Dietary Manipulation of Gut Microbiota for Improvement of Metabolic Health

Bomhof, Marc

doctoral thesis

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Dietary Manipulation of Gut Microbiota for Improvement of Metabolic Health

by

Marc R Bomhof

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

Background: Obesity is a highly complex disease state for which there remains a dearth of effective treatment and prevention strategies. An abundance of research now suggests that gut microbiota plays a role in the pathogenesis of obesity, making it a prime target for obesity management.

Objective: This dissertation examines how dietary agents including prebiotics and probiotics, gut microbiota, and host physiology interact to affect metabolic health in obesity. Specifically, the objectives of this thesis include: 1) assess the individual and combined effects of a prebiotic and probiotic on metabolic health in obese rats; 2) determine the gut microbiota-dependent and -independent actions of the prebiotic oligofructose using a model of selective decontamination with antibiotics in obese rats; 3) examine the effectiveness of the prebiotic oligofructose for treatment of liver-biopsy confirmed non-alcoholic steatohepatitis (NASH) in a pilot clinical trial.

Methods: Animal studies were conducted using diet-induced obese rats. Individuals with NASH were recruited by physicians from the Foothills Medical Centre. Body composition was measured with dual x-ray absorptiometry (DXA). Oral glucose tolerance tests (OGTTs) were conducted to measure glycemia. Markers of satiety, inflammation, and intestinal permeability were measured in blood. Gut microbiota was assessed using qPCR and 16S rRNA gene sequencing. Gene expression was measured using real time RT-PCR. Pre-post study liver biopsies were collected to assess histological changes in NASH.

Results: The primary findings from our three study objectives were: 1) oligofructose, in comparison to *Bifidobacterium animalis* ssp. *lactis* BB-12, provides a more potent stimulus in reducing adiposity and modifying gut microbiota; 2) the ability of oligofructose to reduce adiposity and intestinal permeability is attenuated when *Lactobacillus* and *Bifidobacterium* growth is impeded with ampicillin in an animal model; 3) oligofructose supplementation improves histological measures of steatosis and has a tendency to decrease hepatocellular inflammation in individuals with NASH.

Conclusion: Our results provide evidence for the role of prebiotics in correcting metabolic dysfunction in obesity. The findings from our pilot study provide the rationale for a larger-scale clinical trial assessing the effects of inulin type fructans and other prebiotics in NASH.
Preface

The dissertation is presented in a manuscript-based format.

Chapter 2 Portions of chapter 2 have been published as a book chapter in Probiotics and Prebiotics: Current Research and Future Trends.

Chapter 3 has been published in Obesity.

Chapter 4 has been published in FASEB J.

Chapter 5 – in preparation for submission to a clinical journal.

Copyright permissions are included in the Appendix. Given the manuscript-based format, there is some redundancy in the methods described and background information between chapters.
Acknowledgements

First and foremost, I would like to express my sincere thanks to my supervisor Dr. Raylene Reimer for her continuous support, expert guidance, and mentorship throughout my PhD. I will forever be grateful for the skills and lessons she has taught me. It has been an honour and privilege to work with such a knowledgeable and committed supervisor.

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Finally, I would like to thank my parents – for being wonderful role models, offering a lifetime of love and support, and encouraging me to pursue higher education. Did you have any idea that it would last for this many years?
Dedication

To my wonderful, supportive, and loving wife Tara and our boys Emmet and Thomas
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<td>alanine aminotransferase</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>AM</td>
<td>ampicillin</td>
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<td>Angptl4</td>
<td>Angiopoietin-like protein 4</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>aspartate aminotransferase</td>
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<td>BB-12</td>
<td><em>Bifidobacterium animalis</em> subsp. <em>lactis</em> BB-12</td>
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<td>BP</td>
<td>blood pressure</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>body weight</td>
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<td>bone mineral density</td>
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<td>CFU</td>
<td>colony forming units</td>
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<td>CISI</td>
<td>composite insulin sensitivity index</td>
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<td>CLA</td>
<td>conjugated linoleic acid</td>
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<td>CONV-R</td>
<td>conventionally raised</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DP</td>
<td>degree of polymerisation</td>
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<td>DIO</td>
<td>diet-induced obese</td>
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<td>DXA</td>
<td>dual-energy X-ray absorptiometry</td>
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<td>eCB</td>
<td>endocannabinoid</td>
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<td>FXR</td>
<td>farnesoid x-receptor</td>
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<td>FIAF</td>
<td>fasting-induced adipocyte factor</td>
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<td>FD-4</td>
<td>fluorescein isothiocyanate-dextran-4000 daltons</td>
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<td>FISH</td>
<td>fluorescent in situ hybridization</td>
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<td>GIP</td>
<td>glucose-dependent insulino tropic polypeptide</td>
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<td>HDL</td>
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<td>HOMA2-%β</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IGN</td>
<td>intestinal gluconeogenesis</td>
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<td>KO</td>
<td>knockout</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>operational taxonomic units</td>
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<td>ZO-1</td>
<td>zona occludens-1</td>
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Epigraph

I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving

Antony van Leeuwenhoek, 1683
Chapter One: **Introduction**

1.1 **Background**

The environmental changes that have occurred over the last 40 years have made it challenging for the majority of the population to maintain a healthy weight. Presently 67% of men and 54% of women in Canada suffer from overweight and obesity(1). As evidence of the alarming increase in obesity prevalence, between 1978 and 2011 the national obesity rate climbed from 14% to 26%(1). According to the Organisation for Economic Cooperation and Development, Canada now ranks fifth in the world for highest obesity prevalence, trailing only behind the United States, Mexico, New Zealand, and Australia(2). Highlighting the rapid increase in obesity rates in the past two decades is the finding that of the individuals with overweight and obesity today, less than 2/3rds were overweight or obese in their adolescent years(1). Presently in Canada 13% of children aged 5-17 are obese and 20% are overweight(3). Given that obesity tends to persist from childhood to adulthood, there is concern that obesity rates will continue to rise(4). Furthermore, it is now clear that obesity is not just a problem of westernized nations. Worldwide, obesity rates have increased from 3.2% to 10.8% in men and from 6.4% to 14.9% in women between 1975 and 2014(5). For the first time in history, obesity now surpasses underweight as the major nutritional concern globally(5).

Although the cause of obesity can be distilled down to positive energy balance, it is clear that factors contributing to positive energy balance are extremely complex and not easily mitigated or reversed(6). Historically, humans have had to contend with a food scare environment, necessitating a physiological system biased towards weight gain and energy conservation for survival. It is purported that the homeostatic and hedonic energy regulating pathways evolved to facilitate this very important objective, with the homeostatic pathway regulating food intake to ensure adequate energy for day to day activity and the hedonic system increasing our desire for highly palatable, calorie-dense foods in order to store extra calories for unforeseen periods of food scarcity(7,8). Whereas highly palatable foods were once scarce, ultra-processed foods that appeal to the hedonic brain now make up approximately 60% of North America diet(9). The relatively rapid transformation in our food environment over the past 40 years has created a mismatch between our biology and the current food environment. This mismatch is believed to
be one of the primary factors responsible for the obesity epidemic of today(7). Furthermore,
some individuals, relative to others, are known to have perturbed neurohormonal signalling
resulting in heightened food seeking behaviour, which is another factor that contributes to
obesity risk(10,11). Regrettably, based on the difficulty that individuals face trying to stave off
weight gain, particularly after weight loss, it is clear that overriding these prominent appetite
regulating energy systems is extremely difficult.

Unfortunately, while research demonstrates that obesity is the result of complex genetic,
epigenetic, physiological, sociocultural and environmental factors, it is still a commonly held
belief amongst the general population as well as the medical community that obesity is a lifestyle
choice, driven by overeating and lack of willpower(12,13). This misunderstanding has
perpetuated a growing problem of weight bias and stigmatization in obesity that is adversely
affecting mental health, work and education opportunities, as well as physical health in
individuals with obesity(14). When health care professionals were surveyed, characteristics
attributed to individuals with obesity included laziness, non-compliance, decreased intelligence,
and lack of honesty(15). As a consequence of weight bias and stigma, physicians are less likely
to provide sufficient medical care to patients with obesity and patients are less likely to seek-out
medical care(13). In part due to the decreased quality of life issues faced by individuals with
obesity, in 2013 the American Medical Association officially classified obesity as a chronic
disease and the Canadian Medical Association followed suit in 2015(16,17). Although this
decision was somewhat controversial, given the belief that the body’s ability to gain weight is
not indicative of impaired physiological function(18), the decision to label obesity as a disease
hinges on the need to end the stigma and social discrimination of obesity and increase access to
quality care for patients(16).

Notwithstanding weight bias and social stigma, one of the major concerns with obesity is the
fact that it is the leading contributor to a variety of metabolic diseases including type 2 diabetes,
chronic kidney disease, cardiovascular disease, and non-alcoholic fatty liver disease
(NAFLD)(19). These comorbidities of obesity are known to decrease anticipated life span(20)
such that a BMI between 30-35kg/m² decreases median survival by 2-4 years and a BMI
between 40-45 kg/m² decreases survival by 8-10 years(20). In the year 2000 it was estimated that
that 9.3% of all deaths in Canada could be theoretically attributed to obesity(4), and this number may now be even higher. For these reasons, finding effective treatment strategies for obesity is urgently required.

It is known that a 5-10 % weight loss can yield significant improvement in metabolic risk factors (e.g. hypertension, insulin resistance, and dyslipidemia) that are associated with obesity(1). First line treatments to achieve this goal include behavioural therapies, improved diet, and increased physical activity, all of which are aimed at reducing caloric intake and/or increasing energy expenditure. Unfortunately, weight loss is self-limiting due to changes in satiety hormones, altered sympathetic tone, and enhanced intrinsic muscle efficiency(6,21,22). Collectively, these metabolic adaptations to weight loss serve to slow down energy expenditure and increase energy intake. Consequently, the first line therapy in obesity treatment to “Eat Less and Move More” has had little impact on obesity prevalence, in part because this strategy fails to address the underlying biological factors that contribute to weight gain and obesity(23). More promising obesity treatments target the underlying biology affecting energy intake.

Pharmacotherapy is one approach that has been used successfully to achieve clinically relevant weight loss, but unfortunately several obesity drugs have had to be recalled due to adverse psychological and cardiovascular effects(24). In contrast to the numerous pharmacological agents available to treat other chronic disease such as hypercholesterolemia and type 2 diabetes, in Canada, only two medications are currently available for obesity treatment, orlistat and liraglutide(24). The intervention that has proven to be most effective for obesity treatment is bariatric surgery. Although the mechanisms have not been fully elucidated, bariatric surgery is purported to achieve long term weight loss by affecting numerous variables that collectively decrease appetite(25). Bariatric surgery, however, carries with it significant surgical risk and is only available to a small fraction of eligible candidates(26). For these reasons, alternative treatment approaches are necessary.

Research over the past decade has implicated a new player, the gut microbiota, in the pathogenesis of obesity(27). Ley et al., in 2006, published a paper in *Nature* demonstrating that individuals with obesity had an altered gut microbiota community, with obesity being associated with increased Firmicutes and decreased Bacteroidetes(28). The altered profile of gut microbiota
in obesity has a lower microbiota gene richness (decreased diversity) and an increased capacity to extract energy from food(29,30). Interestingly, Dr. Rob Knight, a prominent gut microbiota researcher, showed that it was possible to predict to within 90% accuracy whether a gut microbiota donor had obesity or not by assessing the genes represented in a sample of gut microbiota(27). Alternatively, based on the genes represented within the donor’s own genome, the prediction accuracy was only 58%(27). The gut microbiota and the genetic capacity represented within (the microbiome) can influence the host by altering gene expression and neurohormonal factors regulating appetite(31,32). Although the etiology of an altered gut microbiota in obesity is debated, it is known that diet plays a prominent role in shaping the gut microbiota. Sonnenburg et al. purports that a perturbed gut microbiota in obesity is likely the result of diminished fiber in the Western diet(33). Dietary fiber, by resisting digestion in the upper part of the gastrointestinal tract, is an important substrate for maintaining the health and the diversity of the gut microbiota. It is believed that as fiber is removed from the diet, certain health-promoting bacterial species are being diminished and perhaps even becoming extinct, a process that is reducing the overall diversity of the gut microbiota(33,34). Comparison between countries in which a traditional high fiber diet is consumed and nations where low fiber diets are prominent reveal that gut microbiota diversity is compromised. This decreased microbial diversity is thought to be partially responsible for some of the adverse metabolic effects observed with a Western diet(35).

With evidence that a low fiber diet promotes gut microbiota ‘dysbiosis’ in obesity, there is significant interest in whether the gut microbiota can be targeted to restore the gut microbiota-host symbiosis. While there are numerous approaches that can be used to alter the gut microbiota, the most common strategies include the use of prebiotics and probiotics. Prebiotics function by feeding the endogenous ‘health-promoting’ bacteria within the gut(36), whereas probiotics are live ‘health-promoting’ organisms that can be introduced to the gut via the diet/dietary supplements(37). Both prebiotics and probiotics have the capacity to reshape the gut microbiota in a manner favouring improved body composition, insulin resistance, and appetite control(38,39). While the evidence for prebiotics and probiotics is very promising, our understanding of how these dietary factors function remains limited. Furthermore, the full
potential of these gut microbiota modifiers to treat metabolic comorbidities in obesity has yet to be fully elucidated.

1.2 Purpose of research

The overall aim of this thesis is to complete basic and clinical research exploring the mechanisms by which prebiotics and probiotics interact with the gut microbiota and host physiology to impact body composition and metabolic health in obesity. Given the current challenges with effectively managing obesity, translation of the information generated from this research into future clinical trials will potentially yield effective, non-invasive treatment strategies for individuals with obesity. Ultimately, it is hoped that the aspects of obesity that are attributed to gut microbiota dysbiosis can be mitigated with effective prebiotics and probiotics. If this goal was achieved, a prebiotic/probiotic strategy could be utilized within a non-invasive treatment approach to effect long-term weight loss and improved health for individuals afflicted with obesity.

1.3 Overview of separate chapters

This manuscript-based thesis is composed of 6 chapters. Chapter 1 introduces the dissertation. Chapter 2 is an overview of the role that gut microbiota plays in the pathogenesis of obesity. Evidence for the use of prebiotics and probiotics in the treatment of obesity is reviewed. These sections were published as a book chapter entitled *Pro and prebiotics: The role of gut microbiota in obesity*. An additional section in chapter 2 provides an overview of NAFLD and discusses the potential of prebiotics and probiotics to improve metabolic outcomes in NAFLD. Chapter 3 examines the synergistic potential of a prebiotic and probiotic to elicit improvements in metabolic health in obese rats. The manuscript from chapter 3 was published in *Obesity*. Chapter 4 examines the role of gut microbiota in mediating the beneficial metabolic effects of oligofructose in obese rats. This manuscript was published in *FASEB J*. Chapter 5 details the results of the pilot clinical study that assessed the effectiveness of oligofructose for improving histological parameters of NASH. This chapter will be submitted for publication shortly after the
thesis defence. Chapter 6 provides an overall discussion of the results from chapters 3-5 and suggests areas for future research in the field.
Chapter Two: **Pre & probiotics – The role of gut microbiota in obesity**

2.1 Abstract

Obesity is a multifactorial disease that is widespread and continuing to increase in prevalence worldwide. The composition of the gut microbiota is altered in obesity and is associated with impaired gut barrier, enhanced proinflammatory response, and metabolic disturbances. Manipulation of the gut microbiota as a therapeutic intervention in obesity and other chronic diseases including non-alcoholic fatty liver disease is of major interest. Diet plays a profound role in shaping the composition of the gut microbiota and prebiotics and probiotics have received much attention in this regard. This chapter summarizes the evidence for the effect of prebiotics and probiotics on obesity with special attention given to body weight and adiposity, appetite regulation, inflammation and gut barrier integrity, glucose and lipid metabolism, and hepatic steatosis and inflammation.

2.2 Introduction

Overweight (BMI ≥ 25) and obesity (BMI ≥ 30) are defined as “abnormal or excessive fat accumulation that may impair health”(40). Whereas obesity was once considered a problem that was limited to developed nations, obesity has seen a dramatic increase worldwide. Global obesity rates have nearly doubled over the past 35 years and today more than 10% of the adult population is classified as obese(40) and nearly 40 million preschool children are considered overweight(41). Excess body fat predisposes individuals to a host of chronic health conditions including type 2 diabetes, hypertension, cardiovascular disease, non-alcoholic fatty liver disease, and some types of cancer(19,42), making obesity one of the leading causes of death worldwide(40).

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The increasing incidence of obesity around the world reflects the vulnerability of the human biological system to alterations in the physical and nutritional environment. While the human body is physiologically adept at dealing with periodic food scarcity, an environment in which energy is ubiquitous threatens overall metabolic homeostasis. Body weight is ultimately defined by energy balance, where calibration between ‘energy in’ and ‘energy out’ dictates whether weight is lost, gained, or stable. Given the limited success of countless regimes that promote weight loss through caloric restriction and/or increased physical activity, it is clear that simply trying to manipulate energy intake and expenditure through diet and exercise has limited efficacy for long term weight loss. Over the past few decades, extensive research has demonstrated that the human body is very efficient at defending against sustained weight loss through the regulation of physiological processes that control hunger, energy expenditure, and metabolic efficiency(43). Given the powerful influence of these control mechanisms, it is now acknowledged that obesity is not just a lifestyle choice but rather a complex disease that is driven by biological, environmental, and psychological factors(23). Targeting the underlying physiological factors that affect energy balance, rather than the behaviours and activities that result from these factors is perhaps the best approach to developing effective treatments and prevention strategies for obesity.

In recent years it has been demonstrated that the gut microbiota plays a prominent role in the regulation of host energy balance and metabolism(28). Gut microbiota has both the ability to affect ‘energy in’ as well as the metabolic efficiency by which energy substrates are processed. Considered an environmental factor for the body, the gut microbiota offers a component of the human biological system that can be tailored to fit the demands and needs of the human body. Given these qualities, gut microbiota has become a very promising target in the restoration of energy homeostasis and metabolic health.

The gut microbiota is the product of millions of years of co-evolution between bacteria and host that has led to a complex ectosymbiotic relationship yielding benefits for both bacteria and host(44). Microbes benefit from the delivery of an abundant supply of energy in the digestive tract, while the host benefits from the competitive exclusion of opportunistic and pathogenic organisms and the adoption of various metabolic and digestive functions mediated by
bacteria\(^{(44,45)}\). It is estimated that there are approximately one hundred trillion microbes that inhabit the gut, outnumbering the ten trillion eukaryotic cells that comprise the human body by a factor of ten\(^{(46,47)}\). The collection of genes that are coded in the makeup of all the microbiota, referred to as the microbiome, exceeds that of the human genome by \(\sim 150\) times\(^{(47)}\). While enhancing our ability to culture more of the gut microbiota is currently a new frontier in microbiota research\(^{(48)}\), the advent of sequencing technologies, utilizing the highly conserved, intra-species 16S ribosomal RNA (rRNA) gene region as a taxonomic marker, has allowed for a more extensive characterization of the various phylotypes (including the non-cultured variation) that dwell within the gut. It has only been in recent years that much of the diversity of the microbiota has been identified, due to the fact that approximately 70% of the organisms that lived in the gut could not be cultured via traditional culture techniques. Altogether, an estimated 1150 species or phylotypes have been identified\(^{(47)}\). Firmicutes and Bacteroidetes are recognized as the most prominent phyla representing \(\sim 90\)% of the bacteria, whereas Proteobacteria (e.g. genus *Escherichia*), Actinobacteria (e.g. genus *Bifidobacterium*), Fusobacteria, and Verrucomicrobia are found in lower abundance\(^{(49)}\). Eckburg and colleagues, by harvesting fecal samples from various locations within the colon, established that there is spatial variability in bacterial phylotypes\(^{(49)}\). Proximal versus distal and mucosal-associated versus luminal domains contain unique communities of bacteria, varying according to phylotype make-up and complexity\(^{(50,51)}\). The duodenum/stomach, jejunum/ileum, and colon contain \(10^1\)-\(10^3\), \(10^4\)-\(10^7\), and \(10^{11}\)-\(10^{12}\) colony forming units (CFU)/ml, respectively\(^{(52)}\). Approximately 70% of the gut microbiota associated with the body resides in the colon\(^{(52)}\). Altogether, the total mass of the gut microbiota is approximately 1.5kg\(^{(44)}\). It has been demonstrated that despite interpersonal phylotype diversity, a core set of genes exists in all microbiomes. Referred to as functional redundancy, a core genome ensures that key processes are not affected by changes in diversity\(^{(50,53)}\).

Given the vast metabolic capacity of the gut microbiota and the close proximity that the gut microbiota occupies to the intestinal epithelium, gut microbiota has been described as a ‘metabolic organ’ within the host providing critical physiological functions\(^{(54)}\). The vast genetic potential of the microbiota has led some to draw parallels between the gut microbiota and the
liver in regards to metabolic capacity(50). Known functions of the gut microbiota include immune system development, epithelial barrier function, protection against pathogens through production of antimicrobial factors such as bacteriocins and organic acids, synthesis of vitamin K and B, dehydroxylation and de-conjugation of bile acids, enterocyte differentiation, xenobiotic metabolism, regulation of intestinal mobility, and cation absorption(44,54-56). Another prominent function of the gut microbiota, and perhaps the most relevant to a discussion on energy balance, is fermentation of non-digestible substrate which leads to a range of metabolic by-products that provide energy to the host. Plant polysaccharides are a diverse group of carbohydrates comprised of unique sugars, such as xylan, pectin, and arabinose, each with unique glycosidic bonds that cannot be digested by human enzymes. Anaerobic microbes, however, contain various glycoside hydrolases, which permit fermentation of these complex carbohydrates. This process produces a range of metabolic by-products, including the short chain fatty acids acetate, propionate, and butyrate, which are absorbed via passive diffusion and by mono-carboxylic acid transporters along the epithelium(38). Butyrate is primarily used as fuel for colonocytes, whereas propionate and acetate are delivered via the portal vein to the liver. Propionate is a gluconeogenic substrate utilized by hepatocytes and intestinal cells while acetate is metabolized by other tissues(38). Proteins that enter the colon are fermented by proteolytic bacteria and yield by-products such as isobutyrate and isovalerate. The by-products of protein fermentation are reported to be more toxic to the host and may be involved in pathophysiological conditions(38). Cud-chewing animals are able to harness ~70% of their energy requirements from plant food because they have an abundance of cellulolytic bacteria(57,58). Although humans do not rely on bacterial derived energy to the same extent, it is estimated that humans acquire ~10% of total daily energy from gut fermentation(58). Additionally, through production of metabolites, gut microbiota is reputed to affect the host’s ability to store and metabolize nutrients by influencing host-gene expression(59). Genes that are involved in satiety and lipogenesis are notable examples of host-processes that can be altered through microbiota activity(60). With a vast array of microbial metabolic potential at the disposal of the host, researchers are only beginning to understand the complex interactions between microbial metabolism and human physiology.
Early neonatal influences play a prominent role in sculpting gut microbiota. Although the digestive tract was historically thought to be sterile at birth, evidence that bacteria are present in meconium of neonates demonstrates that colonization may be initiated prior to birth(61). Prominent influences on early microbiota development include mode of delivery (vaginal versus caesarean-section), feeding type (breastfed versus formula fed), gestational age, and antibiotic use(62,63). It is theorized that a ‘healthy’ microbiota develops from early exposure to the bacteria that are present in the birth canal, and subsequent ‘feeding’ of these bacteria by substrates that are present in breast milk. Deviations from an optimal bacteria profile may increase susceptibility to disease states in later life(63). During the weaning period, gut microbiota undergoes significant change and transitions into an adult-like gut microbiota(64). Across the lifespan, gut microbiota are highly influenced by diet and the host innate and adaptive immune system(44,65). Altogether, the combination of these variables contributes to the development of a very complex and individualized network of gut microbiota. Over time each human being develops a distinctively unique microbial fingerprint within the intestine that appears very stable over time(53,66).

### 2.3 Gut microbiota and energy balance

Given that the gut microbiota can unlock energy supplies that are inaccessible to the host based on their mammalian enzymatic capacity, Gordon and colleagues employed a germ free (GF) mouse model to study the role of gut microbiota on host metabolism. Although GF does not represent a physiological state, the model offers important insight into the mechanisms by which gut microbiota influences whole body energy homeostasis. Formative work by Backhed et al.(67) revealed that gut microbiota, regardless of food intake, can affect body composition and body weight. Their first study in 2004 demonstrated that GF mice, relative to conventionally raised (CONV-R) mice, had 42% less body fat despite consuming approximately 30% more rodent chow than their CONV-R counterparts(67). Interestingly, based on decreased oxygen consumption measurements, GF mice did not display an elevated metabolic rate(67). Furthermore, when GF animals were colonized with the cecal matter from CONV-R mice, referred to as CONV-D mice, body fat increased by 57%. Alongside increased adiposity, it was
also demonstrated that CONV-D mice had elevated plasma glucose levels during insulin and glucose tolerance tests, suggesting that gut microbiota decreased insulin sensitivity (67). This finding coincided with elevated intestinal saccharide uptake, measured via 2-deoxyglucose gavage, in CONV-D animals. Additionally, CONV-D mice had increased liver triglyceride (TG) levels (67). In a second study completed in 2007, GF and CONV-D mice were fed a Western diet. In this study, it was revealed that gut microbiota increases body weight, in addition to affecting adiposity (32). Calculations showed that the mice had similar fecal energy excretion, suggesting that underlying mechanisms beyond energy harvest were mediating the reduced body weight (32). Overall, these studies reveal that an absence of gut microbiota protects against increased adiposity, liver steatosis, and insulin resistance. In a somewhat contradictory study, Fleissner et al. demonstrated that GF mice are not necessarily protected against weight gain (68). The authors used three different diets in their study: one low fat diet and two high fat diets – a standard high fat diet and Western diet, which was enriched in hydrogenated oils and sucrose and similar to the diet used in the Gordon et al. studies (68). GF mice fed the high fat diet had increased weight relative to conventional mice. Interestingly, the GF mice that received the Western diet had decreased adiposity relative to the GF mice fed standard high fat diet (68).

Based on evidence from the GF model, it appears that microbiota influence adiposity by means and mechanisms that go beyond simple energy extraction. One mechanism explaining the decreased adiposity in GF animals was an up-regulation of fasting-induced adipocyte factor (FIAF), also known as angiopoietin-like protein 4 (Angptl4), in the intestinal epithelium. FIAF is an inhibitor of the enzyme lipoprotein lipase (LPL) which is responsible for cleaving fatty acids from lipoproteins in circulation for uptake by other tissues (67). No changes in FIAF expression were observed in white adipose tissue and liver thereby suggesting that intestinal microbes were responsible for the observed change in intestinal FIAF. To confirm the proposed link between gut microbiota and FIAF-mediated changes in adiposity, GF FIAF−/− knockout (KO) mice were studied. Without the FIAF gene, the reductions in adiposity seen with GF were absent. Furthermore, the GF FIAF−/− KO mice had a 67% increase in LPL activity compared to GF wildtype mice. As further confirmation, plasma triglyceride concentrations remained elevated longer in response to an olive oil gavage in GF animals, suggesting greater LPL suppression
relative to CONV-D animals(32). In the absence of markers of adipogenesis and lipogenesis(67), the increased adiposity is consistent with hypertrophied adipose tissue secondary to elevated LPL activity in CONV-D mice. LPL activity was found to be elevated in both epididymal and heart tissue in CONV-D animals(67), demonstrating the FIAF regulation at the level of the gut affects multiple tissues.

Apart from FIAF, gut microbiota alters the expression and modification of numerous adiposity-associated genes and proteins. CONV-D mice had increased expression of the hormone leptin, a long-term regulator of appetite and body weight, which is one possible explanation for why food intake was reduced in the CONV-D group(67). In the liver, associated with the 2.3 fold increase in liver triglycerides, there was an increase in the expression of carbohydrate response element binding protein and sterol regulatory element binding protein-1, both transcriptional factors that regulate fat synthesis. As verification, two genes that are regulated by these transcriptional factors, acetyl-CoA carboxylase and fatty acid synthase were also elevated in CONV-D mice. Additionally, it was found that compared to CONV-D mice, GF mice had increased levels of phosphorylated AMP-activated protein kinase (AMPK), an enzyme commonly known to be the fuel gauge of the cell, in skeletal muscle and liver tissue(32). When activated, AMPK limits lipogenesis and enhances fatty acid oxidation. The gene expression pattern observed in the gastrocnemius muscle of GF animals was consistent with increased fat oxidation. Furthermore, in the liver, increased P-AMPK was associated with decreased glycogen synthase activity alongside depressed glycogen storage(32). It is clear from this data that gut microbiota plays a role in regulating host metabolism by affecting various physiological processes involved in energy partitioning and adiposity.

