunology/Morrison_Group/Nida/metabat
/dmf/uqdi/Immunology/Morrison_Group/Nida/checkm
```

7.7 Ethics approval letters

Supernova 1 study (Chapter 3 and 4)

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Microbiome in Highly Trained Athletes and Response to Dietary Change
Chief Investigator:	Prof Louise Burke
Supervisor:	None
Co-Investigator(s):	Prof Mark Morrison, Dr Meg Ross, Dr Nicole Vlahovich, A/Prof Katrina Campbell, Dr Hayley O'Neill
School(s):	UQDI; AIS; Bond University
Approval Number:	2015001965
Granting Agency/Degree:	Bond University - CRN-AESS Research Capacity Building Seeding Grant Scheme
Duration:	31st December 2016

Comments/Conditions:

Expedited review on the basis of approval from the AIS Ethics Committee dated 17/08/2015

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 16-12-15

Using diet rich in dietary fibre to improve bowel habit and sense of well-being (Chapter 5)

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Using Diet Rich in Dietary Fibre to Improve Bowel Habit and Sense of Wellbeing
Chief Investigator:	Dr Jane Muir
Supervisor:	None
Co-Investigator(s):	Prof Mark Morrison, Dr Paraic O Cuiv, Prof Peter Gibson
School(s):	Central Clinical School Gastroenterology; UQ Diamantina Institute
Approval Number:	2015000317
Granting Agency/Degree:	
Duration:	26th November 2019

Comments/Conditions:

Expedited review on the basis of approval from Monash University HREC dated 26/11/2014 & 26/02/2015

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 4 MAR 2015

Chapter 8

Chapter 8 References

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