It is well established that gut microbiota play a critical role in maintaining host health. In recent years, it has been observed that gut microbiota differs between the obese and lean state(28). It is theorized that in the context of obesity, the symbiotic relationship between gut microbiota and host shifts to a dysbiotic state, such that the microbiota become pathogenic or cause harm to the host(69). The first studies that looked at the profile of gut microbiota in the lean versus obese state observed a shift in the relative abundance of the taxa present, with a profile characterized by increased Firmicutes and decreased Bacteroidetes in obesity(28,70). Ley
et al. found that genetically obese ob/ob mice had a 50% reduction in Bacteroidetes and a proportional increase in Firmicutes(70). A subsequent study revealed that human obesity was also associated with increased Firmicutes and decreased Bacteroidetes(28). Interestingly, weight loss in obese individuals was associated with an increase in the overall proportion of Bacteroidetes(28). A study by Nadal et al., using fluorescent in situ hybridization (FISH), confirmed that *Bacteroides-Prevotella* was increased in obese adolescents after 4kg weight loss(71).

While other studies have confirmed the decreased Bacteroidetes to Firmicutes ratio in obesity(53,68,72), some studies do not show this characteristic shift. Vrieze et al. found that individuals with obesity had increased Bacteroidetes and lower *Clostridium* cluster XIVa relative to lean subjects(73). Schwieritz et al. found that overweight and obesity favoured an increase in Bacteroidetes relative to Firmicutes(74). A study by Duncan et al., using FISH, did not find a relationship between the major bacterial phyla and BMI or weight loss(75). The authors did not rule out that more detailed analysis at the community level could yield insight into species differences in lean and obese individuals. Some comorbid conditions of obesity, such as type 2 diabetes and non-alcoholic fatty liver disease also appear to be associated with phylum level compositional changes in gut microbiota(76,77).

Another prominent genus of bacteria that is affected in obesity is *Bifidobacterium*, which belongs to the phyla Actinobacteria(78). Cani et al., feeding a high fat diet to mice to induce obesity, observed that this diet led to a decrease in bifidobacteria(78). Further demonstrating the association between obesity and decreased bifidobacteria, a prospective human study found that young children with low counts of bifidobacteria and high counts of *Staphylococcus aureus*, as measured by FISH, were at increased risk for excess weight gain by age 7(79).

Apart from altered abundance of particular microbial taxa, it has also been observed that aberrations from the core-microbiome are associated with obesity. In a study of monozygotic and dizygotic twins concordant for leanness or obesity, Turnbaugh et al. observed that the microbiomes of individuals with obesity versus normal weight, although containing a core set of microbial functions, showed alterations in bacterial genes and metabolic pathways(53). For instance, an obese microbiome was enriched in genes involved in carbohydrate, lipid, and protein
metabolism. A notable example was an increase in enzymes involved in the phosphotransferase system, which mediates carbohydrate metabolism. Interestingly, it was observed that many of the enriched genes in obesity belonged to the Actinobacteria and Firmicutes phyla. It is hypothesized that deviations from the core-microbiome may predispose to altered metabolic and physiological states, such as obesity(53).

Alterations in the functional capacity of the microbiome are likely an important part of increased energy extraction in obesity. Research by Turnbaugh and colleagues established in ob/ob mice that an obesity-associated gut microbiota was more efficient at extracting energy from food(80). The obese microbiome was found to be enriched for genes that facilitate breakdown of dietary saccharides, including glucosidases and galactosidases. Furthermore, SCFA concentrations were increased in the cecum. Confirming that these SCFA were absorbed, bomb calorimetry experiments demonstrated that ob/ob mice had decreased energy in their fecal matter. Additionally, a recent human study found that bacterial groups commonly found in obesity are associated with increased energy extraction from stool(30). Jumpertz et al. in a highly controlled inpatient, randomized, cross-over study found that a 3400kcal diet, balanced for macronutrients (24% protein, 16% fat, 60% carbohydrate), promoted an increase in Firmicutes and decrease in Bacteroidetes, relative to a 2400kcal diet. This bacterial shift was accompanied by a 150kcal increase in energy extraction(30). In another study, Schwiertz et al., studying lean and obese subjects, elucidated that overweight and obese individuals had increased concentrations of fecal SCFA relative to lean counterparts(74). It was observed that the proportion of the SCFA pool that was represented by propionate was increased in obese individuals. Energy extraction appears to be a prominent factor in the link between a dysbiotic gut microbiota and obesity.

One of the current topics of debate is the precise etiology of the characteristic microbial shift in obesity and whether or not a dysbiotic microbiota is a cause or consequence of metabolic disease. Diet is recognized as one of the primary factors that affects the gut microbiota. Whereas diets were historically filled with whole grains and plant based carbohydrates in an agrarian-type society, today many foods are higher in fat, sugar, and energy and low in fiber. To study whether a switch to a high fat diet is responsible for an altered microbiota, Hildebrandt et al. used
RELMβ−/− KO mice, a mouse model known for its resistance to developing obesity(81). When RELMβ−/− KO mice were switched from a lean to a high fat diet, they did not develop obesity, unlike their wildtype counterparts(81). Despite the weight differences between the wildtype and KO mice, both groups of mice developed a dysbiotic microbiota profile, with decreased Bacteroidetes and increased Firmicutes and Proteobacteria on the high fat diet(81). Similarly, a study by Ravussin et al. set out to determine if diet or body weight was primarily responsible for gut microbiota shifts(82). To study this, 4 different groups of mice were compared: 1) diet-induced obese (DIO), 20% weight reduced on a high fat diet (60% of calories from fat); 2) DIO mice *ad libitum* on high fat diet; 3) Lean control fed *ad libitum* on a lean diet (10% calories from fat); 4) Lean control, 20% weight reduced on a lean diet. After the weight reduction in the DIO mice, body composition and body weight was equal to the mice fed the lean control diet *ad libitum*. Based on 16S rRNA gene sequencing, the authors determined that diet, rather than weight reduction, had the greatest impact on bacterial composition(82). Altogether, these studies provide strong evidence that diet, rather than body weight, plays a significant role in regulating the microbiota environment.

Alternatively, there is evidence that a dysbiotic profile of microbiota is not diet related but the result of genotype or metabolic disease itself. As evidence of this, *ob/ob* mice, a mouse model that develops obesity due to a lack of leptin production, developed dysbiosis despite eating the same polysaccharide-rich diet as wildtype animals(70). Furthermore, in a study by Geurts et al., *db/db* mice, which are deficient for the leptin receptor, were found to develop an altered microbial profile, irrespective of diet(83). The *db/db* genotype was found to have a phyla level decrease in Bacteriodetes and an increase in Firmicutes, Proteobacteria, and Fibrobacteres. Overall, this evidence suggests that there is a specific host-level manipulation of the gut microbiota, such that the microbiota is specifically tailored to meet the demands and needs of the host, as dictated by host genotype. The precise mechanisms by which the host genotype manipulates the gut microbiota are not completed understood. One hypothesis is that the microbiota is regulated by the immune system. However, given that the changes in bacteria are phyla wide, and not just species specific, the pattern of microbiota shift does not correlate with typical immune-mediated bacterial change(80).
Despite the proposed role of host genotype affecting the gut microbiota, there is also evidence that the gut microbiota is causative in determining host phenotype. When adult GF C57BL/6J mice were inoculated with bacteria from either an obese or lean host, the phenotype that developed in recipient mice was found to match that of the donor mice(80). Furthermore, in another transplantation experiment, it was found that while body weight might not be affected by microbiota, some of the metabolic complications of obesity such as diabetes or non-alcoholic fatty liver disease (NAFLD) can be transmitted. Le Roy et al. selected the microbiota from two C57BL/6J donor mice based on their response to a high fat diet(84). The responder was found to have developed hyperglycemia and inflammation whereas the non-responder did not develop these complications in response to a high fat diet. After inoculating GF mice with microbiota from either the responder or non-responder, it was found that mice receiving the responder microbiota developed fasting hyperglycemia and insulinemia and NAFLD(84). Although mice that received the non-responder microbiota still developed the same level of obesity as the mice receiving the responder bacteria, the additional metabolic complications did not develop. Altogether, this evidence suggests that gut microbiota play a causative role in NAFLD and other metabolic disease. While animal models can be used to assess the causal role of gut microbiota in obesity, it is not yet clear whether gut microbiota plays a causal role in human disease(46).

In addition to diet and host genotype influence on gut microbiota in obesity, additional environmental influences such as antibiotics are also thought to play a critical role. In the agricultural industry, sub-therapeutic doses of antibiotics have been used for decades to facilitate weight gain and increase fat mass in animals(85). Despite the effectiveness of antibiotics for increasing adiposity, the precise mechanisms mediating these changes were unknown. With these data, Martin Blaser and colleagues hypothesized that modern day exposure to antibiotics is potentially contributing to obesity in humans(86). To study whether this could be true, Cho et al. employed an animal model in which young mice were administered sub-therapeutic levels of antibiotics. The results from the study indicated that low doses of antibiotics did indeed lead to increased adiposity. Furthermore, the antibiotics induced both phyla-level and gene-level changes in the microbiome, such that the capacity of the microbiome to generate SCFAs was increased. This study provides evidence that the indiscriminate use of antibiotics may have
detrimental effects on host-health and metabolism by disturbing the equilibrium of health-promoting bacteria in the gut. Previous studies in humans have identified that the antibiotic ciprofloxacin rapidly alters the gut microbiota with approximately one third of the bacterial taxa populations affected\(^{(87,88)}\). While the pattern of microbial shift has some inter-individual similarity, the changes in gut microbiota with ciprofloxacin varied amongst individuals\(^{(88)}\). Furthermore, even though some taxa returned to baseline levels within a week, ciprofloxacin was demonstrated to alter baseline state, suggesting that antibiotics may lead to long-term changes in gut microbiota.

There are several mechanisms that have been proposed to explain the association between dysbiosis and the pathogenesis of obesity and associated metabolic disease including NAFLD. The underlying physiological processes that are involved in the development of obesity that are directly affected by the gut microbiota include satiety hormone secretion, gut permeability, adipose tissue metabolism, and bile acid signalling. To some extent all of these mechanisms can be manipulated and restored through the actions of prebiotics and probiotics. The following sections will review evidence for the effectiveness of prebiotics and probiotics in the restoration of gut microbiota and metabolic health that has been established to date.

### 2.4 Prebiotics

A prebiotic is by definition a non-digestible food ingredient that elicits 'the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus (era)/species in the gut microbiota that confer(s) health benefits to the host'\(^{(38)}\). While dietary fiber is classified as a non-digestible food ingredient, not all fiber is considered a prebiotic, owing to the fact that not all dietary fibers can be selectively fermented by one or a few members of the microbiota and thereby confer health benefits. Saccharolytic bacteria, which are able to break down and ferment complex oligosaccharides containing glucosyl, fuctosyl, galactosyl, and xylosyl moieties\(^{(89)}\), are generally considered to be beneficial due to the metabolic by-products that they produce\(^{(90)}\). Proteolytic bacteria, which utilize undigested proteins as a source of energy, are considered to be more toxic to the host. Therefore, food ingredients that contain the nondigestible oligosaccharide substrates that match the enzymatic glycosidic hydrolase capacity of health promoting bacteria
are primary prebiotic candidates. The main bacterial genera that are targeted by prebiotics are *Bifidobacterium* and *Lactobacillus*, which are both known for health promoting qualities(38). To date, two chemical groups that have been identified for their prebiotic effects include inulin-type fructans and galacto-oligosaccharides(38). Inulin-type fructans and galacto-oligosaccharides are known for their ability to stimulate bifidobacteria and to some extent lactobacilli(38), at the expense of other harmful bacteria(91).

Inulin-type fructans and galacto-oligosaccharides are characterized by their degree of polymerisation (DP). Inulin-type fructans are composed of linear chains of β2-1 fructosyl-fructose linkages; those with a DP of 10-60 are classified as inulin and a DP of 2-9 is fructo-oligosaccharide (FOS) or oligofructose (OFS)(38). Galacto-oligosaccharides are composed of β1-6, β1-3, and β1-4 galactosyl-galactose linkages and has a DP of 2-8(38). The shorter polymers have been found to be highly fermentable in the caecal-colon(92,93). Inulin is found naturally in plant foods, including onions, leeks, garlic, asparagus, bananas, Jerusalem artichokes, chicory root, wheat, oats, and soybeans(89,92). Inulin-type fructans, due to their reputed health benefits and because intake from food sources is relatively low, are being increasingly incorporated as functional ingredients in a variety of foods. The fermentation of prebiotics is approximately 25-35% as efficient as metabolism of conventional starch and yields approximately 2kcal/gram, or 8.4KJ/g(94). Although considered to be safe for consumption, some of the side effects of prebiotic include infrequent bloating, flatulence, and soft stools.

A substantial amount of research has been conducted in both animals and humans that highlight the antiobesogenic potential of prebiotics. OFS supplementation in animal models consistently reduces energy intake, body weight, and adipose tissue mass and improves metabolic parameters such as blood lipids and insulin resistance(72,78,95,96). Human studies have yielded similar results, showing that prebiotics help to facilitate weight loss, enhance satiety, and improve glucose tolerance(97-99). While our understanding of how prebiotics mediate improvements in metabolic health is still not completely understood, there are several mechanisms that are believed to play a prominent role.

Upon consumption of nutrients the gastrointestinal tract secretes hormones and gut peptides that communicate satiety centrally. In obesity, the production and secretion of satiety hormones
in the basal and/or nutrient-stimulated state is impaired (100-103). Prebiotics on the other hand can augment the secretion of key satiety hormones and thereby enhance satiety. Several key gut derived appetite signals include glucagon like peptide-1 (GLP-1) and peptide YY (PYY); both produced in enteroendocrine L-cells and known to be anorexigenic. Ghrelin, chiefly produced in the stomach, is known for its orexigenic effects. Generated from post-translational modification of the precursor proglucagon, GLP-1, an incretin hormone, delays gastric emptying, reduces food intake, increases β-cell proliferation, enhances insulin secretion, and promotes glucose homeostasis (96,104). Cani et al. demonstrated that OFS supplementation increased both serum GLP-1 and proglucagon mRNA expression in the proximal colon of rats and this was associated with a two fold increase in GLP-1 producing L-cells in the proximal colon (96,105). Additionally, OFS supplementation resulted in a 2.5 fold increase in colonic proglucagon mRNA levels compared to high fat fed mice (78). Everard et al. showed that prebiotic supplementation increased both L-cell number and proglucagon expression and subsequent GLP-1 secretion in \textit{ob/ob} mice (72). Concomitantly, adipose tissue mass was decreased and glucose control and leptin sensitivity were improved.

Animal studies that demonstrate enhanced satiety hormone response in prebiotic fed animals is consistent with the research in human trials. Cani et al., in a double-blind, parallel group, placebo controlled trial showed that 16g of prebiotics/day (oligofructose-enriched inulin, Orafti Synergy 1) for two weeks increased fermentation, as indicated by a hydrogen breath test, as well as decreased hunger in response to a meal tolerance test. Prebiotic treatment in healthy individuals increased plasma PYY and GLP-1 secretion in addition to decreased postprandial glucose (97). These findings were supported by Parnell et al. in a randomized, double-blind placebo controlled trial in overweight and obese individuals (98). Supplementation with 21g/day of OFS for 12 weeks resulted in increased PYY levels and blunted ghrelin response compared to placebo. Self-reported caloric intake was reduced and glycemic control improved with the OFS supplementation (98). Unlike the study by Cani et al., however, no changes in GLP-1 concentrations were observed (98). Another single-blind, cross-over study examined the effect of 8g OFS consumed at breakfast and dinner for two weeks in healthy adults (106). At the end of the experimental phase, energy intake and satiety were measured upon consumption of a free-access
buffet breakfast, lunch, and dinner meal. Using a visual analogue scale (VAS), it was demonstrated that OFS versus placebo increased satiety during breakfast and dinner(106). At dinner, OFS also reduced hunger and prospective food consumption. Combined energy intake at breakfast and lunch was decreased by 5% with OFS(106). In a similar cross-over study, Verhoef et al. measured the effect of 2 weeks supplementation of 16g, 10g, or 0g OFS on satiety and energy intake in healthy human subjects(107). In response to a standardized, free-access meal, 16g OFS produced the most significant reduction in energy intake (~11%). This effect corresponded with increased PYY and GLP-1 area under the curve relative to the lower 10g dose of OFS(107). The authors suggest that a 16g/day dose of OFS is more effective at decreasing energy intake than 10g/day.

These longer term prebiotic human studies demonstrated a clear effect on satiety. However, short term studies with prebiotics have not always shown the same effects. Using subjective ratings, Hess et al. assessed the effect of short-chain fructo-oligosaccharide (scFOS) in 20 healthy subjects using a double-blind, crossover study. Each subject underwent three different days of testing. On each day, the subjects received a dose of either 0, 10, or 16g scFOS, with half of the dose consumed in the morning and the other half consumed 2 hours before dinner. The subjective ratings failed to show that scFOS enhanced acute satiety or decreased hunger, despite hydrogen breath tests confirming that prebiotic fermentation occurred 240 min post fiber consumption(108). These findings were supported in a study by Peters et al., in which the satiating effects of fructo-oligosaccharide and beta-glucan were studied in 21 healthy individuals. Three meal replacement bars, each containing 8g fructo-oligosaccharide, consumed over the course of 1.5 days did not elicit an increase in satiety relative to control treatments(109). The authors speculate that a longer term trial with fructo-oligosaccharide may be required to elicit satiety-enhancing effects.

The mechanisms by which prebiotics elicit these changes in gut satiety hormones are not completely understood. Cani et al. proposed that precursor cells may be differentiated into GLP-1 producing L-cells via expression of neurogenin 3 and NeuroD in response to the prebiotic fermentation products(96). Recent work has confirmed that metabolic by-products of prebiotic fermentation play a vital role in GLP-1 secretion. Tolhurst et al., using mixed colonic cultures in
vitro, found that SCFAs led to increased secretion of GLP-1(110). SCFA also increased the expression of the G-protein-coupled receptors, GPR43 and GPR41 in enteroendocrine L-cells. The known role of SCFA in the activation of GPR41 and GPR43(111) was confirmed by evidence that SCFA increased cytosolic calcium concentrations in the intestinal L-cells, a response consistent with the GPR43-Gq signal transduction pathway(110). Furthermore, in a FFAR2−/− KO and FFAR3−/− KO mouse model, it was observed that GLP-1 secretion was blunted, demonstrating that FFAR is vital for GLP-1 release(110). Consistent with these findings, butyrate has also been found to stimulate GLP-1 secretion from enteroendocrine L-cells in cell culture(112). Altogether, these data provide strong evidence for the role of SCFA in the differentiation of enteroendocrine L-cells and associated GLP-1 secretion.

Like GLP-1, PYY appears to be under a similar SCFA-induced control mechanism(113,114). Samuel et al. employed a GPR41−/− KO mouse model to study the effects of these receptors on host metabolism(113). GPR41−/− KO mice, relative to wildtype, had decreased secretion of PYY. Additionally, it was identified that in the absence of PYY, gastric motility was increased, leading to reduced energy extraction from the diet. These effects were reported to elicit a reduction in body weight and adiposity. To confirm this signalling mechanism, when GPR41−/− KO mice and wildtype mice were rendered GF, the differences in gut hormone secretion and anthropometrics disappeared, confirming that fermentative by-products are critical for PYY signaling. Although SCFA production appears to be a likely mechanism by which prebiotics augment satiety hormone secretion, it has yet to be determined if prebiotics have the capacity to enhance satiety hormone secretion in the relative absence of gut microbiota and SCFA production.

Interestingly, the same G-protein coupled receptors (GPR41 and GPR43) that are found on enteroendocrine L-cells are also expressed in adipocytes(114). It has been demonstrated by Ge et al. that activation of these receptors with the SCFA acetate inhibits lipolysis in both cell culture and in vivo(115). This mechanism was confirmed using a GPR43−/− KO mouse model, which showed that in the absence of the receptor, the decreased lipolytic effect was abolished(115). Given the known effects of prebiotic fermentation on GPR43 in the gut, Dewulf et al. conducted a study to assess the effects of prebiotics on GPR43 and associated metabolism in white adipose tissue(116). Prebiotic treatment significantly increased bifidobacteria and decreased Roseburia.
spp. and *Clostridium cluster* XIVa. Alongside decreased adipocyte size, expression of GPR43 was reduced by OFS, which corresponded with decreased markers of adipogenesis. Furthermore, it was observed that basal lipolysis was increased with OFS, which is somewhat contradictory to the findings by Ge et al. However, it was observed that OFS improved insulin-induced lipolysis inhibition, demonstrating that OFS improved adipocyte insulin sensitivity. Overall, there appears to be clear impacts of prebiotic fermentation and associated SCFA production on adipose tissue, however the precise mechanisms mediating these processes requires further investigation.

Altogether, the evidence points to the production of SCFA as the primary mechanism by which prebiotics mediate the beneficial effects on satiety and adipose tissue metabolism. Paradoxically, the generation of these same molecules has been cited as the mechanism by which microbiota contributes to weight gain. To explore this dichotomy, De Vadder et al. examined the effects of fructo-oligosaccharide and SCFA on intestinal gluconeogenesis (IGN), which is a mechanism that has been linked to beneficial effects on glucose control and energy homeostasis(117). The researchers observed that mice given fructo-oligosaccharide or SCFA had decreased weight gain and improved glucose and insulin tolerance(117). Highlighting the role of IGN in mediating these improvements, it was observed that propionate and butyrate both stimulated intestinal gluconeogenesis through independent mechanisms. While butyrate activated IGN via a cAMP dependent mechanism, it is believed that propionate increases IGN via activation of FFAR3 in the portal vein which activates peripheral neural signalling pathways. Coinciding with these effects, increased neural activity was observed in the dorsal vagal complex, an area of the brain associated with regulation of energy homeostasis(117). Confirming the necessity of the IGN mechanism for mediating the benefits of prebiotic, it was observed that the improvements in weight, adiposity, and glycemia were abrogated when IGN function was disrupted using glucose 6 phosphatase (G6pc) −/− KO mice. Overall, this study provides novel mechanistic insight into the ability of SCFA, produced through the fermentation of prebiotics, to mediate beneficial, antiobesogenic effects on the host(117).

A study by Respondek et al. using obese dogs examined the effect of prebiotics in the mediation of insulin sensitivity in obesity(118). Obese beagle dogs were provided with a diet containing 1% (wt:wt dry diet) short-chain fructo-oligosaccharide (scFOS) for a period of 6
weeks. Hyperinsulinemic-euglycemic clamp studies confirmed that insulin sensitivity was improved in obese dogs that received scFOS.(118) scFOS increased expression of uncoupling protein-2 (UCP-2) and tended to increase carnitine palmitoyltransferase-1 in adipose tissue, suggesting that these proteins are involved in prebiotic-induced improvements in insulin sensitivity. Furthermore, highlighting the importance of gut microbiota in mediating insulin sensitivity, a study by Vrieze et al. found that fecal transplants from lean human donors improved insulin sensitivity in obese human males.(73) Autologous ‘from self’ fecal transplants failed to elicit the same improvements. Analysis of microbiota revealed that increased insulin sensitivity was associated with an increase in butyrate producing bacteria.

Associated with a ‘dysbiotic’ shift in the bacteria profile, high fat feeding has been linked with impaired gut integrity and increased circulatory levels of the pro-inflammatory molecule lipopolysaccharide (LPS).(119,120) LPS, a cell-wall component of gram negative bacteria that can cross the epithelium and initiate a low grade inflammatory state that is 10-50 times less than would be experienced during septic shock, is thought to be involved in the development of insulin resistance and diabetes in obesity.(119) In circulation, LPS binds to receptors Cluster of Differentiation 14 and Toll-like receptors (TLRs) on innate immune cells (mature monocytes, macrophages, neutrophils), which triggers the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, Interleukin (IL)- 6, and IL-1, all of which are associated with the propagation of insulin resistance.(119,121) A high fat diet is believed to increase the number of gram negative bacteria in the gut and impair gut permeability, thus allowing increased LPS translocation into circulation.(120) Furthermore, one of the hallmarks of a high fat diet is a decrease in the proportion of the gut microbiota represented by the health promoting Bifidobacterium species.

Given that prebiotics elicit a potent ‘bifidogenic’ effect, Cani et al. investigated whether increasing bifidobacteria in the colon would ameliorate endotoxemia associated with high fat feeding.(78) As hypothesized, supplementing high fat fed mice with OFS for 14 weeks restored bifidobacteria. Measuring plasma levels of IL-1α, IL-1β, and IL-6 and adipose tissue derived proinflammatory cytokines, IL-1, TNF-α, and plasminogen activator inhibitory type 1 (PAI-1), bifidobacteria was found to be inversely associated with metabolic endotoxemia. Furthermore,
OFS supplementation improved glucose control and glucose-induced insulin secretion(78). In additional studies, obese ob/ob mice treated with a prebiotic had reduced plasma LPS concentrations and oxidative stress compared to control(72,122). To confirm the link between LPS and metabolic endotoxemia, Cani et al. used broad spectrum antibiotics to manipulate the microbiota and decrease the supply of LPS in the gut. The researchers found that treating high fat-fed and ob/ob mice with antibiotics reduced metabolic endotoxemia associated with LPS and down-regulated visceral adipose tissue PAI-1, IL-1, and TNF-α expression(120). Altogether, this evidence highlights the potential for prebiotics to mitigate inflammation and maintain glucose homeostasis and insulin sensitivity. However, while the research suggests that prebiotics likely mitigate endotoxemia through selective increases in bifidobacteria, it is not yet clear if prebiotics maintain the ability to improve endotoxemia in the relative absence of bifidobacteria and other bacterial genera impacted by prebiotic supplementation.

With evidence that prebiotics improved endotoxemia, Cani et al. completed a range of experiments that looked at how microbiota affects intestinal permeability. Genetically obese ob/ob mice were supplemented with OFS, which resulted in significant increases in lactobacilli, bifidobacteria, and Clostridium coccoides–Eubacterium rectale(122). More recent evidence also shows that OFS elicits increases in Bacteroidetes and decreased Firmicutes in the same mouse model(72). Ob/ob mice supplemented with a prebiotic had decreased intestinal permeability relative to control, determined by measuring the amount of DX-4000-FITC that passed from the gut into circulation(122). Having previously identified that high fat feeding disrupts intestinal integrity via reduced expression of epithelial tight junction proteins zona occludens-1 (ZO-1) and occludin(120), Cani et al. demonstrated that mRNA of the tight junction proteins ZO-1 and occludin in the jejunum were up-regulated in response to prebiotic. As evidence of the intimate link between metabolic endotoxemia and gut barrier function, plasma DX-4000-FITC concentrations were found to be positively correlated with portal plasma LPS concentrations(122).

GLP-2, a 33 amino acid peptide that is co-secreted with GLP-1 from the L-cells in the intestine, is thought to play a pivotal role in mediating intestinal adaptation, epithelial cell proliferation, and maintenance of gut permeability(122,123). With an established link between
endotoxemia, intestinal permeability, gut microbiota, and GLP-1, Cani et al. hypothesized that GLP-2 might play a critical role in mediating beneficial prebiotic effects. As predicted, ob/ob mice fed OFS had increased portal plasma GLP-2 relative to control diet. Blocking GLP-2 action with a GLP-2 antagonist blunted the prebiotic-mediated improvements on metabolic endotoxemia and gut integrity(122). To further probe these mechanisms, a pharmacological infusion of GLP-2 was administered to mice which produced similar improvements in gut permeability and endotoxemia(122). Similarly, Hadjiyanni et al. demonstrated that treatment with a GLP-2 analogue decreased jejunal transepithelial permeability in non-obese diabetic mice(124).

While bifidobacteria are associated with improved intestinal permeability, the precise bacteria that are mediating the beneficial effects on intestinal permeability and LPS are not completely understood. The mucin-degrading bacteria *Akkermansia muciniphila* represents approximately 3-5% of the gut microbial community. Given its inverse association with body weight, Everard et al. investigated the role of this specific organism in mediating the host metabolism using ob/ob and DIO mice(125). Both groups of obese mice were found to have low levels of *A. muciniphila*. With OFS supplementation, levels of *A. muciniphila* were restored alongside reductions in body weight and adiposity. Additionally, OFS reduced LPS, a marker of endotoxemia, and macrophage infiltration into adipose tissue(125). To elucidate further the role of *A. muciniphila* in mediating host metabolism, live *A. muciniphila* was provided via gavage to obese mice. The results of live *A. muciniphila* treatment included improved body weight and composition, decreased hepatic expression of glucose 6 phosphatase (a gluconeogenic enzyme), and increased markers of adipocyte differentiation and lipid oxidation(125). Moreover, *A. muciniphila* colonization increased levels of endocannabinoids in the ileum which was speculated to mediate overall improvements in gut permeability and endotoxemia(125). Overall, *A. muciniphila* may represent another organism that plays an important role in regulating intestinal permeability and endotoxemia.

Animal studies have established a link between disturbances in the gut microbiota and low grade endotoxemia in obesity. Several human studies have sought to determine whether prebiotic supplements mediate the same improvements in inflammation, gut integrity, and adiposity as
seen in animal studies. Deghan et al. conducted a randomized control trial in women with type 2 diabetes evaluating the effect of 10g inulin/day versus maltodextrin on inflammation and endotoxemia(126). Supplementation with inulin decreased energy intake, fasting blood glucose, HbA1c, fasting insulin, and HOMA-IR relative to the placebo. Furthermore, at the end of the 8 week trial, plasma concentrations of high-sensitivity C-reactive protein, TNF-α, and LPS were reduced. In another double-blind trial Lecerf et al. randomized sixty healthy participants to receive either xylo-oligosaccharide, xylo-oligosaccharide + inulin, or control for a period of four weeks(127). Supplementation with a combination of xylo-oligosaccharide and inulin increased fecal concentrations of SCFA with specific increases in propionate and butyrate. Furthermore, individuals that received xylo-oligosaccharide + inulin had decreased plasma LPS(127). In a randomized, cross-over trial, Russo et al. provided twenty healthy males with inulin-enriched pasta (~11g inulin/day) or placebo for a period of 5 weeks(128). The washout period between control and treatment was 8 weeks. Intestinal barrier function was determined by analyzing lactulose-mannitol excretion in urine. Inulin decreased lactulose in the urine, indicating improved intestinal permeability. Moreover, this change was associated with enhanced fasting GLP-2, a gut trophic hormone, and decreased plasma Zonulin, a marker positively associated with paracellular intestinal permeability.

A prebiotic galacto-oligosaccharide mixture [(Bi2muno (B-GOS)], was used by Vulevic et al. in a double-blind, randomized, placebo controlled, crossover study in 45 overweight adults to assess the effect of this prebiotic on parameters of metabolic syndrome, gut microbiota profiles, and immune function(129). Consumption of 5.5g/day B-GOS increased bifidobacteria and led to decreases in Bacteroides spp. and C. histolyticum. Although there were no changes in plasma cytokines with the prebiotic, B-GOS increased fecal secretory slgA and lowered both fecal calprotectin (marker of intestinal inflammation) and plasma C-reactive protein. Additionally B-GOS decreased insulin, total cholesterol (TC), TG, and TC:high density lipoprotein (HDL)-C although no changes were noted in glucose, HDL-C, or LDL-C(129).

In another double-blind, placebo controlled trial, 30 women with obesity were treated with a 16g/day (8g twice daily) prebiotic mixture of inulin and OFS for a period of 3 months(130). Using HITChip analysis, it was identified that prebiotic treatment resulted in an increase in
Firmicutes and Actinobacteria and a decrease in Bacteroidetes. At the genus level, prebiotic led to increased bifidobacteria and *Faecalibacterium prausnitzii*. Furthermore, qPCR confirmed the increase in bifidobacteria and also demonstrated an increase in lactobacilli. In support of the animal studies which showed the prebiotic supplementation decreased endotoxemia, it was demonstrated that bifidobacteria and *F. prausnitzii* were inversely correlated with serum LPS. Inulin and OFS improved post-OGTT glycemia but no changes in body weight, HbA1c, fasting glycemia and insulinemia, post-OGTT insulinemia, HOMA index, adiponectinemia, cholesterol, or TG were identified(130). Metabolomic analysis of plasma and urine was also performed, revealing positive correlations between decreased *Bacteroides intestinalis*, *Bacteroides vulgatus*, and *Propionibacterium* and suppressed plasma lactate and phosphatidylcholine with inulin and OFS. Overall, despite some of the subtle metabolic effects of prebiotic treatment, several of the measured parameters in regards to inflammation and endotoxemia parallel observations in animal models.

One of the other mechanisms by which microbiota are believed to elicit metabolic change in the host is via the signalling actions of bile acids(131). Primary bile acids are synthesized in the liver through enzymatic modification of cholesterol and conjugated to either taurine or glycine, which serves to enhance hydrophilicity of the amphipathic molecule(132). In the intestines bile acids are subject to bacterial modification, with the primary modifications being deconjugation, oxidation, and dehydroxylation(56). Additionally, free bile acids, by disrupting phospholipid membranes, elicit bactericidal effects on gut microbiota(133). Bile acids are important endogenous signalling molecules for numerous receptors such as farnesoid x-receptor (FXR), G-protein coupled bile acid receptor TGR5, and pregnane X receptor(134). Through these receptors bile acids are able to regulate whole body energy homeostasis through the modulation of lipid metabolism, satiety, and to some extent glucose metabolism(132,134,135). Currently one of the proposed mechanisms by which bariatric surgery is thought to mediate beneficial metabolic improvements is via alterations to bile acid metabolism and signalling(136).

Several animal studies have assessed the effects of prebiotic supplementation on bile acid metabolism. Respondek et al. completed fecal metabolomic analysis on human microbiota inoculated C57BL/6J mice fed a 10% short-chain fructo-oligosaccharide (scFOS) HF diet.
Metabolomic analysis revealed that bile acids were one of the primary metabolites affected by prebiotic supplementation(137). In another study, male Wistar rats supplemented with scFOS for two weeks were found to have altered hepatic FXR and PPARα expression(138). Moreover, healthy humans that consumed 10g/day scFOS for three months had significantly altered bile acids, with an overall increase in primary bile acids(139). Van Meer et al. fed rats a mixture of galacto-oligosaccharides and fructo-oligosaccharide for three weeks to elucidate the potential effects of the prebiotics on bile acid metabolism, citing implications for fat absorption(140). Although supplementation yielded significant increases in both lactobacilli and bifidobacteria, no changes were observed in bile salt composition or bile flow. Overall, given the complex interaction between bile acids and microbiota and the effects that bile acid can elicit on host, targeting bile acid metabolism with prebiotics offers potential for halting the pathogenesis of obesity, although further research in this area is required.

Overall, an abundance of animal research and a growing list of human clinical trials have demonstrated beneficial anthropometric and metabolic effects with prebiotics. Kellow et al. conducted a systematic review of human trials to evaluate the metabolic outcomes associated with prebiotic supplementation(99). A total of 26 randomized control trials were deemed eligible for inclusion, with the sum of the participants from all studies totaling 831. The duration of the prebiotic trials included in the analysis spanned 2 days to 28 weeks. Meta-analysis provided confirmation of enhanced self-reported satiety with prebiotics relative to placebo(99). Although, multiple trials demonstrated increased GLP-1 and PYY as well as decreases in energy intake with prebiotics, the reviewed evidence did not support these claims but did show significant improvements in subjective satiety. The meta-analysis did indicate that prebiotics significantly reduced postprandial glucose and insulin(99). Decreases in fasting glucose, fasting insulin, and insulin resistance were not supported by the data. Furthermore, there was insufficient evidence for the role of prebiotics in the reduction of total cholesterol, LDL, TG, and inflammatory markers. The meta-analysis did not support findings for decreased CRP with prebiotic supplementation. Based on the available evidence, the authors suggest that prebiotics are beneficial for improving satiety and decreasing postprandial glucose and insulin concentrations(99).
Figure 2.1: Summary of potential mechanisms by which prebiotics mediate improvement in body composition and metabolic health in obesity. Prebiotics, by changing the composition and activity of the gut microbiota, have been demonstrated to improve gut barrier function, reduce inflammation, decrease adiposity, improve glycemic control, reduce appetite and increase satiety, and reduce hepatic steatosis and inflammation.

ACC, acetyl co-A carboxylase; FAS, fatty acid synthase; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; LPS, lipopolysaccharide; NEFA, non-esterified fatty acids; PPARα, peroxisome proliferator-activated receptor-α; PYY, peptide YY; SCFA, short chain fatty acids; SREBP-2, sterol regulatory element-binding protein-2; VAT, visceral adipose tissue; ZO-1, zona occludens-1.

2.5 Probiotics

Probiotics are defined as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host”(37). Nobel prize winner Elie Metchnikoff (1845-1916) first conceptualized the idea of probiotics after hypothesizing that intestinal bacteria were causing ‘autoinfection’ which accelerated aging by releasing toxins into the vascular and neural system(141). Metchnikoff inferred that the consumption of fermented milk was responsible for prolonging the health and lives of Bulgarian peasants, and therefore speculated that ‘implanting’ a lactic acid producing bacteria would kill certain harmful proteolytic bacteria and contribute to improved health and longevity. Today probiotics are consumed in yogurt, yogurt drinks, kefir, and dietary supplements for their purported benefits on amongst others, intestinal health.
Although not all studies are consistent in their findings, some animal and human studies have identified that probiotics elicit antiobesogenic effects. In changing the gut microbiota, probiotics have the ability to change the metabolic function of the gut microbiome, enhance gut barrier function, and influence host satiety and metabolic function through various endocrine and signalling mechanisms. In particular *Bifidobacterium* spp. and *Lactobacillus* spp. have the potential to affect energy homeostasis, adiposity, lipidemia, glycemia, and body weight.

Research suggests that probiotics elicit a positive influence on blood lipids which are commonly elevated in obesity and the metabolic syndrome. A 1998 study by Taranto et al. identified that supplementation with *L. reuteri* CRL 1098 (10^4 cells/day) for 7 days in Swiss albino hypercholesterolemic mice reduced serum cholesterol and triglycerides by approximately 40% and increased the ratio of HDL to LDL by 20% relative to control mice(142). Furthermore, in a randomized, double-blind study by Bukowska et al. it was observed that 1 × 10^{10} CFU/day *L. plantarum* 299v consumed for a period of six weeks reduced both total cholesterol and LDL(143). In another randomized, double-blind, cross-over study, a fermented milk beverage (375ml daily) containing *L. acidophilus* DN 112.053 and DN 112.096 (10^7 -10^8 CFU/ml) was demonstrated to elicit improvements in total cholesterol, LDL, and the LDL/HDL ratio(144).

As an explanation for improved lipidemia with probiotics, it is theorized that probiotic-induced changes in gut microbiota limit the uptake of lipid substrates by the host(145). Wang et al., using *L. plantarum* MA2 that was isolated from traditional Chinese kefir, provided high fat fed male Sprague-Dawley rats with 10^{11} cells/day *L. plantarum* MA2 daily for five weeks. Probiotic reduced serum cholesterol, triglycerides, and LDL(145). These findings corresponded with increased fecal cholesterol, triglycerides, and propionate, thus supporting the idea that probiotics inhibit absorption of lipids in the gut. In support of this mechanism, a study by Hamad et al. found that *L. gasseri* SBT2055 blocked absorption of fat in the intestinal tract(146). When fed to lean and obese Zucker rats for 4 weeks, *L. gasseri* SBT2055 significantly reduced total serum cholesterol as well as hepatic cholesterol. Furthermore, the probiotic led to reductions in mesenteric adipose weight, adipocyte size, and serum leptin(146). It was confirmed that the probiotic increased fecal fatty acids and cholesterol. To assess whether the actions of *L. gasseri* SBT2055 limited uptake of fat by the host a second experiment was conducted in Sprague

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Dawley rats using thoracic cannulas. A 4 hour collection of lymph demonstrated that rats given the probiotic had reduced transport of triglycerides and phospholipid and tended to have reduced transport of cholesterol(146). The authors speculate that lactobacilli might elicit these effects by binding intestinal lipids.

In addition to the proposed mechanism by which probiotics inhibit lipid absorption, it is believed that probiotics might affect blood lipids via bile acid metabolism. Martin et al., using a human baby flora mouse model which was derived by inoculating GF animals with microbiota from a newborn infant, assessed the metabolic effects of the probiotics *L. paracasei* NCC2461 and *L. rhamnosus* NCC4007(147). Probiotics elicited reductions in LDL and VLDL as well as increased plasma triglycerides. Fecal matter had decreased concentrations of the SCFA acetate and butyrate as well as marked changes in unconjugated bile acids and tauro-conjugated bile acids, with an overall decrease in fecal excretion of bile acids. Furthermore, the ileum was found to have increased concentrations of the bile acids ursodeoxycholic acid and chenodeoxycholic acid. Altogether, given this evidence, the authors speculate that some of the observed metabolic effects in lipid metabolism may be mediated by gut microbiota - induced changes in enterohepatic circulation and bile acid signalling(147).

To assess whether a bile acid modifying probiotic could improve lipid profiles in hypercholesterolemic humans, Jones et al. conducted two studies using microencapsulated bile salt hydrolase-active *L. reuteri* NCIMB 30242. In the first study, the probiotic was administered in a yogurt beverage (5×10^{10} CFU twice daily for 6 weeks)(148), whereas in the second study the probiotic was provided in capsule form (2.9×10^{9} CFU twice daily for 9 weeks)(149). In these double-blind, placebo-controlled, randomized studies, each with more than 100 participants, it was demonstrated that both modes of supplementation resulted in reduced total cholesterol, LDL, as well as apoB-100. No changes in HDL or triglycerides were observed (148,149). *L. reuteri* NCIMB 30242 increased plasma deconjugated bile acids. Additionally, it was observed that the probiotic reduced plasma non-cholesterol sterols, suggesting an inhibition of sterol absorption at the level of the gut(149). Overall, the authors purport a beneficial effect of *L. reuteri* NCIMB 30242 for improvement of hypercholesterolemia in humans.
In addition to improved lipid profiles with probiotics, a number of studies demonstrate that probiotics from the genus *Lactobacillus* and *Bifidobacterium* can elicit effects on adiposity and body weight. The effect of probiotic on body weight, however, appears to be species and strain specific. Noting that probiotics from various *Lactobacillus* species have been used for decades in the agricultural industry to enhance growth of farm animals, Million et al. conducted a meta-analysis of clinical trials and experimental models on the effect of *Lactobacillus* spp. probiotics on body weight(150). A total of 17 randomized control trials, 51 farm animal studies, and 14 experimental models were included in the meta-analysis. Although the extent to which conclusions can be made about species-level effects on body weight is limited, given that body weight changes are differentially affected by bacterial strains, the authors identified that *L. acidophilus* was associated with increased weight gain whereas *L. gasseri* decreased body weight in both humans and animals(150). *L. ingluviei* or *L. fermentum* were both associated with weight gain in animals. Additionally, *L. plantarum* was noted to have an anti-obesity effect in animals. Interestingly, it has also been observed that some endogenous *Lactobacillus* species are correlated with impaired glucose control. Karlsson et al., in a study of European women, identified that while *L. gasseri* was not correlated with BMI, there was a positive correlation between *L. gasseri* and fasting glucose and hemoglobin A1c(151). Overall, it appears that different *Lactobacillus* strains elicit differential effects on body weight and host metabolism.

A variable response on body weight is also characteristic of *Bifidobacterium* spp. Yin et al. compared the effects of four different endogenous, naturally-occurring *Bifidobacterium* strains (L66-5, L75-4, M13-4 and FS31-12) on obese male Sprague-Dawley rats. Of the four strains tested, M13-4 resulted in weight gain relative to control, L66-5 decreased body weight gain, and L75-4 and FS31-12 remained weight neutral(152). Despite the differential effects on body weight, the authors noted that all four strains tended to lower serum and liver cholesterol. Chen et al. assessed the effects of *B. adolescentis* (unknown strain) on body weight, adiposity, and metabolic health in high fat fed male Wistar rats(153). Twelve weeks of supplementation decreased body and adipose tissue weight gain. Furthermore, *B. adolescentis* mitigated high fat diet induced increases in liver steatosis and insulin resistance. Kondo et al. demonstrated that *B. breve* B-3 dose dependently decreased body and epididymal fat weight gain in C57BL/6J mice.
fed a high fat diet(154). Additionally, fasting glucose and insulin levels were improved, which may relate to the finding that *B. breve* B-3 increased expression of adiponectin in epididymal adipose tissue. Altogether, there appears to be unique strain specific responses on body weight and adiposity with different probiotics.

Evidence for antiobesogenic properties of a probiotic in an animal model does not always equate to benefits in human studies. In a study conducted by Yun et al. *db/db* mice were fed $2 \times 10^{10}$ CFU/day *L. gasseri* BNR17, a probiotic isolated from breastmilk, for twelve weeks(155). Although no differences were observed in body weight, *L. gasseri* BNR17 improved fasting and postprandial glucose levels. A subsequent study by Kang et al. identified that *L. gasseri* BNR17 reduced body weight gain and adipose tissue mass in male Sprague-Dawley rats fed a high carbohydrate diet(156). These changes were associated with increased expression of genes involved in fat oxidation and a decrease in expression of genes involved in fatty acid synthesis(157). With convincing evidence for the antiobesogenic properties of *L. gasseri* BNR17, Jung et al. conducted a study evaluating the effectiveness of *L. gasseri* BNR17 for treating obesity in humans(158). In the randomized, double-blind trial fifty-seven individuals with obesity received either $6 \times 10^{10}$ CFU/day *L. gasseri* BNR17 or placebo for a period of 12 weeks. At the end of the study, subjects that consumed *L. gasseri* BNR17 failed to achieve a significant reduction in body weight or plasma lipids relative to the control group(158).

In a somewhat serendipitous discovery, Woodard et al. found that probiotics promote sustainable weight loss after Roux en Y gastric bypass surgery(159). In the study, 44 patients were randomized to either a control or probiotic. Post-surgery, the experimental group consumed $2.4 \times 10^9$ CFU/day *Lactobacillus*. Unfortunately, the authors did not specify which species and strain of *Lactobacillus* were provided to patients. Relative to control, probiotic administration reduced bacterial overgrowth and significantly increased weight loss at 3 months and 6 months post-operatively(159). The authors noted that increased weight loss was an unexpected benefit. As an interesting note, the authors speculate that individuals who have undergone gastric bypass surgery may be an ideal group to study the use of probiotics as the survival of probiotics is more prominent in individuals without a gastric pouch(159).
A study by Andreasen et al. evaluated the potential of the probiotic *L. acidophilus* NCFM for improving insulin sensitivity(160). In the randomized, double-blind, control trial, male subjects with either type 2 diabetes, impaired glucose tolerance, or normal glucose tolerance underwent a baseline and post-intervention hyperinsulinemic-euglycemic clamp. Participants received either *L. acidophilus* NCFM or placebo for a period of 4 weeks. It was observed that *L. acidophilus* NCFM preserved insulin sensitivity relative to the group that received the placebo. The authors suggest that the probiotic *L. acidophilus* NCFM may be useful for preventing aberrations in insulin sensitivity.

While some studies show significant benefit with probiotics on body weight, adiposity, and glucose control, the precise mechanisms mediating these changes are not completely understood. A variety of mechanisms have been proposed, including synthesis of SCFA, production of conjugated linolenic acid, altered tissue gene expression, changes in cytokine and satiety hormone secretions, as well as modulation of gut permeability and associated low grade inflammation.

In a study by Yadav et al. the probiotic VSL#3, a combination of *B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. paracasei, L. bulgaricus, and Streptococcus thermophilus*, was administered to both high fat fed C57J/B6 mice, DIO mice, as well as *ob/ob* mice for approximately 8 weeks(112). It was demonstrated that VSL#3 decreased body weight gain, fat mass, adipocyte size, liver steatosis, as well as improved glucose and insulin tolerance, inflammation, and adipokine secretion from adipose tissue(112). Furthermore, VSL#3 decreased food intake in high fat fed animals, which was associated with reduced expression of agouti-related protein and neuropeptide Y and increased expression of proopiomelanocortin in the hypothalamus, suggesting a role of VSL#3 in decreasing central hunger cues. It was observed that GLP-1 was significantly increased with VSL#3 treatment. Additionally, VSL#3 increased fecal and plasma concentrations of the SCFA butyrate(112). A subsequent *in vitro* experiment using human L-cells demonstrated that butyrate stimulates GLP-1 secretion(112). The authors purport that VSL#3-induced SCFA production and consequent GLP-1 release is a probable mechanism regulating anthropometric and metabolic improvements observed with probiotic treatment.
Previous studies have identified that gut microbiota affects adipose tissue mass by regulating the activity of lipoprotein lipase. Given the established link between the increased expression of angiopoietin-like protein 4 (Angptl4) and decreased activity of LPL in GF mice(67), Aronsson et al. investigated the potential role of Angptl4 expression in the mediation of \textit{L. paracasei} ssp. \textit{paracasei} F19-induced changes in host adiposity(161). Mice supplemented with \textit{L. paracasei} F19 gained significantly less body fat and were found to have an increased VLDL triglyceride load, although no changes in cholesterol levels were noted. It was observed that \textit{L. paracasei} F19 induced levels of circulating Angptl4, suggesting that increased TG was secondary to decreased LPL activity. Co-culture experiments with colonic cell-lines confirmed that \textit{L. paracasei} F19 has the ability to stimulate Angptl4 expression. GF mice monocolonized with \textit{L. paracasei} F19 also demonstrated enhanced circulating levels of Angptl4, thus providing confirmation for the potential role of Angptl4 expression in mediating probiotic-induced changes in adiposity. A study by Fak et al., however, did not observe changes in the expression of ileal Angptl4 with the probiotic \textit{Lactobacillus reuteri} ATCC PTA 4659, despite the fact that body, adipose, and liver mass in Apoe-/- mice were significantly reduced(162). Instead, it was observed that hepatic expression of carnitine palmitoyltransferase 1a was increased, suggesting that β-oxidation in the liver was a mediating factor.

It has also been proposed that probiotics mediate improved metabolic effects on the host through the synthesis of conjugated linoleic acid (CLA). CLA is purported to reduce adiposity via suppression of appetite, increasing energy expenditure, inhibiting lipogenesis, increasing lipolysis, and increasing adipocyte apoptosis(163). Lee et al. conducted a study in DIO mice using the probiotic \textit{L. rhamnosus} PL60, which is a human derived strain known for its ability to synthesize trans10, cis12 CLA(164). \textit{L. rhamnosus} PL60 supplementation elicited reductions in white adipose tissue and a down-regulation of TNF-α and fatty acid synthase mRNA and an up-regulation of UCP-2. Although CLAs have previously been linked to increased liver steatosis, it was found that \textit{L. rhamnosus} PL60 ameliorated liver steatosis(164). It has been identified that \textit{Bifidobacterium} species have the ability to convert linoleic acid into CLA and alpha-linolenic acid into conjugated linolenic acid, which is another fatty acid associated with health benefits(165). Gorissen et al. confirmed that \textit{B. breve} (LMG 11040, 11084, 11613, & 13194). \textit{B.}
bifidum LMG 10645, and B. pseudolongum subsp pseudolongum LMG 11595 were able to synthesize both CLA and conjugated linolenic acid(165). Potentially, synthesis of CLA is a mechanism by which Bifidobacterium spp. stimulate health promoting effects.

It is believed that maintenance of gut permeability and barrier function is another mechanism by which probiotics contribute to improved energy homeostasis. As demonstrated by Cani et al. impaired intestinal permeability is associated with increased endotoxemia due to translocation of LPS into circulation(119). Given that the development of NAFLD is linked with impaired intestinal permeability, Ritze et al. assessed the effects of L. rhamnosus GG (5.2×10^7 CFU/g of body weight per day) in female C57BL/6 mice fed a high fructose diet(166). In addition to mitigating inflammation and steatosis in the liver, L. rhamnosus GG prevented reductions in the tight junction proteins occludin and claudin-1 and decreased portal plasma LPS concentrations by a factor of 3 relative to the control group. In another study, Naito et al. found that the probiotic L. casei Shirota improved insulin resistance and glucose tolerance in diet-induced obese C57BL/6J mice(167). Although L. casei Shirota did not elicit a decrease in adipose tissue mass, probiotic treatment significantly decreased plasma concentrations of LPS-binding protein, evidence that L. casei Shirota decreased endotoxemia. It has also been demonstrated in diet-induced obese Sprague Dawley rats that the probiotic B. animalis ssp. lactis BB-12 stimulates the secretion of the gut trophic hormone GLP-2(168), a hormone associated with increased tight junction integrity and reduced intestinal permeability(122). The increase in GLP-2 was associated with improved glycemia and lower insulin, however, the result was not corroborated by decreased intestinal permeability or inflammatory markers. There is also evidence that the probiotic VSL#3 maintains intestinal barrier function through the production of mucin, which provides a protective barrier against pathogens. Daily supplementation with 3×10^9 CFU/day VSL#3 in male Wistar rats increased luminal mucin by 60% which was associated with up-regulation of MUC2 expression in goblet cells(169). Overall, these studies highlight the potential for probiotics to protect against the pathogenesis of obesity by regulating gut permeability and barrier function.

Altogether, there is an abundance of animal and human studies that demonstrate the influence of specific probiotics on the regulation of energy balance, body weight, and metabolic health.
The mixed results associated with various species and strains of probiotic in both animal models and clinical studies emphasize that the metabolic effects are strain specific and have the potential to contribute to weight loss or weight gain. Given the current evidence, it is difficult to conclude which probiotic supplements are most beneficial for managing obesity. Defining an optimal probiotic for prevention and treatment of obesity and associated metabolic disease will require further research and a better understanding of the mechanisms by which individual probiotics mediate their metabolic effects. Furthermore, while probiotics and prebiotics both demonstrate the ability to improve metabolic health in obesity, little research has focused on the combined effects of a probiotic and prebiotic in obesity. Given the inherent synergistic potential that exists between probiotics and prebiotics, synbiotic strategies (probiotic + prebiotic) may provide a more efficient and effective means to target gut microbiota dysbiosis. A synbiotic is generally defined as a prebiotic substrate that selectively stimulates the competitiveness, survival, or metabolic activity of one or a limited number of health promoting probiotic organisms in the gastro-intestinal ecosystem(170,171). Although synbiotics offer potential for obesity treatment, few synbiotic studies have assessed the effects of the synbiotic alone in relation to the individual effects of the constituent prebiotic or probiotic. Without such comparisons, elucidation of the synergistic potential between the prebiotic and probiotic is not possible. Future research studies would benefit from assessing the individual effects of a probiotic and supporting prebiotic in relation to the synergistic potential of the probiotic and prebiotic in combination.
Figure 2.2: Summary of potential mechanisms by which certain probiotic strains mediate improvement in body composition and metabolic health in obesity. Specific strains of probiotic organisms have been demonstrated to improve gut barrier function, reduce inflammation, decrease adiposity, improve glycemic control, reduce appetite and increase satiety, and reduce hepatic steatosis and inflammation.

AgRP, agouti-related protein; Angptl4, angiopoietin-like protein 4; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; LPS, lipopolysaccharide; NEFA, non-esterified fatty acids; POMC, pro-opiomelanocortin; PPAR-α, peroxisome proliferator-activated receptor-α; PYY, peptide YY; SCFA, short chain fatty acids; SREBP-2, sterol regulatory element-binding protein-2; UCP-2, uncoupling protein-2; VAT, visceral adipose tissue.

2.6 Non-alcoholic fatty liver disease (NAFLD)

One of the metabolic consequences of obesity is the deposition of fat in organs that are not classically associated with adipose storage. The liver, an organ that generally stores less than 5% triglycerides, is vulnerable to abnormal accumulation of fat in obesity due to increased flux of free fatty acids from insulin resistant adipose tissue, increased dietary fat, and in some individuals, increased de novo lipogenesis(172,173). A triglyceride content in excess of 5% of the liver, if not secondary to excessive alcohol intake, is referred to as NAFLD(174). With the rise in obesity, NAFLD is now the most common liver disease in the world(175). It is currently estimated that 30% of Americans have NAFLD and in those individuals with obesity this number...
increases to 75% of adults and 34% of children(175-177). While the accumulation of fat in the liver is not always a threat to liver function, in approximately 10-25% of individuals with NAFLD a more severe form of the disease develops, referred to as non-alcoholic steatohepatitis (NASH). NASH is marked by hepatic inflammation, hepatocellular ballooning, and fibrosis and predisposes individuals to cirrhosis, hepatocellular carcinoma, and ultimately end stage liver failure(175). Liver failure secondary to NASH is now one of the primary indicators for liver transplantation(178). It is estimated that 2-5% of the general population has NASH(175,176).

The pathophysiology of NASH is still not completely understood. Originally, a ‘two-hit’ hypothesis was proposed wherein increased hepatic steatosis (the first hit) and a resultant increase in oxidative stress and lipid peroxidation (second hit) initiates a pro-fibrotic cascade in the liver(179). The two hit theory, however, failed to recognize that peripheral factors also play a significant role in the propagation of simple steatosis to NASH. The theory was hence expanded to the ‘multiple parallel hit’ theory wherein metabolic insults from a variety of sources in the body contribute to the pathogenesis of NASH(180). Some of the peripheral factors involved include inflammatory cytokines arising from dysregulated adipose tissue, altered leptin, resistin, and insulin hormone concentrations, as well as the gut microbiota and various pro-inflammatory agents including endotoxin derived from the gut(181-183). Through hepatic resident kupffer cells and the release of transforming growth factor β-1 (TGF-β1), it is purported that the collective influence of peripheral and local pro-inflammatory factors leads to transformation of quiescent hepatic stellate cells into myofibroblast-like cells which actively produce extracellular matrix, in particular type 1 collagen, in the space of Disse, creating the ‘chicken-wire” fibrosis that characterizes advanced NASH(182,183). Currently, the gold standard for diagnosing NASH is a liver biopsy, which permits direct measurement of steatosis, inflammatory and hepatocellular ballooning, a marker of cell injury(184). Given the invasive nature of the liver biopsy, the less-invasive Fibroscan® (vibration-controlled transient elastography) technique is being increasingly utilized to diagnose and monitor NASH(185). While there is interest in using various biomarkers (e.g. TGF-β1, alanine aminotransferase (ALT), cytokeratin 18) to assess NASH, these biomarkers are less reliable(175).
Although NASH is becoming increasingly prominent, there are few treatment options available for this disease (186). Numerous treatment strategies for NASH have been studied including vitamin E, pioglitazone, obeticholic acid (an FXR agonist), omega-3 polyunsaturated fatty acids, and resveratrol (187-191). Vitamin E and pioglitazone both demonstrated histological improvement in steatosis and inflammation, but the pioglitazone was associated with significant weight gain relative to placebo (190). Obeticholic acid also improved histological parameters of NASH, but subjects reported a high likelihood of developing pruritus (189). Omega-3 polyunsaturated fatty acid supplementation in a double-blind randomized trial demonstrated a small benefit for reducing steatosis, but overall there was little histological changes with omega-3 supplementation relative to control (191). In children, omega-3 docosahexaenoic acid was shown to significantly improve histological markers of NASH, however this study was limited by the fact that there was no control group (188). High dose resveratrol, a naturally occurring polyphenol that activates AMPK, provided no benefit for reducing lipid content or the expression of lipogenic genes in the liver (187). With no drugs approved for the treatment of NASH, the primary treatment for this disease is lifestyle management and weight loss (175). In a prospective study conducted by Vilar-Gomez et al., which followed 293 patients with liver biopsy diagnosed NASH, it was demonstrated that 10% weight loss was very effective for eliciting histological improvement in NASH (192). Furthermore, 10% weight loss achieved through an intensive lifestyle intervention produced the same result (193). Unfortunately, given the challenges with weight loss recidivism (194), lifestyle intervention by itself it not effective on a population level for long term treatment of NASH (195). Based on this evidence, there still remains a need for effective NASH treatment strategies.

With a wealth of evidence now demonstrating that gut microbiota plays a significant role in the pathogenesis of obesity, affecting energy extraction, metabolic programming, gut permeability, inflammation, and endotoxemia, there is significant interest in the contribution of gut microbiota to the development of NAFLD (173, 181). Early experiments conducted by Backhed et al. using GF mice demonstrated that the gut microbiota affects the expression of numerous hepatic transcription factors and genes controlling oxidation and lipogenesis (32, 67). It has also been demonstrated by Henao-Mejia et al. that an altered gut microbiota, induced in
NLRP6 and NLRP3 inflammasome deficient mice, exacerbated hepatic steatosis and inflammation via mechanisms related to activation of innate immune receptors and release of TNF-α(196). Furthermore, Le Roy et al., elegantly demonstrated in an animal model that gut microbiota is causally implicated in the development of NAFLD. Using high fat fed C57BL/6J mice, researchers identified ‘responders’ and ‘non-responders’ based on the glycemic and inflammatory profile of the mice. Gut microbiota from both groups was collected and used to inoculate GF mice. Mice that received a dysbiotic ‘responder’ versus ‘non-responder’ gut microbiota developed a metabolic phenotype consistent with their donor(84). Highlighting the fact that gut microbiota may predispose to NAFLD, the GF mice that received ‘responder’ gut microbiota developed hepatic steatosis and were noted to have increased expression of genes involved in de novo lipogenesis. From this evidence, it can be inferred that altered gut microbiota profiles might exert a differential effect on hepatic metabolism such that the liver is at increased risk of developing NASH.

In the last few years, with the recognition that gut microbiota is implicated in the development of NAFLD, numerous studies have examined the composition and metabolic characteristics of a NAFLD-associated gut microbiota. Raman et al., using multitag pyrosequencing, demonstrated that certain genera of Firmicutes including Dorea, Robinsoniella, and Roseburia as well as species within the genus Lactobacillus were elevated whereas Oscillibacter was under-represented in NAFLD(197). In addition to these microbial changes, NAFLD was associated with increased fecal-ester volatile organic compounds. Similarly, Jian et al., using 16S rRNA next-generation sequencing, reported an increase in Lactobacillus in addition to increases in Escherichia, Anaerobacter, and Streptococcus. Associated with these bacterial changes, individuals with NASH had increased intestinal permeability, as evidenced by larger tight junction gaps(198). In another study, Mouzaki et al. provided evidence that individuals with NASH have elevated levels of Clostridium coccoides and a lower proportion of Bacteroides. No differences in absolute levels of Bacteroidetes, Bifidobacterium, Clostridium leptum, Escherichia coli, or total bacteria were noted(199). In contrast, Boursier et al. showed that individuals with NASH have increased levels of Bacteroides and decreased levels of Prevotella(200). Wong et al. demonstrated that NASH was associated with lower fecal
abundance of \textit{Faecalibacterium} and \textit{Anaerosporobacter} but higher abundance of \textit{Parabacteroides} and \textit{Allisonella} (201). In addition to these noted changes, it has also been observed that individuals with NAFLD tend to have an overgrowth of bacteria in the small intestine, which is associated with an impairment in intestinal permeability (202, 203). Overall, while it is clear that NAFLD is characterized by an altered gut microbiota, there is not yet an overall consensus as to which organisms are over- or under-represented.

Children with NAFLD have also been identified to have disturbed gut microbiota profiles. Given that NAFLD manifests histologically in a manner consistent with alcoholic fatty liver disease, it has been postulated that gut microbiota in NAFLD produce higher levels of ethanol. Zhu et al. provided evidence that children with NASH have increased levels of ethanol – producing Proteobacteria, Enterobacteriaceae, and \textit{Escherichia} as well as increased serum ethanol concentrations (204). A similar increase in serum ethanol in children with NAFLD was reported by Michail et al. (205). The association between increased ethanol production in NAFLD remains somewhat controversial, however. Engstler et al. provided evidence that increased steatosis in children may not arise from increased production of ethanol in the gut, but rather decreased expression of alcohol dehydrogenase in the liver (206). Regardless, whether children are at increased risk for NAFLD due to increase ethanol production or a decreased ability to metabolize the alcohol, potentially cutting off ethanol production at the gut microbiota level could be beneficial for preventing NAFLD pathogenesis in children.

With evidence of a dysbiotic gut microbiota in NAFLD, microbiota-based interventions using prebiotics and probiotics is receiving considerable attention. Prebiotics, by shifting the gut microbiota away from a dysbiotic state, have been demonstrated in animal models to elicit significant improvement in adiposity, intestinal permeability, endotoxemia, and hepatic steatosis (207). In an animal model of NAFLD, the mechanisms by which oligofructose mediates improvements in steatosis include inhibition of hepatic SREBP-2-induced cholesterol synthesis and increased peroxisome proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\))-mediated fatty acid oxidation (208). To date, there is only one study that has examined the effects of prebiotics in NASH. Daubioul et al., in a double-blind, cross-over design study, demonstrated that 16g/day oligofructose/day for 8 weeks reduced aspartate aminotransferase (AST) and reduced insulin
levels in 7 patients with NASH(209). Unfortunately, no histological outcomes were included in the study. As prebiotic fermentation yields SCFAs, it is likely that SCFAs play some role in mediating the potential benefits of prebiotics in NAFLD. Chambers et al., using an inulin-propionate ester as a novel SCFA delivery system, investigated the potential for inulin-propionate to improve metabolic health in overweight and obesity. Interestingly, after 24 weeks of consuming the 10g/day inulin-propionate ester, intrahepatocellular lipid content (assessed with MR spectroscopy), weight gain, and deterioration of insulin sensitivity was reduced(210). Although there are few studies assessing the effects of prebiotics in NAFLD, limited evidence suggests that prebiotics may be an effective treatment strategy.

Several studies have been conducted that have assessed the effects of a probiotic and small dose of prebiotic for treatment of NAFLD. As one of the few studies to include histological outcome measurements, in a randomized clinical trial, Malaguarnera et al. assessed the effects of *Bifidobacterium longum* (dose unspecified) and 2.5g/day fructo-oligosaccharide in addition to lifestyle intervention for a period of 24 weeks in 66 individuals. After 24 weeks of treatment, the addition of the synbiotic to lifestyle intervention resulted in greater improvement in serum AST, LDL, CRP, TNF-α, HOMA-IR, serum endotoxin, steatosis, and NASH activity index(211). In another study, Eslamparast et al. demonstrated in a group of 52 individuals with NAFLD that the synbiotic Protexin containing ~ 200million CFU *Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, and Lactobacillus bulgaricus* and an unspecified dose of fructo-oligosaccharide and hydroxypropyl methyl cellulose for 28 weeks improved ALT, gamma-glutamyl transferase (GGT), AST, high-sensitivity CRP, TNF-α, and fibrosis score assessed with transient elastography(212).

Numerous studies have also been completed assessing the effectiveness of probiotics for treatment of NAFLD. Aller et al. demonstrated that 500 million CFU *Lactobacillus bulgaricus* and *Streptococcus thermophilus* versus placebo significantly improved ALT, AST, and GGT, despite no changes in anthropometric measures in 28 patients with NAFLD(213). Wong et al. conducted a 6 month pilot clinical trial in 20 individuals with liver biopsy confirmed NASH using the probiotic Lepicol, which contains ~200million CFU (combined) *Lactobacillus*
*Lactobacillus plantarum* (ATCC 14917), *Lactobacillus delbrueckii* ssp. *bulgaricus* (ATCC 11842), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus rhamnosus* (ATCC 7469) and *Bifidobacterium bifidum* (ATCC 29521). At the end of the study, Lepicol was demonstrated to improve intrahepatic triglyceride (assessed with proton-magnetic resonance spectroscopy) and AST relative to control in individuals with liver biopsy proven NASH(214). As one of the few probiotic-based studies to assess the effect of the intervention on gut microbiota, it was observed that the reduction in intrahepatic triglyceride was associated with a reduction in Firmicutes and an increase in Bacteroidetes(201). In children, Alisi et al. conducted a randomized clinical trial to examine the effectiveness of the probiotic VSL#3 (~450 billion CFU/day *Streptococcus thermophilus*, bifidobacteria [*B. breve, B. infantis, B. longum*], *Lactobacillus acidophilus, L. plantarum, L. paracasei*, and *L. delbrueckii* subsp. *bulgaricus*) for treating of NASH. Four months of VSL #3 supplementation was demonstrated to reduce NASH severity, as assessed by ultrasonography. An increase in the secretion of GLP-1 was cited as one possible mechanism by which VSL#3 mediates its beneficial effects(215).

With compelling evidence that the gut-liver axis exerts a significant influence on the development of NAFLD, new strategies using prebiotics and probiotics have been investigated. Overall, prebiotic-based research studies that have been conducted in NAFLD and NASH are scarce and do not utilize the gold standard histological follow-up measurements. Although some beneficial effects using probiotic and synbiotics have been observed, few of these studies have provided evidence of the microbial shifts eliciting the noted physiological effects. As such, the mechanistic link between the consumption of the probiotic and the beneficial changes in hepatic metabolism is not entirely clear. Gut microbiota-based interventions using follow-up histological measurements and assessment of microbiota and metabolites is warranted for future investigations.

### 2.7 Conclusion

Gut microbiota plays a profound role in body weight and metabolic health, in part through caloric extraction as well as influence on the various biological systems that mediate appetite, metabolism, and inflammation. As the gut microbiota provide a genetic pool that can be non-
invasively manipulated through dietary and pharmaceutical means, the gut microbiota offers an intriguing intervention target. Both prebiotics and probiotics have been demonstrated to elicit significant improvement on the biological factors mediating obesity and associated metabolic disease including NAFLD. Developing effective interventions using prebiotics and probiotics requires a better understanding of how prebiotics and probiotics interact with gut microbiota and host physiology to impact health in obesity. Furthermore, given evidence that prebiotics may be able to alter the pathophysiology of NASH, a randomized controlled trial utilizing gold standard histological measurements is warranted to determine whether prebiotics may offer an effective treatment strategy for NASH.

2.8 Research Objectives and Hypotheses
Overall thesis objective: Examine how dietary agents including prebiotics and probiotics, gut microbiota, and host physiology interact to affect body composition and metabolic health in obesity.

The three specific objectives and hypotheses in this dissertation include:

**Objective #1:**
Determine the combined and individual effects of the bifidogenic prebiotic oligofructose and the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 on gut microbiota, glycemia, appetite regulation, and adiposity in obese rats.

_Hypothesis:_ Given the inherent synergistic potential between a bifidogenic prebiotic and probiotic, we hypothesized that oligofructose and *Bifidobacterium animalis* subsp. *lactis* BB-12 in combination would elicit greater improvement in adiposity, glucose control and appetite regulation than oligofructose or *Bifidobacterium animalis* subsp. *lactis* BB-12 alone

**Objective #2:**
Using antibiotics as a means to selectively knock down bacterial groups, identify the gut microbiota-dependent and -independent effects of oligofructose on body weight, adiposity, inflammation, and intestinal permeability in obese rats.
**Hypothesis:** With evidence demonstrating that oligofructose mediates improvements in metabolic health by selectively increasing health-promoting bacteria, including *Bifidobacterium* and *Lactobacillus*, we hypothesized that selective decontamination of certain bacterial groups would mitigate some of the microbiota-mediated metabolic improvements in obesity.

**Objective #3:**
In a pilot clinical trial, investigate the therapeutic potential of the prebiotic oligofructose for treatment of liver biopsy confirmed non-alcoholic steatohepatitis (NASH).

*Hypothesis:* We hypothesized that oligofructose would improve histological and biochemical markers of NASH through combined effects on inflammatory cytokines and gut microbiota.
3.1 Abstract

Prebiotics and probiotics may be able to modify an obesity-associated gut microbiota.

Objective: To examine the individual and combined effects of the prebiotic oligofructose (OFS) and the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) on gut microbiota and host metabolism in obese rats.

Design and Methods: Adult male, diet-induced obese Sprague Dawley rats were randomized to: 1) Control (C); 2) 10% OFS; 3) BB-12; 4) OFS + BB-12 for 8 weeks (n=9-10 rats/group). Body composition, glycemia, gut permeability, satiety hormones, cytokines, and gut microbiota were examined.

Results: Prebiotic, but not probiotic reduced energy intake, weight gain and body fat mass (*P*<0.01). OFS, BB-12 and the combined OFS + BB-12 improved glycemia (*P*<0.05). Individually, OFS and BB-12 reduced insulin levels (*P*<0.05). Portal GLP-1 was increased with OFS while probiotic increased GLP-2 (*P*<0.05). There was a marked increase in bifidobacteria and lactobacilli (*P*<0.01) with OFS which was not observed with probiotic alone.

Conclusions: The impact of prebiotic intake on body composition and gut microbiota was of greater magnitude than the probiotic BB-12. Despite this, an improvement in glucose AUC with both prebiotic or probiotic demonstrates the beneficial role of each of these ‘biotic’ agents in glycemic control.

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3.2 Introduction

The human biological system, while capable of coping with food scarcity, proves vulnerable to chronic food excess(216). Among the many factors that influence the development of obesity, there is a growing body of research demonstrating the prominent role of gut microbiota in energy balance and metabolism. Through centuries of co-evolution, a complex ectosymbiotic relationship has developed between gut microbiota and humans(44). In recent years it has been shown that gut microbiota differ between the lean and obese states(28). It is theorized that in the context of obesity, the symbiotic relationship between gut microbiota and host shifts to a ‘dysbiotic’ state, such that the microbiota impair host metabolism. Some but not all reports have shown that obesity is characterized by an increased ratio of Firmicutes to Bacteroidetes and reduced bifidobacteria cell numbers(28,73,79).

While fulfilling important biological and metabolic functions for the host, the microbiome offers a component of the human metagenome that can be altered through minimally invasive means. Strategies to restore gut microbiota include the use of prebiotics and probiotics. Prebiotics are non-digestible food ingredients that beneficially alter the composition and metabolism of the gut microbiota in such a way that it confers a health benefit to the host(217). Oligofructose (OFS) is highly fermentable in the caecal-colon and has been demonstrated to selectively enhance Bifidobacterium spp. and Lactobacillus spp. in favour of a lean phenotype(89,218). Probiotics are defined as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host”(37). Mounting evidence suggests that probiotics, such as Bifidobacterium spp. and Lactobacillus spp. are able to reduce adiposity, cholesterol, and body weight as well as improve glycemia(39,152-154,160,219).

The combination of a prebiotic and a probiotic is referred to as a synbiotic. Several studies have suggested that synbiotics have antiobesogenic properties(220,221). These studies, however, did not compare the effect of a synbiotic to a prebiotic or probiotic alone and conclusions about synergistic potential could not be evaluated. Our objective was to determine the synergistic, as well as singular effects and mechanisms by which the prebiotic OFS and the probiotic Bifidobacterium animalis subsp. lactis BB-12 alter gut microbiota profiles, glycemia, satiety hormone secretion, and adiposity in obese rats.
3.3 Methods and procedures

3.3.1 Animals and treatments

The study protocol was approved by The University of Calgary Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals. Eighty male Sprague-Dawley rats (10 wks of age) were obtained from Charles River (Charles River, St. Constant, PQ) and housed three per cage on a 12hr light–dark cycle in a temperature (20-22°C) and humidity controlled (41-60%) room. Rats were fed a high fat/sucrose diet *ad libitum* for eight weeks (Dyets, Inc., Bethlehem, PA) to induce obesity. The 40 rats with the greatest weight gain were transitioned to individual housing and randomized into 1 of 4 groups: 1) Control (C, AIN-93M diet); 2) 10% (wt/wt) OFS (Orafti P95, BENEQ-Orafti Inc.); 3) *Bifidobacterium animalis* subsp. *lactis* BB-12® (Chr. Hansen, Milwaukee, WI) (1×10¹⁰ CFU/d) (BB-12); 4) 10% OFS + BB-12 (OFS + BB-12) for a period of eight weeks (n=10 rats/group). The 10% dose of OFS was selected based on previous literature showing reductions in fat mass at this level(105). The dose of BB-12 was selected based on previous studies in rodents using a similar dose and evidence that it survives gastrointestinal transit in rats(222). Composition of experimental diets is provided in Table 3.1. Diets containing the probiotic *B. lactis* BB-12 were prepared fresh three times weekly. Freeze-dried probiotic BB-12 powder (3×10¹⁰ CFU/g) was mixed with AIN-93M diet to ensure that each rat received 1×10¹⁰ CFU/d BB-12 daily (~1.2-1.7g/100g AIN-93M). Body weight was measured once every week and food intake was measured three times per week. One day prior to sacrifice, rats were lightly anaesthetized with isoflurane and body composition was measured via dual-energy X-ray absorptiometry scan with software for small animals (Hologic ODR 4500; Hologic).
Table 3.1 Composition of experimental diets

<table>
<thead>
<tr>
<th></th>
<th>HFS</th>
<th>C^1</th>
<th>OFS^1</th>
<th>BB-12^1</th>
<th>OFS + BB-12^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>140</td>
<td>126</td>
<td>140</td>
<td>126</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.8</td>
<td>1.62</td>
<td>1.8</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>499.48</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Dyetrose</td>
<td>155</td>
<td>139.5</td>
<td>155</td>
<td>139.5</td>
<td></td>
</tr>
<tr>
<td>Cornstarch</td>
<td>465.7</td>
<td>419.1</td>
<td>465.7</td>
<td>419.1</td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>100</td>
<td>40</td>
<td>36</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>t-butylhydroquinone</td>
<td>0.02</td>
<td>0.008</td>
<td>0.0072</td>
<td>0.008</td>
<td>0.0072</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>45</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>35</td>
<td>35</td>
<td>31.5</td>
<td>35</td>
<td>31.5</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.25</td>
<td>2.5</td>
<td>2.25</td>
</tr>
<tr>
<td>OFS</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>BB-12 (3×10^{10} CFU/g)</td>
<td></td>
<td>15</td>
<td></td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>BB-12 (CFU/day)/rat</td>
<td></td>
<td>~1×10^{10}</td>
<td></td>
<td>~1×10^{10}</td>
<td></td>
</tr>
<tr>
<td>Energy (KJ/g)</td>
<td>19.2</td>
<td>15.1</td>
<td>14.2</td>
<td>15.1</td>
<td>14.2</td>
</tr>
<tr>
<td>Protein (% energy)</td>
<td>15.9</td>
<td>14.1</td>
<td>14.0</td>
<td>14.1</td>
<td>14.0</td>
</tr>
<tr>
<td>Carbohydrate (% energy)</td>
<td>45.0</td>
<td>76.0</td>
<td>76.0</td>
<td>76.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Fat (% energy)</td>
<td>39.1</td>
<td>9.9</td>
<td>10.0</td>
<td>9.9</td>
<td>10.0</td>
</tr>
</tbody>
</table>

^1Based on AIN – 93M diet (Dyets, Inc., Bethlehem, PA). BB-12, *Bifidobacterium animalis* ssp. *lactis* BB-12; C, control; HFS, High fat/high sugar; OFS, oligofructose.

3.3.2 *In vivo intestinal permeability*

Based on methods previously described(120,122), movement of fluorescein isothiocyanate-dextran-4000 daltons (FD-4) (Sigma-Aldrich, St. Louis, Missouri, USA) across the epithelium was used to assess intestinal permeability. On week 6, following 16h food deprivation, rats were gavaged with 500 mg/kg FD-4. At 1h and 4h post-gavage, 300 µl of blood was collected from the tip of the tail and centrifuged at 4°C for 3 min (12,000×g). Plasma samples were diluted in equal volumes of PBS and 100 ul loaded in duplicate into a 96 well plate. Standards were made by serial diluting FD-4 in a 1:3 v/v mixture of plasma:PBS. FD-4 concentrations were measured with a fluorescence spectrophotometer (excitation of 485 nm and emission of 535 nm).
3.3.3 Oral glucose tolerance test

One week prior to sacrifice, following 16h feed deprivation, rats were given an oral gavage of 2g/kg glucose. Blood was collected via tail nick at 0, 15, 30, 60, 90, and 120 min post gavage into a chilled tube containing diprotinin-A (0.034 mg/ml blood; (MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Blood glucose was measured immediately with a blood glucose meter (OneTouch Glucose Meter, Lifescan Inc.). Plasma was stored at -80°C until analysis for satiety hormones and cytokines. The insulinogenic index and composite insulin sensitivity index (CISI) was calculated as previously described(223). The early phase and total insulin response for the insulinogenic index was calculated using the formula (Insulin AUC15)/(Glucose AUC15) and (Insulin AUC120)/(Glucose AUC120), respectively. CISI was calculated with the formula:

\[
\frac{1000}{((\text{Glucose}_0 \times \text{Insulin}_0) \times \text{Average Glucose}_0-120 \times \text{Average Insulin}_0-120)}
\]

3.3.4 Plasma and tissue samples

Following 16 h feed deprivation, rats were anaesthetized with isoflurane. A portal blood sample was collected with inhibitors described above. Following the blood draw, rats were killed by over-anesthetization and aortic cut. The liver, stomach, small intestine, cecum, and colon were excised, weighed, and snap frozen in liquid nitrogen. Fecal and cecal matters were collected. All samples were stored at -80°C until analysis.

3.3.5 Plasma satiety hormones, cytokines, and LPS

Portal plasma glucagon-like peptide-1(active), glucagon-like-peptide 2 (GLP-2), and adiponectin were measured using ELISA kits (Millipore, Billerica, MA). Ghrelin (active), amylin (active), insulin, leptin, glucose-dependant insulinitropic polypeptide (GIP) (total), GLP-1 (active), and PYY (total) were quantified using a Milliplex Rat Gut Hormone Panel. TNFα, MCP-1, IL-1β, PAI-1(total), and IL-6 concentrations were quantified using a Milliplex Rat Adipokine Panel (Millipore). Portal plasma LPS was measured using a PyroGene Recombinant Factor C Endotoxin Endpoint Fluorescent Detection assay (Lonza) according to manufacturer directions.

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3.3.6 *Hepatic triglyceride content*

Triglycerides (TG) were measured using TG (GPO) reagent set (Point Scientific Inc., Lincoln Park, Michigan, USA) according to our previous work(95).

3.3.7 *Gut microbiota profiling using qPCR*

Microbial profiling was performed according to our previous work(218). Briefly, total bacterial DNA was extracted from fecal/cecal samples using FastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada) and quantified using PicoGreen DNA quantification kit (Invitrogen, Carlsbad, CA, USA). All samples were brought to a concentration of 4 ng/µl prior to storage at -20°C for later analysis. Amplification and detection were conducted in 96-well plates with SYBR Green 2 × qPCR Master Mix (BioRad). Samples were run in duplicate with a final volume of 25 µl containing 0.3 µM primer and 20 ng template DNA. The primers for *Lactobacillus* spp. do not cover the entire genera, but are specific for 15 species of lactobacilli (*L. acidophilus, L. amyloyticus, L. amylovorus, L. crispatus, L. fornicalis, L. gallinarum, L. hamsteri, L. helveticus, L. intestinalis, L. jensenii, L. kefiranofaciens ssp.kefirgranum, L. kitasatonis, L. psittaci, L. suntoryeus, and L. ultunensis*) (224). The specificity of the primers and the limit of detection were determined according to Louie et al.(225). The standard for *B. animalis* was obtained by extracting DNA from BB-12 freeze dried powder(226) and quantified. The 16S rRNA gene copies value was calculated according the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. Standard curves were normalized to the copy number of the 16S rRNA gene obtained from the following webpage: http://rrndb.mmg.msu.edu/index.php.

3.3.8 *Expression of genes related to intestinal permeability*

Gene expression for tight junction protein-1 (TJP1) and occludin were analyzed according to previous real-time PCR work(227). The primers were designed using Beacon Designer 3 software. TJP1: Sense Primer CCATGCCTCCTCCTCCTC, Anti-sense Primer ACGGAATTGCCTTCACCTCCTG; occludin: Sense Primer GAGGACTGGCTCAGGGAATATC, Anti-sense Primer TTGTGGACCTCGTGGTGTTCTG. Actin was confirmed to be a suitable
reference gene and is not changed in response to the treatment. PCR amplification efficiency was established for actin, occludin and TJP1 by means of calibration curves. The $2^{\Delta\Delta Ct}$ calculation was used to determine the relative difference in mRNA expression.

3.3.9 Determination of BB-12 viability

The concentration of *B. lactis* BB-12 supplied by Chr. Hansen was verified via traditional culture technique. Freeze dried BB-12 powder was suspended in autoclaved PBS containing 0.05% filter sterilized cysteine-HCl, followed by preparation of a 10-fold dilution series which was spread plated under anaerobic conditions. 0.1 ml of each of the dilution series samples were plated on MRS agar plate supplemented with 0.05% cysteine-HCl. After 72 hr incubation, the plates were removed and the CFU were counted to verify the stated concentration of the product.

3.3.10 BB-12 gavage study

To determine whether the storage time and food matrices had an impact on the inoculation potential of the BB-12 a brief gavage study was conducted. Sixteen male Sprague-Dawley rats (10 wk of age) were randomized to receive a daily gavage of either: 1) PBS control (Gavage - C) or 2) $1 \times 10^{10}$ CFU/d BB-12 suspended in PBS (Gavage BB-12) for two weeks while consuming AIN-93M diet *ad libitum*. Gut microbiota was analyzed as above.

3.3.11 Statistical analysis

All data are presented as mean ± SEM. A 2-way ANOVA was used to determine the main effects of prebiotic (OFS vs control-containing treatments), probiotic (BB-12 vs control-containing treatments) and their interaction. If a significant interaction was identified, a one way ANOVA with all 4 treatments was performed with Tukey’s *post hoc* test to determine differences between experimental groups. For measurements with repeated measures, a 2-way repeated measures ANOVA was used with time as the within-subject factor and prebiotic and probiotic as between-subject factors. For comparisons between the treatment groups in the BB-12 gavage study a one-way ANOVA was used. Analysis was completed using SPSS V19.0 software (SPSS Inc., Chicago, IL, USA). Data was considered significant at p<0.05.
3.4 Results

3.4.1 Food intake, body composition, hepatic triglyceride & cecum weight

Prebiotic but not probiotic reduced energy intake ($P=0.003$; Table 3.2). While body weight increased with time in all rats ($P<0.001$), only prebiotic reduced weight gain ($P=0.002$) (Figure 3.1), fat mass ($P=0.003$) and body fat % ($P=0.006$) (Table 3.2). Bone mineral density ($P=0.013$) and cecum weight ($P<0.001$) was higher in rats consuming prebiotic. Prebiotic reduced hepatic TG ($P=0.001$).
Table 3.2 Energy intake and body composition in obese rats treated with OFS, BB-12, both or neither for 8 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C</th>
<th>OFS</th>
<th>BB-12</th>
<th>OFS + BB-12</th>
<th>Pre</th>
<th>Pro</th>
<th>Pre × Pro</th>
<th>Two way ANOVA P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake², g/d</td>
<td>23.9 ± 0.3</td>
<td>24.3 ± 0.4</td>
<td>23.5 ± 0.5</td>
<td>23.0 ± 0.5</td>
<td>0.92</td>
<td>0.07</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Energy intake, KJ/d</td>
<td>360.7 ± 5.0</td>
<td>349.4 ± 5.7</td>
<td>354.7 ± 7.8</td>
<td>331.2 ± 6.5</td>
<td>0.01</td>
<td>0.07</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>485 ± 7.6</td>
<td>479 ± 6.9</td>
<td>490 ± 6.8</td>
<td>470 ± 7.2</td>
<td>0.08</td>
<td>0.76</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Total weight change, g/8 wk</td>
<td>60.0 ± 4.05</td>
<td>52.6 ± 4.34</td>
<td>65.1 ± 4.39</td>
<td>56.3 ± 4.50</td>
<td>0.003</td>
<td>0.77</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>60.0 ± 5.28</td>
<td>49.6 ± 3.51</td>
<td>65.4 ± 4.61</td>
<td>49.5 ± 2.95</td>
<td>0.003</td>
<td>0.53</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Percent fat, %</td>
<td>12.3 ± 1.05</td>
<td>10.4 ± 0.76</td>
<td>13.3 ± 0.88</td>
<td>10.5 ± 0.62</td>
<td>0.006</td>
<td>0.49</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³</td>
<td>0.169 ± 0.001</td>
<td>0.178 ± 0.002</td>
<td>0.174 ± 0.003</td>
<td>0.177 ± 0.001</td>
<td>0.013</td>
<td>0.42</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Liver, g</td>
<td>12.6 ± 0.49</td>
<td>12.0 ± 0.33</td>
<td>11.9 ± 0.29</td>
<td>12.0 ± 0.73</td>
<td>0.67</td>
<td>0.48</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Liver TG, µg/mg</td>
<td>29.7 ± 0.99</td>
<td>28.03 ± 0.68</td>
<td>30.0 ± 1.28</td>
<td>24.6 ± 0.58</td>
<td>0.001</td>
<td>0.10</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Cecum weight, g</td>
<td>0.93 ± 0.05</td>
<td>1.91 ± 0.13</td>
<td>0.96 ± 0.07</td>
<td>2.11 ± 0.19</td>
<td>0.001</td>
<td>0.35</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

¹Values are means ± SEM, n = 9-10. ²Refers to mean daily food intake over the 8 wk study. BB-12, *Bifidobacterium animalis* subsp. *lactis* BB-12; C, control; OFS, oligofructose; Pre, prebiotic; Pro, probiotic
Figure 3.1: Body weight of obese rats treated with OFS, BB-12, both or neither for 8 wk. Values are mean ± SEM, n=9-10. BB-12, *Bifidobacterium animalis* ssp. *lactis* BB-12; C, control; OFS, oligofructose.

### 3.4.2 Glycemic and insulinemic response

Glucose and insulin during the OGTT were affected by time (*P*<0.001) and the interaction of time and prebiotic (*P*<0.05; Figure 3.2A). The interaction between prebiotic and probiotic affected blood glucose levels during the OGTT (*P*=0.024). At fasting, both OFS (*P*=0.028) and OFS+BB-12 (*P*=0.022) were lower than C. At 15 min, OFS + BB-12 (*P*=0.009) was lower than C. At 90 min, both OFS (*P*=0.007) and BB-12 (*P*=0.003) were lower than C. Glucose AUC was affected by the interaction between prebiotic and probiotic (*P*=0.012), whereby OFS, BB-12 and OFS+BB-12 (*P*<0.015) were all lower than C. Independently, prebiotic (*P*=0.019) and probiotic (*P*=0.035) reduced insulin levels during the OGTT (Figure 3.2B). Only probiotic (*P*=0.007) reduced fasting insulin concentrations. Insulin AUC was significantly reduced by prebiotic (*P*=0.035) and probiotic (*P*=0.048)(Table 3.3).
Plasma Insulin, pmol/L

Prebiotic P = 0.019
Probiotic P = 0.035

Blood Glucose, mmol/L

Prebiotic X Probiotic P=0.024

Plasma Amylin, pmol/L

Prebiotic P=0.004
Probiotic P=0.063

Plasma Leptin, pmol/L

Prebiotic P<0.001
Probiotic P=0.079

Plasma Ghrelin, pmol/L

continued on next page
Figure 3.2: Blood glucose (A), plasma insulin (B), amylin (C), leptin (D), ghrelin (E), GIP (F), PYY (G) and portal GLP-1 (H) of obese rats treated with OFS, BB-12, both or neither for 8 wk.

Values are mean ± SEM, n=9-10. Labeled means at a time without a common letter differ, P<0.05. BB-12, *Bifidobacterium animalis* subsp. *lactis* BB-12; C, control; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; OFS, oligofructose; PYY, peptide tyrosine tyrosine.
Table 3.3 AUC for blood glucose and plasma satiety hormones of obese rats treated with OFS, BB-12, both or neither for 8 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C</th>
<th>OFS</th>
<th>BB-12</th>
<th>OFS + BB-12</th>
<th>Pre</th>
<th>Pro</th>
<th>Pre × Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, nmol/L X 120 min</td>
<td>1050 ± 35.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>908 ± 24.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>917 ± 24.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>921.4 ± 21.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.017</td>
<td>0.039</td>
<td>0.012</td>
</tr>
<tr>
<td>Insulin, nmol/L X 120 min</td>
<td>57 ± 6.6</td>
<td>45 ± 5.0</td>
<td>46 ± 2.3</td>
<td>38 ± 3.6</td>
<td>0.035</td>
<td>0.048</td>
<td>0.69</td>
</tr>
<tr>
<td>Amylin, nmol/L X 120 min</td>
<td>2.30 ± 0.17</td>
<td>2.18 ± 0.09</td>
<td>2.29 ± 0.12</td>
<td>1.81 ± 0.08</td>
<td>0.023</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td>Leptin, nmol/L X120 min</td>
<td>23.9 ± 2.13</td>
<td>14.1 ± 1.54</td>
<td>19.6 ± 2.01</td>
<td>12.8 ± 1.05</td>
<td>0.001</td>
<td>0.11</td>
<td>0.40</td>
</tr>
<tr>
<td>Ghrelin, nmol/L X120 min</td>
<td>7.4 ± 1.1</td>
<td>8.5 ± 1.0</td>
<td>6.4 ± 0.7</td>
<td>8.4 ± 0.8</td>
<td>0.09</td>
<td>0.56</td>
<td>0.60</td>
</tr>
<tr>
<td>PYY, nmol/L X 90 min</td>
<td>0.75 ± 0.07</td>
<td>1.07 ± 0.10</td>
<td>0.76 ± 0.06</td>
<td>0.91 ± 0.13</td>
<td>0.030</td>
<td>0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>GIP, nmol/L X 120 min</td>
<td>3.51 ± 0.38</td>
<td>4.04 ± 0.32</td>
<td>3.34 ± 0.29</td>
<td>3.79 ± 0.20</td>
<td>0.11</td>
<td>0.49</td>
<td>0.90</td>
</tr>
<tr>
<td>(InsulinAUC&lt;sub&gt;15&lt;/sub&gt;)/(Glucose AUC&lt;sub&gt;15&lt;/sub&gt;)&lt;sup&gt;, pmol/mmol&lt;/sup&gt;</td>
<td>71 ± 6.0</td>
<td>64 ± 8.5</td>
<td>64 ± 7.2</td>
<td>54 ± 5.0</td>
<td>0.23</td>
<td>0.25</td>
<td>0.90</td>
</tr>
<tr>
<td>(InsulinAUC&lt;sub&gt;120&lt;/sub&gt;)/(Glucose AUC&lt;sub&gt;120&lt;/sub&gt;)&lt;sup&gt;, pmol/mmol&lt;/sup&gt;</td>
<td>53 ± 4.9</td>
<td>50 ± 5.2</td>
<td>50 ± 3.4</td>
<td>42 ± 3.7</td>
<td>0.20</td>
<td>0.26</td>
<td>0.54</td>
</tr>
<tr>
<td>CISI, score</td>
<td>0.51 ± 0.07</td>
<td>0.70 ± 0.05</td>
<td>0.68 ±0.04</td>
<td>0.89 ± 0.09</td>
<td>0.004</td>
<td>0.011</td>
<td>0.89</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means ± SEM, n = 9-10. Labeled means in a row without a common letter differ, P≤0.05. BB-12, *Bifidobacterium animalis* subsp. *lactis* BB-12; C, control; CISI, composite insulin sensitivity index; GIP, glucose-dependent insulinotropic polypeptide; (Insulin AUC<sub>15</sub>)/(Glucose AUC<sub>15</sub>), ratio of insulin AUC:glucose AUC from 0 to 15 min of the oral glucose tolerance test; (Insulin AUC<sub>120</sub>)/(Glucose AUC<sub>120</sub>), ratio of insulin AUC:glucose AUC from 0 to 120 min of the oral glucose tolerance test; OFS, oligofructose; PYY, peptide tyrosine tyrosine; Pre, prebiotic; Pro, probiotic
3.4.3 Insulin sensitivity and surrogate indexes of beta-cell function

Given the fact that type 2 diabetes is both a function of insulin resistance at the tissue level as well as an impairment of pancreatic beta-cell function (228), we utilized proxy measures from OGTT data to assess these parameters. Insulin sensitivity, according to CISI calculations, improved with both prebiotic (P=0.004) and probiotic (P=0.011) (Table 3.3). No effects of the treatments were seen in early phase or total insulinogenic index calculations.

3.4.4 Plasma satiety hormones

For concentrations of hormones during the OGTT, there was a significant effect of time (P<0.001) for amylin, leptin, ghrelin, GIP and PYY; a time × prebiotic effect (P<0.01) for GIP and PYY; and time × probiotic (P<0.05) effect for amylin, leptin, ghrelin, GIP, and PYY (Figure 3.2). Amylin was reduced by prebiotic (P=0.004) and probiotic (P=0.063). Prebiotic reduced fasting amylin concentrations (P=0.029; Figure 3.2C) and amylin AUC (P=0.023) (Table 3.3). Prebiotic reduced leptin concentrations at all time points during the OGTT (P<0.001) (Figure 3.2D) and leptin AUC (P<0.001). Fasting GIP was decreased by probiotic (P=0.02) (Figure 3.2F). PYY was increased by prebiotic (P=0.001) (Figure 3.2G) at fasting and for AUC (P=0.030) (Table 3.3).

3.4.5 Portal plasma gut hormones

GLP-1 was increased in portal plasma by prebiotic (P<0.001) (Figure 3.2H). GLP-2 was increased by probiotic (P=0.04) (Figure 3.3A). No difference in adiponectin was detected (data not shown).
Figure 3.3: Portal GLP-2 (A); ileal TJP-1 and occludin mRNA levels (B); serial plasma FD-4 concentrations (C); and FD-4 AUC (D) in obese rats treated with OFS, BB-12, both or neither for 8 wk.

Values are mean ± SEM, n=9-10. BB-12, *Bifidobacterium animalis* subsp. *lactis* BB-12; C, control; FD-4, Fluorescein isothiocyanate-dextran - 4000 daltons; GLP-2, glucagon-like peptide-2; OFS, oligofructose. TJP1, tight junction protein-1.
3.4.6 Intestinal permeability, LPS, cytokines, and tight junction proteins

Probiotic but not prebiotic increased TJP1 mRNA levels in the ileum ($P=0.012$) (Figure 3.3B). No differences were seen in intestinal occludin mRNA levels. *In vivo* intestinal permeability, tested with FD-4, did not show differences between groups (Figure 3.3C,D). No differences were detected in plasma LPS, TNF-α, IL-6, IL-1β, PAI-1, and MCP-1 (data not shown).

3.4.7 Gut Microbiota

Prebiotic increased *Bacteroides* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and *B. animalis* (all: $P<0.002$). Prebiotic decreased *C. coccoides*, *C. leptum*, *Clostridium* cluster XI and I and Enterobacteriaceae (all: $P<0.004$) (Table 3.4). There was no apparent change in the composition of the gut microbiota with the probiotic. However, a direct comparison between C and BB-12 revealed that *B. animalis* was elevated in rats that received BB-12. This was reflected in both fecal samples ($P<0.001$) and cecal matter ($P<0.001$) (Figure 3.4). The ratio of Firmicutes (*C. coccoides*, *C. leptum*, *Clostridium* cluster XI and I, *Roseburia* spp., *Lactobacillus* spp.) to Bacteroidetes (*Bacteroides/Prevotella* spp.) was reduced by prebiotic ($P=0.0015$) and probiotic ($P=0.0011$).

Two weeks of gavaging BB-12 elicited an increase in *B. animalis* similar to that observed with oral ingestion in the diet. *B. animalis* was elevated in both fecal ($P<0.001$) and cecal ($P<0.01$) matter in Gavage BB-12 relative to Gavage-C (Figure 3.4).
Table 3.4 16S rRNA copy number and relative abundance of cecal microbiota of obese rats treated with OFS, BB-12, both or neither for 8 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C (x1000)</th>
<th>OFS (x1000)</th>
<th>BB-12 (x1000)</th>
<th>OFS + BB-12 (x1000)</th>
<th>Pre</th>
<th>Pro</th>
<th>Pre × Pro</th>
<th>Two way ANOVA P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>43831 ± 3984</td>
<td>43103 ± 2619</td>
<td>40594 ± 2481</td>
<td>38626 ± 1698</td>
<td>0.63</td>
<td>0.17</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Bacteroides/Prevotella spp.</td>
<td>3747 ± 133 (8.5)</td>
<td>5074 ± 359 (11.8)</td>
<td>4472 ± 31 (11.0)</td>
<td>5420 ± 462 (14.0)</td>
<td>0.002</td>
<td>0.13</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Clostridium coccoides (cluster XIV)</td>
<td>8898 ± 957 (20.3)</td>
<td>7423 ± 389 (17.2)</td>
<td>8115 ± 441 (20.0)</td>
<td>6070 ± 437 (15.7)</td>
<td>0.004</td>
<td>0.07</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Clostridium leptum (cluster IV)</td>
<td>3787 ± 316 (8.6)</td>
<td>2083 ± 117 (4.8)</td>
<td>3516 ± 454 (8.7)</td>
<td>1565 ± 128 (4.1)</td>
<td>0.001</td>
<td>0.18</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster XI</td>
<td>0.98 ± 0.18 (0.0)</td>
<td>0.20 ± 0.28 (0.0)</td>
<td>0.70 ± 0.09 (0.0)</td>
<td>0.22 ± 0.03 (0.0)</td>
<td>0.001</td>
<td>0.21</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>82.3 ± 8.89 (0.2)</td>
<td>24.0 ± 2.27 (0.1)</td>
<td>76.3 ± 8.90 (0.2)</td>
<td>29.1 ± 5.53 (0.1)</td>
<td>0.00</td>
<td>0.94</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Roseburia spp.</td>
<td>2.48 ± 0.84 (0.0)</td>
<td>4.78 ± 1.79 (0.0)</td>
<td>2.86 ± 1.25 (0.0)</td>
<td>6.00 ± 2.59 (0.0)</td>
<td>0.14</td>
<td>0.66</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>2713 ± 495 (6.2)</td>
<td>6207 ± 964 (14.4)</td>
<td>2419 ± 272 (6.0)</td>
<td>3899 ± 835 (10.1)</td>
<td>0.001</td>
<td>0.08</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>234 ± 97 (0.5)</td>
<td>3795 ± 539 (8.8)</td>
<td>204 ± 52 (0.5)</td>
<td>2958 ± 261 (7.7)</td>
<td>0.001</td>
<td>0.17</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium animalis</td>
<td>0.030 ± 0.004 (0.0)</td>
<td>148.5 ± 27.2 (0.3)</td>
<td>82 ± 1.45 (0.0)</td>
<td>104.4 ± 13.9 (0.3)</td>
<td>0.00</td>
<td>0.19</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Methanobrevibacter spp.</td>
<td>5.44 ± 0.75 (0.0)</td>
<td>4.14 ± 1.26 (0.0)</td>
<td>5.08 ± 0.79 (0.0)</td>
<td>3.96 ± 1.14 (0.0)</td>
<td>0.25</td>
<td>0.79</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>37.3 ± 6.4 (0.1)</td>
<td>14.6 ± 2.4 (0.0)</td>
<td>47.3 ± 10.8 (0.1)</td>
<td>14.2 ± 1.3 (0.0)</td>
<td>0.00</td>
<td>0.46</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Firmicutes/Bacteroidetes ratio</td>
<td>4.08 ± 0.27</td>
<td>3.18 ± 0.28</td>
<td>3.29 ± 0.31</td>
<td>2.39 ± 0.31</td>
<td>0.004</td>
<td>0.011</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

1Values are means ± SEM, n = 9-10. 2 16S rRNA gene copies/20ng total genomic DNA. In order to fit in the table, all values except the ratio were divided by 1000. Therefore data is 16S rRNA gene copies (103)/20 ng genomic DNA. The number in brackets indicates the relative abundance (%) of bacterial taxa per total bacteria (16S rRNA gene copies / total 16S rRNA gene copies). BB-12, Bifidobacterium animalis subsp. lactis BB-12; C, control; OFS, oligofructose; Pre, prebiotic; Pro, probiotic.
Figure 3.4: *Bifidobacterium animalis* in fecal matter from obese rats administered BB-12 via oral gavage or in the feed.
Values are mean ± SEM, n=8-10. BB-12, *Bifidobacterium animalis* subsp. *lactis* BB-12; C, control. *Mean values of rats fed or gavaged BB-12 differ from C (P<0.05)
3.5 Discussion

Our objective was to determine if the combination of a prebiotic and probiotic enhanced the individual effects of these treatments. Based on evidence of singular antiobesogenic effects and luminal synergism between prebiotics and probiotics, we hypothesized that potentiated effects on energy intake, body composition, gut barrier function, and glycemia would be observed. OFS elicited potent effects on gut microbiota and reduced adiposity and glycemia. BB-12 improved glycemia but the effects on gut microbiota were more subtle and absent in regards to adiposity. Despite the noted metabolic improvements with OFS and BB-12, the combination of prebiotics and probiotics did not yield potentiated host metabolic responses.

Classically, prebiotic feeding has been associated with a reduction in energy intake and body fat(105). Our study confirms that OFS supplementation elicits a reduction in energy intake, body fat and weight gain. OFS contributes to reduced energy intake by decreasing the overall caloric density of the diet and by enhancing satiety through the secretion of satiety hormones GLP-1 and PYY(96,105,218,229). In support of this purported mechanism, we observed that OFS increased fasting portal plasma GLP-1 as well as PYY AUC.

Evidence for the ability of probiotics to reduce body weight and adiposity is more limited and controversial(39). Our study indicates that BB-12 does not affect body weight or adiposity. A recent study in DIO Sprague Dawley rats examined the effect of 4 different strains of Bifidobacterium spp. on body weight. Of the 4 strains tested, one was found to reduce weight gain, while the other three were weight neutral or increased weight gain(152). Two studies recently reported that B. breve and B. adolescentis limit weight gain and adipose tissue mass in DIO animal models(153,154). Other studies have found that Lactobacillus gasseri, in both animals and humans, reduces body weight and adiposity(156,219). Altogether, this evidence suggests that probiotic effects on body weight may be strain and model dependent.

Bifidobacterium longum ssp. infantis and other bifidobacteria are some of the first organisms to colonize the gut after childbirth and are recognized as an important commensal group of bacteria(230). Our study demonstrated the classical ‘bifidogenic’ effect of OFS(78), with Bifidobacterium spp. and B. animalis increasing relative to C. Interestingly, the increase in B. animalis elicited by OFS far surpassed that of BB-12. Contrary to our hypothesis, probiotic did
not produce an independent effect for increasing *Bifidobacterium* spp. or *B. animalis* when analyzed against prebiotic. Relative to C, BB-12 did elicit an increase in *B. animalis* but this increase was low compared to the increase observed with the prebiotic. Given these results, we were concerned that BB-12 had been rendered non-viable in the diet matrix. We therefore completed an additional study to determine if administering the BB-12 via oral gavage would increase intestinal *B. animalis*. Virtually no differences in fecal *B. animalis* concentrations were observed with gavage versus feed-administered BB-12. Our findings are consistent with that of another study which found that provision of *B. animalis* spp. *lactis* GCL2505 increased *B. animalis* spp. *lactis* but no other endogenous bifidobacteria(231). These findings likely reflect the vulnerability of probiotics to the harsh environment of the digestive tract(231).

In some but not all reports, obesity has been associated with a decrease in Bacteroidetes and a proportional increase in Firmicutes(70,73). Despite no main effect of probiotic on any one genus or species of bacteria, both prebiotic and probiotic decreased the ratio of Firmicutes to Bacteroidetes. This is relevant in that an obesity associated gut microbiota has been found to be more efficient at extracting luminal energy, potentially contributing to weight gain(80). Prebiotic decreased the ratio between Firmicutes and Bacteroidetes due to an increase in *Bacteroides* spp., and a decrease in prominent members of *Clostridium* spp., including *C. leptum* and *C. coccoides*. Lactobacilli, a group of bacteria belonging to the Firmicutes phylum, also increased in the prebiotic groups, which is important to note as certain lactobacilli have been recognized for health promoting properties. In our study probiotic BB-12 did not increase *Bacteroides* spp. or *Lactobacillus* spp. With *Lactobacillus* spp. belonging to the Firmicutes phylum, the lack of change on *Lactobacillus* spp. with probiotic contributed in part to a reduced ratio of Firmicutes to Bacteroidetes. Therefore, despite seeing beneficial improvements in the ratio between Firmicutes to Bacteroidetes with probiotic treatment, this does not necessarily reflect a more advantageous profile of bacteria with the probiotic relative to the prebiotic.

In type 2 diabetes, hyperglycemia is considered the primary factor propagating metabolic decline(228). For this reason, strategies that improve glucose control are imperative for successful management of the disease. Our study identified an interaction effect between prebiotic and probiotic on glucose levels during an OGTT. The interaction between prebiotic and
probiotic, however, was not a potentiated reduction in glycemia. Rather, OFS, BB-12, and OFS+BB-12 all reduced glycemia to the same extent, suggesting that the dose of prebiotic or probiotic alone saturated the effect. Independently, both prebiotic and probiotic reduced postprandial insulin with the probiotic also reducing insulin levels at fasting. The CISI calculation, with larger numbers indicating improved insulin sensitivity, indicates that both prebiotic and probiotic improved insulin sensitivity. This is an important consideration given the fact that insulin resistance can place additional stress on beta-cells in the pancreas in the context of hyperglycemia(232). While an improvement in glucose control has been reported in overweight and obese adults consuming prebiotics(98), the current data highlights a potential role for probiotics in improving glycemia that should be verified in humans as well.

Gut barrier function plays an important role in halting the development of diabetes and metabolic disease, in part by limiting the passage of pro-inflammatory agents from the gut into the circulation. LPS, a lipoglycan found on the cell surface of gram negative bacteria, interacts with toll-like receptor 4 (TLR4) and initiates the release of proinflammatory cytokines(121,233). GLP-2, a peptide that is co-secreted with GLP-1, plays a pivotal role in intestinal adaptation, epithelial cell-proliferation, and maintenance of gut integrity(122). Previous studies have found that GLP-2 elicits improvements in gut permeability, endotoxemia, and increased expression of zonula occludens (ZO-1) and occludin(122). We observed an increase in GLP-2 specifically with probiotic. While we did not see changes in intestinal permeability with the FD-4 experiments, LPS, or pro-inflammatory cytokines in plasma, we did find that probiotics elevated mRNA TJP1 expression.

Our study was limited by the fact that the rats gained less fat mass than has typically been seen in our DIO model. Following similar durations of high energy diet consumption, our DIO rats typically display body fat percentages ranging from 17-24% which is higher than the 10-13% seen in the current study. This lower body fat % is one possible explanation why we did not find the significant changes in gut barrier integrity and endotoxemia that has been observed in other studies with ob/ob mice(120,122). Furthermore, our microbial 16S rRNA analysis does not allow us to understand the function of the identified bacteria and future studies using a microbial
metagenomic sequencing approach would provide valuable information about the functional diversity of the bacterial community.

In conclusion, despite the hypothesized synergistic effects between prebiotic and probiotic, little potentiation of the effects was observed here in the case of OFS and BB-12. Our results demonstrate that prebiotics, in comparison to probiotics, provide a more potent stimulus in reducing adiposity and modifying gut microbiota. Both prebiotics and probiotics, however, offer benefits in regards to improving glucose tolerance. With a need for more non-invasive strategies for individuals with overweight and obesity, both prebiotic and probiotic treatments offer promise for improving metabolic outcomes, particularly glycemia.

3.6 Acknowledgements
The authors would like to thank Chr. Hansen (Milwaukee, WI) for the generous donation of *Bifidobacterium animalis* subsp. *lactis* BB-12 for our study. Kristine Lee, Faculty of Kinesiology, University of Calgary for technical assistance. Carol Stremich, Biological Sciences, University of Calgary for technical assistance. Dr. D. Morck, Biological Sciences, for allowing us to use his anaerobic hood. Dr. L. McMullen and Dr. M. Gaenzle, Agricultural, Food and Nutritional Science, University of Alberta for technical assistance. This project was funded by Canadian Institutes of Health Research (MOP 115076).
Chapter Four: **Improvement in adiposity with oligofructose is modified by antibiotics in obese rats**

4.1 Abstract

Given the intimate link between gut microbiota and host physiology, there is significant interest in understanding the mechanisms by which diet influences gut microbiota and impacts human metabolic health. Using antibiotics and the prebiotic oligofructose, which has been shown to counteract excess fat mass, we explored the gut microbiota-dependent effects of oligofructose on body composition and host metabolism. Male Sprague-Dawley obese rats, fed a background high-fat/sucrose diet, were randomized to: 1) high energy control (HE); 2) 10% oligofructose (OFS); 3) ampicillin (AM); 4) ampicillin + 10% oligofructose (AM+OFS); 5) ampicillin/neomycin (AM/NE); 6) ampicillin/neomycin + 10% oligofructose (AM/NE+OFS) for 6 weeks. Combining oligofructose with ampicillin treatment blunted the decrease in adiposity seen with OFS. Although ampicillin did not affect total bacteria, ampicillin impeded oligofructose-induced increases in *Bifidobacterium* and *Lactobacillus*. In contrast, the combination of ampicillin and neomycin reduced total bacteria but did not abrogate the oligofructose-induced decrease in adiposity. Oligofructose-mediated effects on host adiposity and metabolic health appear to be, in part, dependent upon the presence of specific microbial species within the gut.

4.2 Introduction

A growing body of research now details the intimate physiological link between gut microbiota and human health. Composed of bacteria, protozoa, archaea, and fungi, the gut microbiota facilitates an important role in immune system development(234) and energy harvest via the fermentative production of short chain fatty acids (SCFA), which serve as important physiological regulators of metabolism and satiety in the human host(110). A perturbed gut microbiota in humans and animals has been linked with the development of inflammatory bowel disease. **

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disease and metabolic conditions including obesity, diabetes, and non-alcoholic fatty liver
disease(235).

The unique mutualistic relationship between gut microbiota and the mammalian host has
developed over millions of years. Historically, with ancient humans consuming a high fiber diet,
the predominant bacteria that fulfil the co-adaptive mutualistic function between gut microbiota
and host evolved to be saccharolytic bacteria(38,236,237). Through fermentation of complex
carbohydrates, saccharolytic bacteria mobilize energy for the host and proliferate at the exclusion
of other harmful bacteria. It is hypothesized that a diet that deviates from the historical high fiber
diet elicits a dysbiotic shift in the gut microbiota composition, such that the activity and
metabolites produced by the gut microbiota may predispose to pathophysiological
conditions(33,38,90,238).

With evidence that a western, low fiber diet elicits deleterious effects on gut microbiota and
human health, there has been intense interest in restoring the mutualistic gut microbiota-host
relationship. Significant interest lies in the use of prebiotics as a means to restore this
equilibrium. Prebiotics are defined as a “selectively fermented ingredient that results in specific
changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring
benefit(s) upon host health”(36). The first prebiotics to be proposed were the fructo-
oligosaccharides due to their ability to increase bifidobacteria and lactobacilli, two bacterial
genera with health promoting properties and some of the early organisms to colonize the infant
gut(239,240). A wealth of research has demonstrated improvements in glycemia, inflammation,
satiety, adiposity and body weight with prebiotic supplementation(241). Underlying mechanisms
mediating these benefits include enhanced satiety hormone secretion(105,218), reduced gut
permeability(122), and altered endocannabinoid tone(242) and adipose tissue metabolism(116).

Despite the wealth of evidence purporting the health benefits of prebiotics, there has been a
lack of research exploring whether the gut microbiota shifts associated with prebiotic
consumption are necessary for mediating improvements in metabolism. In order to assess the
requirement for these bacterial organisms in mediating prebiotic effects, we devised an
experiment using selective decontamination of gut microbes with antibiotics to analyze the
benefits of the prebiotic oligofructose. Our aim was to assess the dependence of oligofructose-
mediated improvement in body weight, adiposity, intestinal permeability, and glycemia on the purported microbiota shifts that oligofructose classically elicits.

4.3 Materials and methods

4.3.1 Animals

Ethical and study protocol approval was granted by the University of Calgary Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals. 120 male Sprague-Dawley rats (10 wks of age) were obtained from Charles River (Saint Constant, QC, Canada) and housed three per cage on a 12hr light–dark cycle in a temperature (20-22°C) controlled room. Rats were fed a high fat/sucrose (HFS) diet ad libitum for eight weeks (Diet #102412, Dyets, Inc., Bethlehem, PA, USA) to induce obesity. Continuing on the background HFS diet, the 60 rats with the greatest weight gain, representing the obesity-prone phenotype, were transitioned to individual housing and randomized into 1 of 6 groups: 1) high energy control (HE); 2) 10% (wt/wt) oligofructose (OFS) (Orafti P95, Beneo-Orafti Inc., Tienen, Belgium); 3) ampicillin (AM); 4) ampicillin + 10% oligofructose (AM+OFS); 5) ampicillin/neomycin (AM/NE); 6) ampicillin/neomycin + 10% oligofructose (AM/NE+OFS); n=10 rats/gp. Composition of the experimental diets is provided in Table 4.1. Diet containing 10% oligofructose was prepared by manually mixing 100g of oligofructose with 900g of HFS diet. Two antibiotic treatments (ampicillin and ampicillin/neomycin) were used to elicit variable effects on gut microbiota. Ampicillin alone was selected given previous evidence that Lactobacillus and Bifidobacterium are susceptible to ampicillin treatment(243), whereas the combination of ampicillin/neomycin is a commonly used model to target the commensal microbes and achieves a significant reduction in bacteria(120,244,245). Both antibiotics are broad spectrum and poorly absorbed. Ampicillin was provided at a dose of 35mg/kg BID and neomycin at 15mg/kg BID via oral gavage Monday–Friday. On weekends, rats received drinking water containing 1mg/ml ampicillin and 0.5 mg/ml neomycin(120,244). The 10% dose of oligofructose was selected based on previous literature showing reductions in fat mass at this level(105,168). Average daily food intake, corrected for any spillage, was measured over a 4 day period in weeks 2, 3, 5 and 6. To ensure that an appropriate number of animals was used in our study, we performed a sample size estimation (β=0.8, α=0.05) based on previously measured
differences\cite{168,246} in serum levels of glucagon-like peptide-1 (GLP-1), a satiety hormone classically increased with prebiotics\cite{38}. One day prior to sacrifice, body composition was measured via dual-energy X-ray absorptiometry with software for small animals (Hologic ODR 4500, Marlborough, MA, USA).

Table 4.1 Composition of background HFS diet and HFS diet containing 10% oligofructose

<table>
<thead>
<tr>
<th>(g/kg)</th>
<th>HFS</th>
<th>HFS + 10% oligofructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>180</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>499.48</td>
<td>449.53</td>
</tr>
<tr>
<td>Lard</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>t-butylhydroquinone</td>
<td>0.02</td>
<td>0.018</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>35</td>
<td>31.5</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.25</td>
</tr>
<tr>
<td>Oligofructose\textsuperscript{1}</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Carbohydrate, % energy</td>
<td>43.3</td>
<td>41.8</td>
</tr>
<tr>
<td>Protein, % energy</td>
<td>17.6</td>
<td>17.0</td>
</tr>
<tr>
<td>Fat, % energy</td>
<td>39.1</td>
<td>37.7</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>4.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\textsuperscript{1}OFS assigned a caloric value of 1.5kcal/g.

4.3.2 Glucose tolerance

Oral glucose tolerance tests (OGTTs) were conducted at baseline and one week prior to sacrifice. Following 16h feed deprivation, a fasted blood sample was taken and rats received an oral gavage of 2g/kg glucose. Additional blood was collected via tail nick at 15, 30, 60, 90, and 120 min post gavage into a chilled tube containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, LLC, Solon, OH, USA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, St. Louis, Missouri, USA) and 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (1mg/ml of blood; MP Biomedicals). Using a blood glucose meter (OneTouch Glucose Meter, Lifescan Inc., Milpitas, CA, USA), blood glucose was measured immediately. Serum was collected and stored at -80°C for satiety hormones analysis. The composite insulin sensitivity index (CISI) was calculated as previously described\cite{168}.
4.3.3 In vivo intestinal permeability
Intestinal permeability was measured using fluorescein isothiocyanate-dextran-4000 (FD-4)(Sigma-Aldrich, St. Louis, Missouri, USA) according to methods previously described(120,122,168).

4.3.4 Serum and tissue samples
On the final day of the study, following 16 h feed deprivation, rats were anaesthetized with isoflurane. After a cardiac blood draw, rats were killed by over-anesthetization and aortic cut. Liver, cecum, and retroperitoneal and inguinal adipose tissue were excised and snap frozen in liquid nitrogen. Fresh fecal samples were collected at baseline, week 3, and sacrifice and cecal contents collected posthumously. All samples were stored at -80°C.

4.3.5 Serum satiety hormones, cytokines, and lipopolysaccharide (LPS)
GLP-1(active) and GLP-2 from cardiac bleed serum were measured using ELISA kits (Millipore, Billerica, MA, USA). Ghrelin (active), amylin (active), insulin, leptin, glucose-dependant insulinotropic polypeptide (GIP)(total), and PYY (total) from the OGTT were quantified using a Milliplex Rat Gut Hormone Panel. Tumor necrosis factor-α (TNFα), monocyte chemoattractant protein-1 (MCP-1), IL-1β, plasminogen activator inhibitor-1 (PAI-1)(total), and IL-6 from cardiac bleed serum were quantified using a Milliplex Rat Adipokine Panel (Millipore). LPS from cardiac bleed serum was measured using a PyroGene Recombinant Factor C Endotoxin Endpoint Fluorescent Detection assay (Lonza, Walkersville, MD, USA) according to manufacturer directions.

4.3.6 Hepatic triglyceride content
Triglycerides (TG) were measured using TG (GPO) reagent set (Point Scientific Inc., Lincoln Park, Michigan, USA) according to previous work(95).
4.3.7 16S rRNA qPCR microbiota analysis

Using ~ 150mg cecal/fecal matter, DNA was extracted using the FastDNA Spin kit for feces (MP Biomedicals, LLC, Solon, OH, USA) and quantified using the Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were diluted to 4ng/µl and stored at negative 20°C until analysis. Microbial profiling using group-specific 16S primers was conducted using an iCycler (Bio-Rad, Hercules, CA, USA) as previously described(168). Purified template DNA from reference strains (ATCC, Manassas, VA, USA) was serially diluted to generate a standard curve. Standard curves were normalized to copy number of 16S rRNA genes using reference strain genome size and 16S rRNA gene copy number values obtained from the rrnDB(247). Group specific primers were used as previously described(248). The specificity of the primers and the limit of detection were determined according to Louie et al(225). Threshold cycle values were used to calculate the number of 16S rRNA gene copies in each sample. Data is expressed as Log₁₀ 16S rRNA gene copy number per mg fecal/cecal matter.

4.3.8 16S rRNA Illumina sequencing analysis

Extracted cecal DNA was purified using sodium chloride ethanol and quantified using the Qubit dsDNA assay (Promega, Madison, WI, USA) and diluted to 5ng/μL. Microbial composition was determined following Illumina’s 16S rRNA amplicon sequencing protocol on the MiSeq platform (Illumina, San Diago, CA, USA). The V3 and V4 region of the 16S rRNA gene was amplified using 2.5 ul (5ng/ul) microbial DNA, 5ul (1uM) of gene specific primers (forward primer -5’TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG and reverse primer -5’GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C), and 12.5ul 2x KAPA HiFi Hotstart Ready Mix (KAPA Biosystems, Boston, MA, USA). PCR thermocycler amplification steps included an initial denaturation step (3:00 @ 95C) followed by 25 cycles of denaturation (00:30 @95C), annealing (00:30 @55C) and elongation (00:30 @72C). The PCR product was purified using Ampure XP beads (Beckman Coulter, Mississauga, ON, Canada) and eluted in 50ul of 10mM Tris pH 8.5. A subset of samples were run on a D1000 Tapestation assay (Agilent Technologies, Santa Clara, CA, USA) to confirm amplicon size (~550bp). Dual-index barcodes were attached to amplicon targets in a second PCR stage using 5ul purified DNA, 5 ul Nextera XT Index Primer 1 and 2,
25μl of 2x HiFi Hotstart Ready Mix (KAPA Biosystems), and 10μl of PCR grade water. Amplification steps included an initial denaturation step (3:00 @ 95°C) followed by 8 cycles of denaturation (00:30 @95°C), annealing (00:30 @55°C), and elongation (00:30 @72°C). The final PCR product was purified using Ampure XP beads (Beckman Coulter) and eluted in 25μl of 10mM Tris pH 8.5. The products were quantified using the Qubit dsDNA assay (Promega). Amplicon size was assessed using a D1000 TapeStation (Agilent Technologies) assay and samples were normalized to 4nM using 10mM Tris pH 8.5. After pooling bar-coded libraries, samples were denatured and diluted to a final concentration of 4pM and a final product containing 10% PhiX underwent dual indexed paired 300bp sequencing on the MiSeq using Reagent kit v3 (Illumina). Paired-end reads were merged using PEAR (Paired-End read merger)(249) and data analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline version 1.9.1(250). Operational taxonomic units (OTUs) were picked using UCLUST(251) with a 97% sequence identity threshold followed by taxonomy assignment using the latest Greengenes database (http://greengenes.secondgenome.com). To evaluate beta-diversity, principle coordinates analysis (PCoA) on weighted UniFrac distances was performed on all OTUs using QIIME. Alpha diversity was measured by calculating the Shannon index, Simpson index, and Chao1 metrics using QIIME(250).

4.3.9 Citrate synthase activity

Citrate synthase (CS) activity was measured according to methods previously described(252). Adipose tissue samples were homogenized 2:1 with NP-40 cell lysis buffer (Invitrogen, Waltham, MA, USA) with protease inhibitor cocktail (Sigma #P-2714, St. Louis, Missouri, USA) in Matrix D tubes in a Fast Prep-24 Tissue Homogenizer (MP Biomedicals, LLC, Solon, OH, USA). Tubes were centrifuged at 1500g at 4°C and lipid carefully removed. Each homogenate was freeze thawed three times using liquid nitrogen to lyse membranes. Homogenate protein concentrations were measured using a DC protein assay (Bio-Rad) and diluted to 1.5μg/μl protein. CS activity was determined by measuring the rate of production of the mercaptide ion spectrophotometrically at 412 nm using a microplate reader (Molecular Devices SpectraMax 190) after the addition of 10 μl of the homogenate in duplicate into a 96
well plate along with 0.3 mM Acetyl CoA, 100 mM Tris buffer (pH 8.0), 0.1 mM DTNB, and 0.5 mM oxaloacetate at 37°C.

4.3.10 Tissue RT-PCR

Gene expression in white adipose tissue (retroperitoneal and inguinal) and liver was analyzed according to previous real-time PCR work(168). The primers were designed using Beacon Designer 3 software and calibration curves completed for all primers to confirm PCR amplification efficiency. TFAM: Sense – GGG GCT AAG GAT GAG TCA CC, Anti-sense – CTT CAG CCA TTT GCT CTT CCC; Cyp7α1: Sense – GCT CTG GAG GGA GTG CCA TTT AC, Anti-sense – GCT GTG CGG ATA TTC AAG GAT GC; PGC-1α: Sense – AGA GGC AGA AGC AGA AAG C, Anti-sense – TGT CTC CAT CCC GCA G; GPR43: Sense – ACC CTC TGC TAT TCT ACT TCT CCT C, Anti-sense – CCT CCA CTG TCT CTT CGG CTC; SREBP-1c: Sense – TTT GTC TAC GGG GAA CCT GT, Anti-sense – GAG GCT GGT TTT GAC CCT TA; LPL: Sense – CCC TAC AAA GTA TTC CAT TAC C, Anti-sense – CCG TGT AAA TCA AGA AGG AG; β-Actin: Sense – AGA TCA AGA TCA TTG CTC CTC C, Anti-sense – ACA TCT GCT GGA AGG TG. Actin was confirmed to be a suitable reference gene and is not changed in response to the treatment. The 2^\(\Delta\Delta C_t\) calculation was used to determine the fold change in mRNA expression relative to HE.

4.3.11 Statistical analysis

All data are presented as mean ± SEM. The analyses were broken down into two models. The first model (model AM) utilized a 2-way ANOVA to determine the main effects of oligofructose, ampicillin, and their interaction. The second model (model AM/NE) utilized a 2-way ANOVA to determine the main effects of oligofructose, ampicillin/neomycin, and their interaction. If a significant interaction was identified, a one way ANOVA with all 4 treatments within either model AM or model AM/NE was performed with Tukey’s post hoc test. For measurements with repeated measures, a 2-way repeated measures ANOVA was used with time as the within-subject factor and oligofructose and antibiotic treatment as between-subject factors. A false discovery rate was used to control for type 1 error in the bacterial analysis. Correlation analysis using data from all six treatment groups was conducted using a Spearman’s correlation.
Analysis was completed using SPSS V20.0 software (SPSS Inc., Chicago, IL, USA). The Anosim method was used to determine differences in beta-diversity between treatment groups. Data was considered significant at p<0.05.

**4.4 Results**

**4.4.1 Effects of treatment on body weight, composition, and energy intake**

Whether treated with ampicillin or ampicillin/neomycin, oligofructose independently lowered body weight and weight gain relative to non-oligofructose containing groups (Figure 4.1A, Table 4.2). Regardless of oligofructose consumption, ampicillin/neomycin but not ampicillin, lowered body weight throughout the study. In model AM, the decrease in body fat with oligofructose was blunted when combined with ampicillin such that OFS had significantly lower body fat% than HE, AM, and AM+OFS (Figure 4.1B). Retroperitoneal fat mass was also independently reduced by oligofructose (Table 4.2). In model AM/NE there was an independent effect of oligofructose for decreasing body fat% which was not affected by ampicillin/neomycin. In model AM, energy intake was independently reduced by ampicillin and oligofructose, whereas in model AM/NE, the interaction of ampicillin/neomycin and oligofructose caused a reduction in energy intake, such that AM/NE, OFS, and AM/NE+OFS decreased energy intake to the same degree (Figure 4.1C). GLP-1, a purported satiety hormone, was elevated as an independent effect of oligofructose in model AM whereas there was an interaction between ampicillin/neomycin and oligofructose affecting GLP-1 in model AM/NE (Figure 4.1E). Interestingly, regardless of oligofructose treatment, serial measurements and area under the curve (AUC) for PYY were independently elevated by ampicillin and ampicillin/neomycin (Figure 4.1F, Table 4.3). In model AM/NE, both ampicillin/neomycin and oligofructose independently reduced liver weight and triglyceride content (Table 4.2).
Figure 4.1: Treatment effects of OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks on A) body weight; B) body fat composition; C) energy intake; D) serial leptin during an OGTT; E) fasting GLP-1 and F) serial PYY during an OGTT. Values are mean ± SEM, n=8-10/gp. When interaction effects were observed between OFS and AM or AM/NE, superscripts indicate significant differences between groups (a,b,c for Model AM and x,y,z for Model AM/NE). Labeled means without a common superscript letter differ, p<0.05.
### Table 4.2 Anthropometrics of obese rats (HE) treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HE</th>
<th>OFS</th>
<th>AM</th>
<th>AM + OFS</th>
<th>AM/NE</th>
<th>AM/NE + OFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight, g</td>
<td>803 ± 17</td>
<td>735 ± 11(*,#)</td>
<td>761 ± 15</td>
<td>721 ± 15(*)</td>
<td>764 ± 9(\dagger)</td>
<td>683 ± 13(*,\dagger)</td>
</tr>
<tr>
<td>Total weight change, g</td>
<td>161 ± 9</td>
<td>87 ± 16(*,#)</td>
<td>123 ± 8(\dagger)</td>
<td>72 ± 16(*)</td>
<td>127 ± 7(\dagger)</td>
<td>51 ± 12(*,\dagger)</td>
</tr>
<tr>
<td>Retroperitoneal fat mass, g</td>
<td>35.5 ± 3.1</td>
<td>23.4 ± 1.7(*,#)</td>
<td>31.6 ± 2.4</td>
<td>28.1 ± 2.3(*)</td>
<td>32.3 ± 2.8</td>
<td>19.2 ± 2.0(#)</td>
</tr>
<tr>
<td>Liver, g</td>
<td>22.5 ± 0.8</td>
<td>22.2 ± 1.1(#)</td>
<td>21.7 ± 0.8</td>
<td>20.0 ± 1.3</td>
<td>21.7 ± 0.7(\dagger)</td>
<td>18.3 ± 0.8(*,\dagger)</td>
</tr>
<tr>
<td>Liver TG, ug/mg</td>
<td>34.2 ± 0.70</td>
<td>31.2 ± 1.31(#)</td>
<td>32.6 ± 1.01</td>
<td>31.5 ± 0.98</td>
<td>31.7 ± 1.25(\dagger)</td>
<td>28.3 ± 0.71(*,\dagger)</td>
</tr>
<tr>
<td>Cecum weight, g</td>
<td>0.68 ± 0.05(\times)</td>
<td>1.00 ± 0.10(*,#)</td>
<td>1.59 ± 0.10(\dagger)</td>
<td>1.72 ± 0.10(*,\dagger)</td>
<td>1.73 ± 0.06(\times)</td>
<td>2.24 ± 0.08(\times)</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SEM, \(n=9-10/gp\). Model AM: *indicates main effect of OFS, \(p<0.05\); † indicates main effect of AM, \(p<0.05\). Model AM/NE: # indicates main effect of OFS, \(p<0.05\); ‡ indicates main effect of AM/NE, \(p<0.05\). When interaction effects were observed between OFS and AM/NE, \(\times,\#\) superscripts indicate significant differences between groups. Labeled means without a common superscript letter differ, \(p<0.05\).
Table 4.3 AUC for serum satiety hormones and glycemic indices of obese rats (HE) treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HE</th>
<th>OFS</th>
<th>AM</th>
<th>AM + OFS</th>
<th>AM/NE</th>
<th>AM/NE + OFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, nmol/L X 120 min</td>
<td>132.6 ± 12.6</td>
<td>102.9 ± 8.3*#</td>
<td>147.2 ± 16.0</td>
<td>130.6 ± 15.6</td>
<td>141.6 ± 14.4</td>
<td>83.7 ± 10.1*#</td>
</tr>
<tr>
<td>Amylin, nmol/L X 120 min</td>
<td>4.13 ± 0.28</td>
<td>2.99 ± 0.28*#</td>
<td>3.47 ± 0.21</td>
<td>3.19 ± 0.30*</td>
<td>3.38 ± 0.12</td>
<td>3.13 ± 0.26*#</td>
</tr>
<tr>
<td>Leptin, nmol/L X 120 min</td>
<td>106.1 ± 7.7</td>
<td>90.3 ± 6.8**#</td>
<td>109.8 ± 5.4</td>
<td>88.4 ± 4.9*</td>
<td>106.5 ± 8.6</td>
<td>70.3 ± 6.6**#</td>
</tr>
<tr>
<td>Ghrelin, nmol/L X 120 min</td>
<td>3.72 ± 0.31x</td>
<td>4.73 ± 0.48y</td>
<td>5.34 ± 0.59</td>
<td>4.89 ± 0.65</td>
<td>5.34 ± 0.53xy</td>
<td>3.50 ± 0.18x</td>
</tr>
<tr>
<td>PYY, nmol/L X 120 min</td>
<td>1.16 ± 0.11</td>
<td>1.57 ± 0.14*</td>
<td>1.90 ± 0.20†</td>
<td>2.05 ± 0.17*†</td>
<td>2.91 ± 0.18†</td>
<td>2.94 ± 0.15†</td>
</tr>
<tr>
<td>GIP, nmol/L X 120 min</td>
<td>2.57 ± 0.20</td>
<td>2.39 ± 0.26</td>
<td>2.89 ± 0.37</td>
<td>2.45 ± 0.28</td>
<td>2.75 ± 0.20</td>
<td>2.09 ± 0.30</td>
</tr>
</tbody>
</table>

1Values are means ± SEM, n = 8-10/gp. Model AM: † indicates main effect of AM, p<0.05; * indicates main effect of OFS, p<0.05. Model AM/NE: ‡ indicates main effect of AM/NE, p<0.05; # indicates main effect of OFS, p<0.05. When interaction effects were observed between OFS and AM/NE, superscripts indicate significant differences between groups. Labeled means without a common superscript letter differ, p<0.05. AM, ampicillin; AM/NE, ampicillin/neomycin; GIP, glucose-dependent insulinojropic polypeptide; HE, high energy; OFS, oligofructose; PYY, peptide tyrosine tyrosine
4.4.2 Intestinal permeability with oligofructose differentially affected by antibiotic treatment

In model AM and AM/NE there were interactions between oligofructose and antibiotic treatment that influenced FD-4 epithelial paracellular permeability (Figure 4.2A,B). In model AM, AM+OFS had significantly elevated FD-4 serum concentrations relative to AM and OFS 1 hr post gavage and for total AUC. Serum LPS concentrations mimicked intestinal permeability changes with AM+OFS having significantly elevated LPS relative to AM (Figure 4.2C). In contrast, in model AM/NE, the interaction was such that OFS, AM/NE, and AM/NE+OFS improved intestinal permeability to the same degree. Independently, ampicillin/neomycin reduced serum LPS. GLP-2, a gut trophic hormone, was independently elevated by oligofructose in both models and independently increased by ampicillin/neomycin (Figure 4.2D). No differences in serum TNFα, MCP-1, IL-1β, PAI-1(total), and IL-6 concentrations were observed in either model (data not shown).
Figure 4.2: Combination of oligofructose and antibiotics affects intestinal permeability. A) Serial serum FD-4 concentrations; B) FD-4 AUC; C) serum LPS; and D) fasting GLP-2 of obese rats treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks. Values are mean ± SEM, n = 8-10/gp. When interaction effects were observed between OFS and AM or AM/NE, superscripts indicate significant differences between groups (a,b,c for Model AM and x,y,z for Model AM/NE). Labeled means without a common superscript letter differ, p<0.05. Labeled means without a common superscript letter differ, p<0.05.
**4.4.3 Antibiotics and oligofructose improve glycemia**

OGTTs conducted at baseline verified that there were no differences in glycemic control between groups prior to treatment (data not shown). OGTTs conducted post treatment demonstrated that oligofructose, ampicillin, and ampicillin/neomycin independently improved serial glucose measurements and reduced AUC (Figure 4.3A,B). Oligofructose independently reduced serial measurements of insulin in model AM and model AM/NE (Figure 4.3C). Insulin sensitivity, assessed using the composite insulin sensitivity index (CISI), with higher numbers representing increased insulin sensitivity, was independently improved by oligofructose in model AM and model AM/NE (Figure 4.3D). In liver tissue, oligofructose increased hepatic expression of cholesterol 7 alpha-hydroxylase-1 (Cyp7α1) in model AM and model AM/NE.
Figure 4.3: Improvements in glycemic control with oligofructose and antibiotics. A) Serial glucose during an OGTT, B) glucose AUC, C) serial insulin during an OGTT, and D) composite insulin sensitivity index of obese rats treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks. Values are mean ± SEM, n = 9-10/gp. When interaction effects were observed between OFS and AM/NE, superscripts indicate significant differences between groups. Labeled means without a common superscript letter differ, p<0.05. Labeled means without a common superscript letter differ, p<0.05.
4.4.4 Oligofructose increases citrate synthase activity in retroperitoneal adipose tissue

Independently, oligofructose increased CS activity in retroperitoneal adipose tissue in model AM (Figure 4.4A) whereas ampicillin/neomycin and oligofructose interacted in model AM/NE, such that CS activity was increased in OFS relative to HE. In retroperitoneal white adipose tissue, Tfam was elevated and G-protein coupled receptor-43 (GPR43) (Figure 4.4B) was reduced by oligofructose. Subcutaneous inguinal adipose tissue did not exhibit any of the noted changes in mRNA expression observed in retroperitoneal adipose tissue.
Figure 4.4: Oligofructose increases markers of mitochondrial activity in retroperitoneal adipose tissue. A) Retroperitoneal white adipose tissue citrate synthase activity and B) fold change gene expression of Tfam, PGC-1α, GPR43, SREBP-1c, and LPL, normalized to actin mRNA expression and expressed relative to HE in obese rats treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks. Values are mean ± SEM, n =9-10/gp. When interaction effects were observed between OFS and AM/NE, superscripts indicate significant differences between groups. Labeled means without a common superscript letter differ, p<0.05.
4.4.5 Characterization of gut microbiota modifications with treatment

Terminal cecal 16S rRNA amplicon analysis

Model AM. The interaction between oligofructose and ampicillin affected relative abundance of Verrucomicrobia (Table 4.4). Independently, oligofructose increased Actinobacteria and reduced the abundance of Firmicutes and Firmicutes/Bacteroidetes ratio. Bifidobacterium and Akkermansia were affected by the interaction between ampicillin and oligofructose, such that the increase in Bifidobacterium elicited with oligofructose was reduced when combined with ampicillin. Akkermansia was uniquely influenced by ampicillin and oligofructose, with AM having higher relative abundance of Akkermansia relative to AM+OFS. Lactobacillus was independently increased by oligofructose and reduced by ampicillin. The interaction of ampicillin and oligofructose affected the Shannon and Simpson index of alpha diversity resulting in a reduction in microbial diversity with OFS, AM, and AM+OFS relative to HE (Table 4.4).

Model AM/NE. Independently, oligofructose increased Actinobacteria and reduced the Firmicutes/Bacteroidetes ratio. The increase in Actinobacteria with oligofructose appeared to be attenuated when combined with ampicillin/neomycin. Ampicillin/neomycin independently increased proportions of Proteobacteria and Enterobacteriaceae whereas oligofructose independently decreased relative abundance of Clostridiales and Enterobacteriaceae. The interaction of ampicillin/neomycin and oligofructose resulted in a reduction of the oligofructose-induced increase in Bifidobacterium (Table 4.4). Lactobacillus was independently increased by oligofructose whereas there tended to be a reduction of Lactobacillus (p=0.053) with ampicillin/neomycin. There was an independent effect of oligofructose for reducing the Shannon index and ampicillin/neomycin for reducing the Chao1 index of alpha diversity (Table 4.4).

PCoA on weighted UniFrac distances demonstrated that the gut microbiota profiles from different treatment groups clustered separately (Figure 4.5A-F).
Table 4.4 Paired read 16S rRNA gene relative abundance of bacterial taxa and alpha diversity indices of obese rats treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HE</th>
<th>OFS</th>
<th>AM</th>
<th>AM + OFS</th>
<th>AM/NE</th>
<th>AM/NE + OFS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phyla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.7 ± 0.2</td>
<td>21.4 ± 4.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.3 ± 0.1</td>
<td>12.3 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 ± 0.4</td>
<td>10.0 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>47.0 ± 4.3</td>
<td>31.9 ± 2.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.4 ± 3.1</td>
<td>33.1 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.3 ± 2.5</td>
<td>27.8 ± 4.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>35.4 ± 4.2</td>
<td>34.2 ± 2.2</td>
<td>28.9 ± 4.8</td>
<td>42.9 ± 2.6</td>
<td>24.1 ± 2.8</td>
<td>34.9 ± 5.0</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>8.1 ± 4.7</td>
<td>2.3 ± 0.3</td>
<td>16.9 ± 9.2</td>
<td>4.4 ± 1.1</td>
<td>14.6 ± 3.3&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>22.1 ± 11.1&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>8.2 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.2 ± 2.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.0 ± 4.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.7 ± 1.5</td>
<td>5.1 ± 1.5</td>
</tr>
<tr>
<td>Firmicutes/Bacteroidetes</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.48&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>2.5 ± 0.5</td>
<td>0.9 ± 0.1&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Order</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridiales</td>
<td>42.6 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.3 ± 3.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>27.6 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.0 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.9 ± 2.2</td>
<td>18.3 ± 2.7&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacteriaceae</td>
<td>0.3 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.6 ± 2.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.0 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>0.7 ± 0.4</td>
<td>6.8 ± 1.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
<td>2.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Streptococcaceae</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.7 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.3 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lachnospiraceae</td>
<td>6.3 ± 1.0</td>
<td>16.2 ± 3.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.7 ± 2.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>18.6 ± 1.4&lt;sup&gt;±&lt;/sup&gt;</td>
<td>7.7 ± 1.1</td>
<td>11.9 ± 2.0&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>13.6 ± 2.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.6 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.9 ± 1.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.6 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 ± 1.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erysipelotrichaceae</td>
<td>0.0 ± 0.1</td>
<td>2.2 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.5 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.3 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.3 ± 0.2</td>
<td>2.7 ± 0.5&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>9.7 ± 2.8</td>
<td>23.4 ± 1.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>15.4 ± 3.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>32.9 ± 3.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.8 ± 1.3</td>
<td>26.8 ± 4.7&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>S24-7</td>
<td>16.3 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 1.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.2 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.7 ± 1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.5&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>2.7 ± 2.0</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.6</td>
<td>0.9 ± 0.6</td>
<td>9.7 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Genus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.3 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.6 ± 2.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.0 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>0.7 ± 0.4</td>
<td>6.8 ± 1.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
<td>2.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blautia</td>
<td>0.1 ± 0.0</td>
<td>5.3 ± 1.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.2 ± 0.5</td>
<td>3.5 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.9 ± 0.2</td>
<td>3.1 ± 0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>9.7 ± 2.8</td>
<td>23.4 ± 1.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>15.4 ± 3.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>32.9 ± 3.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.8 ± 1.3</td>
<td>26.8 ± 4.7&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>0.5 ± 0.1</td>
<td>5.1 ± 1.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.8 ± 0.3</td>
<td>4.5 ± 1.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.7 ± 0.1</td>
<td>3.1 ± 0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oscillospira</td>
<td>5.7 ± 0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.8 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.3 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.6 ± 0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.9 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morganella</td>
<td>1.1 ± 0.7</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7 ± 0.6</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.4 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>8.2 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.2 ± 2.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.0 ± 3.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.3 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.7 ± 1.5</td>
<td>5.1 ± 1.5</td>
</tr>
<tr>
<td><strong>Alpha Diversity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shannon</td>
<td>7.93 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.97 ± 0.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.58 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.78 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.51 ± 0.39</td>
<td>6.91 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Simpson</td>
<td>0.97 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.02</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td>Chao1</td>
<td>73812 ± 6534</td>
<td>71360 ± 7823&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68473 ± 7260</td>
<td>69821 ± 4636</td>
<td>70569 ± 8850&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61112 ± 7577&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1Values are means ± SEM and expressed as relative proportions, n=10/gp. Model AM: * indicates main effect of OFS, p<0.05; † indicates main effect of AM, p<0.05. Model AM/NE: ‡ indicates main effect of OFS, p<0.05; † indicates main effect of AM/NE, p<0.05. When interaction effects were observed between OFS and AM or AM/NE, superscripts indicate significant differences between groups (<sup>abc</sup> for Model AM and <sup>ab</sup> for Model AM/NE). Labeled means without a common superscript letter differ, p<0.05.
Figure 4.5: Beta diversity between the different experimental groups at time of sacrifice was assessed by weighted Principle coordinates analysis (PCoA) of Unifrac distances. Weighted PCoA analysis of A) all experimental groups B) HE vs OFS; C) AM vs AM+OFS; D) OFS vs AM+OFS; E) AM/NE vs AM/NE+OFS; F) OFS vs AM/NE+OFS. 

n =10/gp. P-values were calculated using the anosim method in QIIME.
4.4.5.1 16S rRNA amplicon correlations

*Bifidobacterium* was positively correlated with *Lactobacillus* ($r = 0.797$, $p<0.01$) and inversely correlated with *Akkermansia* ($r = -0.362$, $p<0.01$). *Lactobacillus* negatively correlated with *Akkermansia* ($r = -0.341$, $p<0.05$). *Bifidobacterium* ($r = 0.797$, $p<0.05$) and *Lactobacillus* ($r = -0.439$, $p<0.01$) were inversely correlated with body fat%. Both *Bifidobacterium* and *Lactobacillus* were positively correlated with GLP-1 ($r = 0.734$, $p<0.01$ and $r = 0.571$, $p<0.05$, respectively) and GLP-2 ($r = 0.465$, $p<0.05$ and $r = 0.328$, $p<0.05$, respectively).

4.4.5.2 Cecal qPCR 16S rRNA gene copies

Independently, ampicillin/neomycin reduced total 16S rRNA gene copies, whereas ampicillin did not (Table 4.5). The finding that ampicillin did not affect total bacteria is consistent with evidence from an *in vitro* study conducted by Johnson et al., which observed that after an initial decrease in bacterial numbers with ampicillin there was a recovery in bacterial growth(253).

*Model AM*. Interactions between oligofructose and ampicillin affected 16S rRNA gene copies of *Bifidobacterium*, *A. muciniphila*, and *Lactobacillus* (Table 4.5). Notably, oligofructose-mediated increases in *Bifidobacterium* and *Lactobacillus* were impeded when combined with ampicillin (Table 4.5). AM had increased *A. muciniphila* relative to AM+OFS.

*Model AM/NE*. Enterobacteriaceae, *Bifidobacterium*, and *Lactobacillus* were affected by the interaction between oligofructose and ampicillin/neomycin (Table 4.5). Ampicillin/neomycin prevented the increase in *Bifidobacterium* and *Lactobacillus* and maintained the decrease in Enterobacteriaceae elicited with oligofructose (Table 4.5). Ampicillin/neomycin independently reduced *A. muciniphila*.

4.4.5.3 Fecal 16S rRNA gene copy relative abundance at week 0, 3, and 6

Figures for fecal 16S rRNA gene copy relative abundance can be found in Figure 4.6.
Table 4.5 qPCR 16S rRNA gene copy number/mg cecal matter of obese rats (HE) treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS for 6 wks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HE</th>
<th>OFS</th>
<th>AM</th>
<th>AM + OFS</th>
<th>AM/NE</th>
<th>AM/NE + OFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bacteria</td>
<td>7.42 ± 0.13</td>
<td>7.55 ± 0.15</td>
<td>7.31 ± 0.11</td>
<td>7.43 ± 0.09</td>
<td>4.99 ± 0.13</td>
<td>5.44 ± 0.22</td>
</tr>
<tr>
<td>Bacteroides/Prevotella</td>
<td>6.57 ± 0.21</td>
<td>6.86 ± 0.16*</td>
<td>6.59 ± 0.30</td>
<td>6.76 ± 0.11*</td>
<td>4.19 ± 0.14</td>
<td>4.61 ± 0.24</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>4.57 ± 0.13 abx</td>
<td>5.81 ± 0.09 ab,y</td>
<td>3.71 ± 0.12a</td>
<td>5.46 ± 0.15c</td>
<td>3.29 ± 0.13x</td>
<td>3.77 ± 0.11x</td>
</tr>
<tr>
<td>Methanobrevibacter</td>
<td>4.82 ± 0.31 ab</td>
<td>4.84 ± 0.22 ab</td>
<td>5.13 ± 0.37a</td>
<td>4.48 ± 0.38b</td>
<td>2.94 ± 0.17‡</td>
<td>2.27 ± 0.17‡</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>4.82 ± 0.13ab</td>
<td>3.28 ± 0.10b,y</td>
<td>3.43 ± 0.11b</td>
<td>3.32 ± 0.06b</td>
<td>2.66 ± 0.05y</td>
<td>2.49 ± 0.03y</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>5.02 ± 0.10x</td>
<td>4.35 ± 0.12* y</td>
<td>5.30 ± 0.17</td>
<td>4.51 ± 0.14*</td>
<td>3.73 ± 0.07y</td>
<td>3.44 ± 0.06y</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>4.08 ± 0.18ax</td>
<td>5.02 ± 0.19b,y</td>
<td>3.35 ± 0.10a</td>
<td>3.71 ± 0.16a</td>
<td>2.60 ± 0.1x</td>
<td>2.54 ± 0.12 x</td>
</tr>
<tr>
<td>Clostridium coccoides</td>
<td>6.76 ± 0.22</td>
<td>6.73 ± 0.23</td>
<td>6.56 ± 0.21</td>
<td>6.55 ± 0.09</td>
<td>4.44 ± 0.14 ‡</td>
<td>4.31 ± 0.15 ‡</td>
</tr>
<tr>
<td>(cluster XIV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td>6.72 ± 0.34ax</td>
<td>5.36 ± 0.19b,y</td>
<td>5.93 ± 0.20b</td>
<td>5.56 ± 0.13b</td>
<td>3.92 ± 0.14 y</td>
<td>3.28 ± 0.16 y</td>
</tr>
<tr>
<td>(cluster IV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster XI</td>
<td>6.87 ± 0.17ax</td>
<td>5.85 ± 0.23b,y</td>
<td>5.18 ± 0.11b</td>
<td>4.94 ± 0.16b</td>
<td>4.40 ± 0.13 y</td>
<td>3.72 ± 0.08 y</td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>5.17 ± 0.12 x</td>
<td>4.55 ± 0.11* y</td>
<td>5.37 ± 0.26</td>
<td>4.73 ± 0.14*</td>
<td>3.46 ± 0.09 y</td>
<td>3.14 ± 0.11 y</td>
</tr>
<tr>
<td>Roseburia</td>
<td>5.61 ± 0.27 ax</td>
<td>4.47 ± 0.15b,y</td>
<td>4.36 ± 0.12b</td>
<td>4.13 ± 0.09b</td>
<td>3.13 ± 0.09 y</td>
<td>2.61 ± 0.05 y</td>
</tr>
</tbody>
</table>

Values are means ± SEM and expressed as log10 16S rRNA gene copies/mg cecal matter, n=9-10/gp. Model AM: * indicates main effect of OFS, p<0.05; † indicates main effect of AM, p<0.05. Model AM/NE: ‡ indicates main effect of OFS, p<0.05; †† indicates main effect of AM/NE, p<0.05. When interaction effects were observed between OFS and AM or AM/NE, superscripts indicate significant differences between groups (ab,c for Model AM and xy,z for Model AM/NE). Labeled means without a common superscript letter differ, p<0.05.
A. Total Bacteria

B. Bacteroides/Prevotella

C. Bifidobacterium

D. Lactobacillus

E. Akkermansia muciniphila

F. Methanobrevibacter

G. Enterobacteriaceae

H. Clostridium cocoides

continued on next page
Figure 4.6: A) 16S rRNA gene copy number for total bacteria and 16S rRNA gene copy relative abundance (group 16S rRNA copy number/total bacteria 16S rRNA) for B) Bacteroides/Prevotella; C) Bifidobacterium; D) Lactobacillus; E) Akkermansia muciniphila; F) Methanobrevibacter; G) Enterobacteriaceae; H) Clostridium coccoides (cluster XIV); I) Clostridium leptum (cluster IV); J) Clostridium cluster XI; K) Clostridium cluster I; and L) Roseburia in obese rats treated with OFS, AM, AM/NE, AM+OFS, or AM/NE+OFS at baseline (wk 0), mid-point (wk 3), and sacrifice (wk 6).

Values are mean ± SEM, n = 8-10/gp. When interaction effects were observed between OFS and AM or AM/NE, superscripts indicate significant differences between groups (a,b,c for Model AM and x,y,z for Model AM/NE). Labeled means without a common superscript letter differ, p<0.05.
4.5 Discussion

With evidence that a perturbed gut microbiota is implicated in the pathophysiology of human obesity (235), there is significant interest in the use of prebiotics as a means to restore homeostasis and potentially treat metabolic disease. We used broad-spectrum antibiotics to assess whether oligofructose-mediated effects on the host were susceptible to antimicrobial reduction of the commensal microbiota. Ampicillin, although not reducing total bacterial abundance, blunted the decrease in adiposity elicited by oligofructose and impaired intestinal permeability when combined with oligofructose. Ampicillin/neomycin, which drastically reduced bacterial content, did not perturb the benefit of oligofructose on adiposity and intestinal permeability. Despite these changes, there remained an independent benefit of oligofructose, ampicillin, and ampicillin/neomycin on improving glycemia. These results suggest that oligofructose mediates its effects on the host in part through gut microbiota dependent mechanisms and leaves open the possibility of gut microbiota independent mechanisms.

A decrease in body weight and adiposity, or in many studies an attenuation in the rate of weight gain in animals that are still growing, is a consistent finding with oligofructose intake in animal models (72,96,168). Often questioned, however, is whether these effects are the result of decreased energy intake due to energy dilution of the diet or if the effects can be attributed to altered gut microbiota. One of the purported mechanisms for reduced energy intake with oligofructose is increased secretion of the incretin hormone GLP-1 and other satiety hormones such as PYY mediated through the action of SCFAs (110,254). Consistent with previous literature, we observed that oligofructose reduced body weight, adiposity, and energy intake relative to other treatment groups and this was paired with increased GLP-1. When ampicillin was administered with oligofructose, the reduction in adiposity with oligofructose was prevented, suggesting that the oligofructose-mediated benefit on adiposity is vulnerable to a disruption of the gut microbiota by ampicillin. Noteworthy, the decrease in adiposity elicited by oligofructose appeared to be independent of energy consumption, evidenced by the fact that energy intake between OFS and AM+OFS was comparable. The independent ability of ampicillin to increase PYY could explain this reduction in energy intake. Taken together, these findings support a unique adipose tissue reducing effect of oligofructose which occurs
independent of energy consumption. This finding is corroborated by a study completed by Schroeder et al. wherein a lower dose (5% wt/wt) of short chain fructo-oligosaccharide reduced epididymal fat pad weight independent of energy intake(255). Importantly, in humans, oligofructose supplementation over 3 months is associated with a small but significant reduction in fat mass (1 kg) in adults with overweight and obesity(98). This decrease was accompanied by a significant increase in PYY. The two organisms classically increased by oligofructose include bacteria in the genera *Bifidobacterium* and *Lactobacillus*(38). Confirmed through 16S sequencing and qPCR, both of these genera were significantly increased by oligofructose and as hypothesized were decreased in AM. When oligofructose was combined with ampicillin, increases in *Bifidobacterium* and *Lactobacillus* were blunted. Thus, given the inverse correlation between *Bifidobacterium* and *Lactobacillus* and adiposity, these findings provide evidence that the increase in *Bifidobacterium* and *Lactobacillus* is, in part, responsible for mediating the benefits of oligofructose.

In contrast to the results of the AM model, ampicillin/neomycin did not attenuate the oligofructose-mediated decrease in adiposity. Instead the decrease in relative adiposity with oligofructose was maintained when ampicillin/neomycin was paired with oligofructose. Evidence that ampicillin/neomycin by itself did not have a significant effect on adiposity, despite decreased food intake, increased PYY, and a drastic reduction in total bacteria, suggests that oligofructose maintains some capacity to affect adiposity independent of gut microbiota. Indeed, a recent study by Woting et al. demonstrated that oligofructose maintained the capacity to decrease body fat in germ free mice(256). In the absence of fermentation, it is believed that prebiotics can elicit beneficial anti-inflammatory effects on the host through direct immunomodulation(257-259). The purported mechanisms include interaction between oligosaccharides and dendritic cells, ligation of pathogen recognition receptors, and subsequent modulation of pro-inflammatory cytokines. Thus, it is plausible that some oligofructose-mediated benefits on adiposity occur independently of gut microbiota. However, in our antibiotic model, we did not achieve total eradication of bacteria and therefore cannot exclude the possibility that oligofructose may have interacted with an ampicillin/neomycin-modified microbiota to assist in the maintenance of a decreased adiposity with oligofructose. Given
evidence that ampicillin/neomycin affected proportions of *Bifidobacterium* and *Lactobacillus* in a similar manner to that of ampicillin, it is not clear which organisms could be responsible for these changes, although Enterobacteriaceae and Clostridiales are potential candidates based on the independent effects of oligofructose to reduce the relative abundance of these bacteria in model AM/NE.

While others have demonstrated that oligofructose appears to have unique adipose targeting capacity, many of the mechanisms by which this occurs are still not completely understood. It is believed that SCFAs produced by gut microbiota target adipocytes via activation of GPR43(114). Ge et al. showed that activation of these receptors with the SCFA acetate inhibits lipolysis in both cell culture and *in vivo* via a GPR43 dependent mechanism(115). Interestingly, Dewulf et al. showed that oligofructose, while decreasing adipose size, reduced the expression of GPR43, an effect purported to be mediated via decreased activation of peroxisome proliferator activated receptor-γ (PPARγ)(116). Evidence from a subsequent study in which oligofructose was given to high fat fed mice in conjunction with a thiazolidinedione (TZD), an activator of PPARγ, demonstrates that oligofructose maintains the benefits of the TZD without increasing adiposity, suggesting that oligofructose may increase adipose tissue oxidative capacity(260). Here we confirmed that oligofructose reduced expression of GPR43 in retroperitoneal adipose tissue. Although Hong et al. demonstrated that acetate and propionate lead to an increase in GPR43 expression in 3T3-L1 cells(261), our findings suggest that, similar to Dewulf et al.(116) the fermentation of oligofructose *in vivo* reduces GPR43 expression in retroperitoneal adipose tissue. Further support for SCFAs not triggering increased adipose GPR43 expression comes from a study by Brockman et al. that demonstrated that guar gum, another fermentable fiber, fails to elicit changes in GPR43 expression in adipose tissue(262). Interestingly, we observed an independent effect of oligofructose for increasing the transcription factor Tfam, which is involved in mitochondrial biogenesis(263). Furthermore, assessment of CS activity in retroperitoneal adipose tissue revealed an increase in the activity of this enzyme with oligofructose. We did not, however, observe changes in the expression of PPARγ coactivator-1α (PGC-1α), the purported master regulator of mitochondrial biogenesis. While further exploration into the mechanisms by which oligofructose elicits these changes is warranted, this evidence
suggests that oligofructose may mediate improvements in adipose tissue oxidative capacity via increased mitochondrial biogenesis. Considering the greater overall contribution of skeletal muscle to oxidative capacity, further studies should also examine markers of mitochondrial biogenesis in muscle tissue.

Consistent with the trend observed in adiposity, our data suggests that when oligofructose was paired with ampicillin, the improvement in intestinal permeability observed with oligofructose was abrogated, as evidenced by elevated serum FD-4 and LPS. Interestingly, ampicillin by itself did not exhibit this impairment. This suggests that it was the interaction between oligofructose and ampicillin that led to these deleterious effects. While increased GLP-2 is cited as one mechanism by which oligofructose improves intestinal permeability(122), the fact that GLP-2, in model AM, was not grossly depressed by AM + OFS suggests that some other impairment is responsible for this gut leakiness. Conceivably, the abundance of *Akkermansia* could explain this relationship. Whereas *Akkermansia* was elevated with ampicillin, which has been reported with some antibiotic treatments(264,265), when ampicillin was combined with oligofructose the increase was prevented. *Akkermansia* has been demonstrated to decrease endotoxemia and is associated with improved insulin sensitivity in diet-induced obese mice(125,266). Thus, given the close association between intestinal permeability and endotoxemia, the lowering of *Akkermansia* concentrations with AM+OFS could in part be responsible for the noted increase in LPS and intestinal permeability. However, given the known effect of oligofructose to increase *A. muciniphila*(125), it is not clear how oligofructose might elicit a decrease in *Akkermansia* when consumed in combination with ampicillin.

In contrast to what was observed with adiposity and intestinal permeability, ampicillin and ampicillin/neomycin did not alter oligofructose-induced improvement in glycemia. In fact, ampicillin and ampicillin/neomycin both independently improved glucose concentrations during an OGTT. The improvement in glycemia with antibiotics is not novel, with many animal studies demonstrating that insulin sensitivity is improved in obese animals upon antibiotic treatment(267-269). Membrez et al., in *ob/ob* mice, demonstrated that ampicillin and norfloxacin improved glycemia via a mechanism that involved decreased endotoxemia(268). Here, ampicillin/neomycin independently decreased serum LPS concentrations. Furthermore, Chou et
al., noting that decreased steatosis is consistent with enhanced glucose tolerance, observed that liver triglycerides in *ob/ob* mice treated with antibiotics was reduced (243). A similar decrease in hepatic triglycerides was seen with oligofructose and ampicillin/neomycin in our study. Interestingly, in association with this effect, we observed an increase in Cyp7α1 expression with oligofructose and a trend towards increased expression with ampicillin/neomycin. Cyp7α1, a rate limiting enzyme in bile acid synthesis, has been demonstrated to protect against obesity and insulin resistance (270,271). As the expression of Cyp7α1 is susceptible to bile acid signalling, an increase in Cyp7a1 may reflect the influence of oligofructose on bile acid reabsorption in the gut. Potentially, by lowering intestinal pH, oligofructose may decrease the solubility and reabsorption of bile acids, thus leading to an induction of Cyp7a1.

In conclusion, our data provides evidence that the health promoting effects of oligofructose on adiposity and intestinal permeability are negatively affected when *Lactobacillus* and *Bifidobacterium* growth is impeded with ampicillin in an animal model. Moreover, the finding that certain benefits of oligofructose were maintained even when total bacteria were drastically reduced suggests that oligofructose may elicit direct, non-fermentative action in the host. Subsequent studies using gnotobiotic and germ free models are warranted to further elucidate these mechanisms. Furthermore, our data demonstrates that enhanced mitochondrial activity may be an additional mechanism by which oligofructose influences adipose tissue metabolism. While the effects of oligofructose may not be as robust in humans due to the complexity of human obesity, as we learn more about the mechanisms of prebiotics, it is hoped that we will ultimately be able to develop prebiotic strategies that more effectively target the pathogenesis of obesity related to gut microbiota dysbiosis.

### 4.6 Acknowledgements

The authors would like to thank K. Lee, Faculty of Kinesiology, University of Calgary for her technical assistance. S. Wegener, Dr. R. Pon, and Dr. P. Gordon, University Core DNA Services and Alberta Children’s Hospital Research Institute (ACHRI) Genomics facility at the University of Calgary for their technical assistance and support with the 16S rRNA gene sequencing and analysis. The authors also thank Chieh Jason Chou (Nestle Institute of Health Science, Lausanne,
Switzerland). This work was supported by a research grant from the Canadian Institutes of Health Research (CIHR)(MOP 115076).
Chapter Five: **Histological improvement of non-alcoholic steatohepatitis with a prebiotic: a pilot clinical trial.**

5.1 Abstract

**Background:** In obesity and diabetes the liver is highly susceptible to abnormal uptake and storage of fat. In certain individuals hepatic steatosis predisposes to the development of non-alcoholic steatohepatitis (NASH), a disease marked by hepatic inflammation and fibrosis. Although the precise pathophysiology of NASH is unknown, it is believed that the gut microbiota-liver axis influences the development of this disease. With few treatment strategies available for NASH, exploration of gut microbiota-targeted interventions is warranted.

**Methods:** We investigated the therapeutic potential of a prebiotic supplement to improve histological parameters of NASH. In a single-blind, placebo controlled, randomized pilot trial, 14 individuals with liver biopsy confirmed NASH (Non-alcoholic fatty liver activity score (NAS) ≥ 5) were randomized to receive oligofructose (8 g/day for 12 weeks and 16 g/day for 24 weeks) or isocaloric placebo for 9 months. The primary outcome measure was the change in liver biopsy NAS score and the secondary outcomes included changes in body weight, body composition, glucose tolerance, serum lipids, inflammatory markers, and gut microbiota.

**Results:** Independent of weight loss, oligofructose improved liver steatosis relative to placebo and improved overall NAS score (P<0.05). *Bifidobacterium* was enhanced by oligofructose whereas bacteria within *Clostridium* cluster XI and I were reduced with oligofructose. There were no adverse side-effects that deterred individuals from consuming oligofructose for treatment of this disease.

**Conclusions:** Independent of other lifestyle changes, prebiotic supplementation reduced histologically-confirmed steatosis in patients with NASH. Larger follow-up studies are warranted.
5.2 Introduction

The incidence of non-alcoholic fatty liver disease (NAFLD) has increased in parallel with the rise in obesity(175). It is estimated that 75% of individuals with obesity are affected by NAFLD(272). Although the liver is not functionally an adipose depot, the liver is susceptible to increased storage of fat in obesity in part due to metabolic complications associated with insulin resistance in peripheral tissues(273). Emerging evidence also suggests that some individuals are prone to increased hepatic steatosis as a result of elevated de novo lipogenesis(172,274).

Although storage of fat in the liver is usually not a major threat to liver function, in approximately 10-25% of individuals with NAFLD an inflammatory cascade is triggered leading to the activation of fibrosis-promoting hepatic stellate cells. Referred to as non-alcoholic steatohepatitis (NASH), this advanced form of NAFLD jeopardizes liver function and can lead to cirrhosis, hepatocellular carcinoma, and end stage liver failure(175,275). Alarmingly, although ~5% of the population is affected by NASH(175), there are very few treatment options available.

In general, weight loss through diet and exercise is prescribed given evidence that a 10% reduction in weight improves histological parameters of NASH(192,193). However, due to the difficulties in achieving sustainable weight loss with lifestyle management(276), alternative treatment strategies are warranted.

Although the pathophysiology mediating the transition from simple steatosis to NASH is not completely understood, it has been proposed that in NAFLD the liver is vulnerable to parallel ‘hits’ stemming from localized oxidative stress as well pro-inflammatory cytokines derived from peripheral tissues(180). It is believed that one of the major ‘hits’ to affect the pathogenesis of NASH is a dysbiotic gut microbiota(84,181,196). Capable of impairing intestinal permeability, a dysbiotic gut microbiota can induce increased leakage of endotoxin into systemic circulation where it can activate host innate immune system receptors contributing to the release of pro-inflammatory cytokines(181). The liver, being in close association with the gut via the portal vein, is particularly susceptible to the influences of bacterial remnants and metabolites entering circulation(277). Furthermore, in addition to the noted effects of gut microbiota on inflammation, the gut microbiota also exerts a significant influence on host energy extraction, appetite, and adiposity(278), all factors involved in the pathogenesis of NASH. Noteworthy, individuals with
NASH have been identified to have increased intestinal permeability (202) as well as an altered gut microbiota (197, 199). Given this collective evidence, there is considerable interest in exploring ways in which the altered gut microbiota in NASH can be modified to halt and/or reverse the progression of this disease (279).

Prebiotics, by increasing the growth and activity of health promoting bacteria, offer a safe and effective means to elicit a shift in gut microbiota (38). Animal and human research has demonstrated that the prebiotic oligofructose improves several NAFLD-associated metabolic risk factors (98, 207-209). However, studies have not thoroughly investigated the potential of oligofructose to affect histological measures of steatosis, lobular inflammation, and hepatocellular ballooning, the current gold standard for diagnosing NASH (175). The objective of this pilot study was to investigate the effectiveness of the prebiotic oligofructose for improving histological measures in liver biopsy confirmed NASH (NAS ≥ 5) (184). Secondary outcomes included changes in body weight, body fat, glucose tolerance, serum lipids, inflammatory markers, and gut microbiota.

**5.3 Methods**

**5.3.1 Subjects**

Participants with liver biopsy confirmed NASH (NAS score ≥ 5) were identified and recruited between 2012-2015 by gastroenterologists and hepatologists at the University of Calgary Medical Centre. Additional inclusion criteria were: males and females age ≥ 18, BMI > 25 kg/m² for Caucasians and > 23 kg/m² in Asians, previous history of Serum ALT > 1.5X upper normal limit, no changes in lipid-lowering or diabetes medication over previous three months, and ability to provide informed consent. Exclusion criteria were: alcohol consumption >20g/day in women or >30g/day in men, alternate or concomitant etiology for abnormal liver enzymes (drug, total parenteral nutrition cholestasis, etc.), decompensated liver disease (defined as presence of ascites, encephalopathy or jaundice, or known esophageal varices), concomitant use of orlistat or liraglutide, antibiotic use within 3 months prior to enrollment, and concomitant use of a prebiotic or probiotic supplement. Eligibility was assessed using a screening questionnaire and interview conducted by a research coordinator. If a subject met eligibility criteria, written and informed
consent was obtained. A flow diagram of subject recruitment, randomization, and analysis is provided in Figure 5.1. Ethical approval for the study was obtained from the Conjoint Health Research Ethics Board of the University of Calgary, Ethics ID # E-23936.

Figure 5.1: Flow diagram of subject recruitment, randomization, and analysis.

5.3.2 Study design and diet intervention
Following screening, participants were randomly assigned to one of two groups by a senior study investigator not involved in recruiting participants. Participants were randomized into either a treatment group that received oligofructose (Orafti P95, Beneo-Orafti Inc., Tienen, Belgium) prebiotic (PRE) 8g orally (one package) per day for 12 weeks followed by 16g orally (two packages) per day for 24 weeks or a placebo (PLA) control group that received an isocaloric amount of maltodextrin (one package for the first 12 weeks followed by 2 packages for 24 weeks). The maltodextrin powder looks and tastes similar to the oligofructose. The participants and research staff performing analyses were blinded to the treatment allocation. Instruction was provided to each participant on the method to consume the supplements. Adherence to supplement protocol was monitored throughout the study through direct questioning and
counting unused supplement packages. Participants were asked to maintain their usual physical activity and refrain from starting or stopping a new exercise or diet program.

Body weight, waist circumference, blood pressure, body composition via dual energy x-ray absorptiometry (DXA) scan (Hologic QDR 4500, Hologic, Inc. Bedford, MA, USA), dietary intake, fasting blood chemistries (lipids, inflammatory markers, and liver function tests), oral glucose tolerance, and gut microbiota were serially measured at baseline, week 12, week 24 (excluding DXA), and week 36 (Figure 5.2). Regular meetings took place at the Roger Jackson Centre for Health and Wellness at the University of Calgary. Blood work and OGTTs were conducted at one of nineteen Calgary Laboratory Services clinics located throughout the city of Calgary, AB, Canada.

Food intake was assessed using 3 day food records (one weekend day and two weekdays) recorded by participants every 6-12 weeks. To assist with accuracy, each participant was provided with a food scale and instruction from a registered dietitian on how to complete a dietary record. The 3 day food records were analyzed using FoodWorks 14 Software (The Nutritional Company, Long Valley, NJ).

Physical activity patterns at baseline and week 36 were monitored using a modified version of Godin’s leisure time exercise questionnaire (GLTEQ)(280). Metabolic Equivalent of Task (MET)•hours was determined for each subject by multiplying the number of hours of mild, moderate, and strenuous activity by an estimated MET value for each level of activity (Mild e.g. easy walking - 3 METS•hour; Moderate e.g. brisk walking – 5 METS•hour; Strenuous e.g. running – 9MET•hour).

Post-study subjective evaluation of acceptability, convenience, tolerability, and hunger was determined using 100mm visual analog scales (VAS). Scores for each question were added and compared between groups.

At baseline and upon completion of the study, all subjects underwent an ultrasound guided liver biopsy conducted by a radiologist at the University of Calgary Foothills Medical Centre. Steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis scores were determined by the GI pathologist at the University of Calgary Foothills Medical Centre. The NAS score is based on a standardized grading system for steatosis (scale of 0-3), lobular inflammation (scale
of 0–3), and hepatocellular ballooning (scale of 0–2). Higher scores indicate increasing severity of disease, with NAS score of ≥ 5 indicating NASH(184). Additionally, all participants underwent an indirect, non-invasive measure of liver fibrosis using transient elastography (Fibroscan®, Echosens, Cambridge, MA) at baseline and 36 weeks at the University of Calgary Foothills Medical Centre.

5.3.3 Blood biochemistry

Fasting concentrations of alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), serum lipids [total, low-density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol and triglycerides], C-reactive protein (CRP), and serial measurements of glucose from the OGTT were measured by Calgary Laboratory Services (Calgary, AB, Canada). Fasting inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) as well as leptin and serial measures of insulin from serum collected during the OGTT were measured in duplicate using the Human Adipokine Milliplex Panel B kit, 6-plex (HADK2MAG-61K, Millipore, St. Charles, MO, USA). Fasting adiponectin was measured in duplicate using the Human Adipokine
Panel-1, 1-plex (HADK1MAG-61K, Millipore). Luminex analysis was completed by Eve Technologies (Calgary, AB). Insulin resistance and β-cell function were approximated using the updated homeostasis model assessment of insulin resistance (HOMA2-IR) and β-cell function (HOMA2-%β)(281). Lipopolysaccharide (LPS) was measured using a PyroGene Recombinant Factor C Endotoxin Endpoint Fluorescent Detection assay (Lonza, Walkersville, MD, USA) according to manufacturer directions. Serum samples were diluted 1/50 and heated for 15 minutes at 70°C to inhibit active serum proteases(282).

5.3.4 Stool collection and gut microbiota analysis
Stool was collected using a convenient, home-use collection kit (Protocult, Rochester, MN). Participants were instructed to place ~25mls of stool in a 50ml conical tube. Samples were then placed in a biohazard container with icepacks and immediately placed in a home -20°C freezer. Within 48hrs samples were delivered to the Roger Jackson Centre for Health and Wellness and placed into a -80°C freezer until further analysis. Gut microbiota was assessed using qPCR. Using ~500mg stool, DNA was extracted using the FastDNA Spin kit for feces (MP Biomedicals, LLC, Solon, OH, USA), purified using ethanol precipitation, and quantified using the Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were diluted to 4ng/µl and stored at -20°C until analysis. Microbial profiling using group-specific 16S primers was conducted using an iCycler (Bio-Rad, Hercules, CA, USA) as previously described(168). Purified template DNA from reference strains (ATCC, Manassas, VA, USA) was serially diluted to generate a standard curve. Standard curves were normalized to copy number of 16S rRNA genes using reference strain genome size and 16S rRNA gene copy number values obtained from the rrnDB(247). Group specific primers were used as previously described(248). The specificity of the primers and the limit of detection were determined according to Louie et al.(225). Threshold cycle values were used to calculate the number of 16S rRNA gene copies in each sample. Data is expressed as Log_{10} 16S rRNA gene copy number per 20ng DNA.
5.3.5 Statistical analysis

Raw data expressed as mean ± SEM. For outcome variables measured at baseline and week 36, mean difference (post minus pre-treatment) values were used to compare groups using an independent t-test or Mann-Whitney U test. Within group differences were determined using a paired t-test or Wilcoxon signed-rank test. Data with serial measures at week 0, 12, 24, and 36 was analyzed using mixed design (split-plot) one-way repeated measures ANOVA. OGTT data with repeated measures for week (0, 12, 24 and 36) as well as minute (0, 60 and 120min) were analyzed using a mixed design (split-plot) two way repeated measures ANOVA. Data that was not normally distributed (determined using a Shapiro-Wilk test) was log\textsubscript{10} transformed. Correlations were assessed using Spearman’s correlation test in SPSS. Analysis was completed on an intent-to treat basis. P<0.05 was considered statistically significant. Data analysis was performed using SPSS 21.0 software (IBM, Armonk, NY, USA).

5.4 Results

5.4.1 Subject characteristics

As described in Figure 5.1, a total of 14 individuals were recruited and of these, 14 completed the study. Participant demographics and baseline characteristics in the PRE and PLA groups are described in Table 5.1. Baseline age, body weight, BMI, and body composition did not differ between PRE and PLA.

5.4.2 Anthropometrics

There were no differences in repeated measures of body weight, BMI, waist circumference (WC), blood pressure (BP), body fat, fat free mass, body fat %, or bone mineral density (BMD) between PRE and PLA across the 36 week intervention (Table 5.2).
Table 5.1 Demography and baseline characteristics of PRE and PLA participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PRE, n=8</th>
<th>PLA, n=6</th>
<th>Total, n=14</th>
<th>P-value</th>
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<tbody>
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<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5 (63%)</td>
<td>3 (50%)</td>
<td>8 (57%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Female</td>
<td>3 (38%)</td>
<td>3 (50%)</td>
<td>6 (43%)</td>
<td></td>
</tr>
<tr>
<td>Age, y&lt;sup&gt;1&lt;/sup&gt;</td>
<td>45.3 ± 5.6</td>
<td>53.3 ± 4.8</td>
<td>50.3 ± 5.2</td>
<td>0.32</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
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<td>102.1 ± 8.1</td>
<td>101.8 ± 9.8</td>
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<tr>
<td>Height, cm</td>
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<td>170.8 ± 3.7</td>
<td>171.3 ± 4.1</td>
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<tr>
<td>BMI, kg/m&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>34.8 ± 2.2</td>
<td>34.3 ± 2.1</td>
<td>0.79</td>
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<td>Fat mass, kg</td>
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<td>35.6 ± 6.2</td>
<td>33.7 ± 5.5</td>
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<td>Lean mass, kg</td>
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<td>Body fat, % BW</td>
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<td>34.2 ± 3.7</td>
<td>32.6 ± 3.5</td>
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<tr>
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<td>Completed High School</td>
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<td>6 (43%)</td>
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</tr>
<tr>
<td>Some University/College</td>
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<td>2 (33%)</td>
<td>4 (29%)</td>
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</tr>
<tr>
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<td>2 (33%)</td>
<td>4 (29%)</td>
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<tr>
<td>Some or Completed Grad School</td>
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<td>&lt;$20,000</td>
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<td>1 (7%)</td>
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<td>1 (17%)</td>
<td>2 (14%)</td>
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<td>1 (17%)</td>
<td>2 (14%)</td>
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<tr>
<td>&gt;$80,000</td>
<td>5 (63%)</td>
<td>4 (67%)</td>
<td>9 (64%)</td>
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<td>5 (83%)</td>
<td>9 (64%)</td>
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<td>5 (36%)</td>
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<td>7 (50%)</td>
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<td>2 (14%)</td>
<td>4 (29%)</td>
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<tr>
<td>Retired</td>
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<td>3 (50%)</td>
<td>6 (43%)</td>
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</tr>
<tr>
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<td>1 (13%)</td>
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<td>Unemployed</td>
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<tr>
<td>Disability/sick leave</td>
<td>1 (13%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Data represented as mean ± SEM. Chi-squared test was performed to determine differences in age between PRE and PLA. Independent t-test was performed to measure baseline anthropometric differences between PRE and PLA.
Table 5.2 Anthropometrics in the PRE and PLA groups over the course the 36 week study.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment</th>
<th>Week</th>
<th>P-value</th>
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<tr>
<td>Weight</td>
<td>PRE</td>
<td>101.3 ± 11.4</td>
<td>100.7 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>102.1 ± 8.1</td>
<td>101.8 ± 8.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>PRE</td>
<td>33.7 ± 3.0</td>
<td>33.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>34.8 ± 2.2</td>
<td>34.7 ± 2.2</td>
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<td>Waist, cm</td>
<td>PRE</td>
<td>111.5 ± 8.2</td>
<td>110.5 ± 8.1</td>
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<td></td>
<td>PLA</td>
<td>115.6 ± 6.6</td>
<td>113.3 ± 6.7</td>
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<td>Systolic, mmHg</td>
<td>PRE</td>
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<td>132 ± 6.6</td>
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<td>PLA</td>
<td>126 ± 5.7</td>
<td>131 ± 5.9</td>
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<tr>
<td>Diastolic, mmHg</td>
<td>PRE</td>
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<td>88 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>82 ± 3.5</td>
<td>85 ± 3.1</td>
</tr>
<tr>
<td><strong>Adiposity</strong></td>
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</tr>
<tr>
<td>Fat Free mass, kg</td>
<td>PRE</td>
<td>69.3 ± 7.5</td>
<td>69.1 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>66.2 ± 4.6</td>
<td>66.3 ± 4.4</td>
</tr>
<tr>
<td>Adipose mass, kg</td>
<td>PRE</td>
<td>31.9 ± 4.9</td>
<td>31.3 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>35.6 ± 6.2</td>
<td>35.5 ± 6.3</td>
</tr>
<tr>
<td>Body fat, % of total weight</td>
<td>PRE</td>
<td>31.0 ± 2.3</td>
<td>30.7 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>34.2 ± 3.7</td>
<td>34.0 ± 3.7</td>
</tr>
<tr>
<td>BMD, g/cm²</td>
<td>PRE</td>
<td>1.141 ± 0.035</td>
<td>1.138 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>1.130 ± 0.035</td>
<td>1.129 ± 0.031</td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM. n=8 PRE, n=6 PLA. Mixed design (split-plot) repeated measures ANOVA performed to determine main effect of week, treatment, and their interaction.
5.4.3 Liver, lipid, inflammatory, and glycemic control biochemistries

PRE and PLA did not affect serum ALT, GGT, or ALP concentrations (Table 5.3). No benefit of PRE was observed for improving TG, TC, LDL, HDL, or the TC/HDL ratio. There was an independent effect of PRE for increased TC, LDL, and TC/HDL ratio, however these differences were present at baseline and persisted over the course of the trial. Inflammatory blood markers (TNF-α, MCP-1, IL-8, IL-6, CRP, and LPS) were not affected by PRE and PLA (Table 5.4). Furthermore, no improvement in glycemic control indices was observed during the trial with PRE. Prebiotic was associated with lower fasting glucose and improved pancreatic β-cell function according to HOMA-%β calculation over the course of the trial, however this was largely driven by the significantly lower baseline fasting glucose levels and increased HOMA-%β in PRE compared to PLA. Over the course of the 4 OGTTs conducted during the trial, PRE consistently demonstrated lower glycemia relative to PLA (Figure 5.3). Serum insulin levels and insulin sensitivity according to the HOMA2-IR remained the same between PRE and PLA during the study. Insulin levels during the OGTTs, although not statistically different (p=0.133), tended to be lower in the PLA group. Altogether there was a main effect of PRE for reduced glucose AUC (p=0.009) and a main effect of PLA for reduced insulin AUC (p=0.052) which in both cases were largely driven by lower baseline values respectively. There was a main effect of week (p=0.037) for increasing leptin concentrations.
<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>Time</th>
<th>Treatment</th>
<th>Interaction</th>
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<td><strong>Liver function tests</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>PRE</td>
<td>80.4 ± 7.9</td>
<td>91.8 ± 19.5</td>
<td>75.3 ± 13.1</td>
<td>68.1 ± 14.0</td>
<td>0.883</td>
<td>0.222</td>
<td>0.479</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>60.5 ± 14.0</td>
<td>51.8 ± 9.6</td>
<td>62.7 ± 17.2</td>
<td>59.3 ± 14.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>PRE</td>
<td>60.5 ± 10.2</td>
<td>62.4 ± 9.6</td>
<td>62.0 ± 12.4</td>
<td>62.3 ± 14.9</td>
<td>0.979</td>
<td>0.201</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>44.5 ± 8.9</td>
<td>41.7 ± 6.4</td>
<td>41.3 ± 7.2</td>
<td>44.7 ± 7.2</td>
<td></td>
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</tr>
<tr>
<td>ALP, U/L</td>
<td>PRE</td>
<td>75.4 ± 7.5</td>
<td>80.9 ± 7.2</td>
<td>81.3 ± 8.6</td>
<td>78.8 ± 7.8</td>
<td>0.396</td>
<td>0.188</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>64.2 ± 7.6</td>
<td>62.5 ± 8.0</td>
<td>63.2 ± 7.8</td>
<td>64.3 ± 7.9</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood lipids</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>PRE</td>
<td>1.78 ± 0.26</td>
<td>2.10 ± 0.27</td>
<td>2.35 ± 0.36</td>
<td>1.96 ± 0.36</td>
<td>0.406</td>
<td>0.433</td>
<td>0.223</td>
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<td>PLA</td>
<td>1.76 ± 0.26</td>
<td>1.71 ± 0.17</td>
<td>1.72 ± 0.17</td>
<td>1.82 ± 0.20</td>
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<tr>
<td>TC, mmol/L</td>
<td>PRE</td>
<td>5.25 ± 0.24*</td>
<td>5.63 ± 0.19</td>
<td>5.32 ± 0.21</td>
<td>5.29 ± 0.27</td>
<td>0.177</td>
<td><strong>0.030</strong></td>
<td>0.324</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>4.07 ± 0.16</td>
<td>4.23 ± 0.29</td>
<td>4.47 ± 0.35</td>
<td>4.11 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>PRE</td>
<td>3.33 ± 0.22*</td>
<td>3.50 ± 0.25</td>
<td>3.23 ± 0.28</td>
<td>3.35 ± 0.34</td>
<td>0.663</td>
<td><strong>0.013</strong></td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>2.15 ± 0.23</td>
<td>2.31 ± 0.32</td>
<td>2.48 ± 0.36</td>
<td>2.17 ± 0.29</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HDL, mmol/L</td>
<td>PRE</td>
<td>1.08 ± 0.10</td>
<td>1.08 ± 0.08</td>
<td>1.03 ± 0.08</td>
<td>1.05 ± 0.09</td>
<td>0.922</td>
<td>0.510</td>
<td>0.449</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>1.12 ± 0.09</td>
<td>1.14 ± 0.09</td>
<td>1.18 ± 0.09</td>
<td>1.12 ± 0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>PRE</td>
<td>5.04 ± 0.50*</td>
<td>5.44 ± 0.51</td>
<td>5.54 ± 0.66</td>
<td>5.33 ± 0.61</td>
<td>0.587</td>
<td><strong>0.027</strong></td>
<td>0.821</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>3.73 ± 0.27</td>
<td>3.85 ± 0.51</td>
<td>3.87 ± 0.41</td>
<td>3.80 ± 0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM. n=8 PRE, n=6 PLA. Mixed design (split-plot) repeated measures ANOVA performed to determine main effect of week, treatment, and their interaction. *indicates p<0.05 between PRE and PLA at baseline.
Table 5.4 Inflammatory markers and glycemic control indices in the PRE or PLA groups.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment</th>
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<th>12</th>
<th>24</th>
<th>36</th>
<th>Time</th>
<th>P-value</th>
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<th></th>
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<tr>
<td>Inflammatory markers</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α, pmol/L</td>
<td>PRE</td>
<td>0.460 ± 0.100</td>
<td>0.391 ± 0.087</td>
<td>0.416 ± 0.063</td>
<td>0.390 ± 0.064</td>
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<td>0.850</td>
<td>0.701</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>0.447 ± 0.039</td>
<td>0.468 ± 0.046</td>
<td>0.440 ± 0.049</td>
<td>0.448 ± 0.041</td>
<td></td>
<td>0.850</td>
<td>0.215</td>
<td>0.882</td>
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<tr>
<td>MCP-1, pmol/L</td>
<td>PRE</td>
<td>24.9 ± 3.8</td>
<td>22.7 ± 3.6</td>
<td>24.4 ± 4.1</td>
<td>24.6 ± 4.6</td>
<td></td>
<td>0.850</td>
<td>0.215</td>
<td>0.882</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>30.6 ± 1.7</td>
<td>30.6 ± 2.3</td>
<td>30.2 ± 3.7</td>
<td>31.5 ± 3.8</td>
<td></td>
<td>0.655</td>
<td>0.162</td>
<td>0.888</td>
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<tr>
<td>Il-8, pmol/L</td>
<td>PRE</td>
<td>1.83 ± 0.34</td>
<td>1.53 ± 0.22</td>
<td>1.68 ± 0.30</td>
<td>1.58 ± 0.20</td>
<td></td>
<td>0.530</td>
<td>0.894</td>
<td>0.387</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>1.33 ± 0.25</td>
<td>1.21 ± 0.16</td>
<td>1.14 ± 0.18</td>
<td>1.26 ± 0.17</td>
<td></td>
<td>0.366</td>
<td>0.939</td>
<td>0.824</td>
<td></td>
</tr>
<tr>
<td>Il-6, pmol/L</td>
<td>PRE</td>
<td>0.366 ± 0.172</td>
<td>0.267 ± 0.295</td>
<td>0.269 ± 0.080</td>
<td>0.211 ± 0.054</td>
<td></td>
<td>0.530</td>
<td>0.939</td>
<td>0.824</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>0.214 ± 0.047</td>
<td>0.165 ± 0.046</td>
<td>0.329 ± 0.180</td>
<td>0.347 ± 0.164</td>
<td></td>
<td>0.530</td>
<td>0.894</td>
<td>0.387</td>
<td></td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>PRE</td>
<td>2.31 ± 0.85</td>
<td>3.41 ± 1.71</td>
<td>2.40 ± 1.09</td>
<td>3.91 ± 1.92</td>
<td></td>
<td>0.366</td>
<td>0.939</td>
<td>0.824</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>2.22 ± 0.60</td>
<td>1.63 ± 0.46</td>
<td>2.13 ± 0.59</td>
<td>1.88 ± 0.55</td>
<td></td>
<td>0.318</td>
<td>0.634</td>
<td>0.771</td>
<td></td>
</tr>
<tr>
<td>LPS, EU/mL</td>
<td>PRE</td>
<td>9.73 ± 1.63</td>
<td>8.66 ± 0.91</td>
<td>9.79 ± 1.64</td>
<td>8.03 ± 0.80</td>
<td></td>
<td>0.752</td>
<td>0.269</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>9.78 ± 1.86</td>
<td>10.63 ± 1.70</td>
<td>11.63 ± 2.14</td>
<td>12.86 ± 1.89</td>
<td></td>
<td>0.752</td>
<td>0.269</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>Glycemic control indices</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>PRE</td>
<td>4.99 ± 0.18*</td>
<td>5.04 ± 0.28</td>
<td>5.44 ± 0.32</td>
<td>5.61 ± 0.59</td>
<td>0.619</td>
<td>0.022</td>
<td>0.502</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>7.02 ± 0.91</td>
<td>7.68 ± 1.13</td>
<td>6.90 ± 0.96</td>
<td>7.28 ± 0.64</td>
<td></td>
<td>0.318</td>
<td>0.634</td>
<td>0.771</td>
<td></td>
</tr>
<tr>
<td>Fasting insulin, mU/L</td>
<td>PRE</td>
<td>48.6 ± 9.8</td>
<td>51.0 ± 8.4</td>
<td>51.3 ± 9.8</td>
<td>51.8 ± 9.6</td>
<td></td>
<td>0.619</td>
<td>0.022</td>
<td>0.502</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>37.9 ± 6.3</td>
<td>47.8 ± 9.3</td>
<td>46.0 ± 7.8</td>
<td>46.9 ± 10.5</td>
<td></td>
<td>0.318</td>
<td>0.634</td>
<td>0.771</td>
<td></td>
</tr>
<tr>
<td>HOMA2-IR</td>
<td>PRE</td>
<td>5.01 ± 0.97</td>
<td>5.27 ± 0.84</td>
<td>5.41 ± 0.99</td>
<td>5.51 ± 1.02</td>
<td>0.262</td>
<td>0.878</td>
<td>0.755</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>4.38 ± 0.77</td>
<td>5.58 ± 1.17</td>
<td>5.20 ± 0.94</td>
<td>5.27 ± 1.03</td>
<td></td>
<td>0.262</td>
<td>0.878</td>
<td>0.755</td>
<td></td>
</tr>
<tr>
<td>HOMA2-%B</td>
<td>PRE</td>
<td>296 ± 46*</td>
<td>315 ± 52</td>
<td>266 ± 35</td>
<td>267 ± 31</td>
<td>0.736</td>
<td>0.014</td>
<td>0.287</td>
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<tr>
<td></td>
<td>PLA</td>
<td>149 ± 20</td>
<td>150 ± 17</td>
<td>188 ± 35</td>
<td>163 ± 38</td>
<td></td>
<td>0.736</td>
<td>0.014</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>Leptin, nmol/L</td>
<td>PRE</td>
<td>1.43 ± 0.29</td>
<td>1.60 ± 0.41</td>
<td>1.77 ± 0.40</td>
<td>1.68 ± 0.37</td>
<td></td>
<td>0.736</td>
<td>0.014</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>2.53 ± 0.75</td>
<td>2.62 ± 0.78</td>
<td>2.87 ± 0.80</td>
<td>3.24 ± 0.91</td>
<td></td>
<td>0.736</td>
<td>0.014</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>Adiponectin, pmol/L</td>
<td>PRE</td>
<td>16.7 ± 3.0</td>
<td>17.5 ± 3.3</td>
<td>16.3 ± 3.3</td>
<td>16.5 ± 3.5</td>
<td>0.585</td>
<td>0.788</td>
<td>0.375</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>PLA</td>
<td>14.6 ± 2.6</td>
<td>15.3 ± 3.0</td>
<td>15.6 ± 3.1</td>
<td>16.5 ± 2.8</td>
<td></td>
<td>0.585</td>
<td>0.788</td>
<td>0.375</td>
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</tbody>
</table>

Data presented as mean ± SEM. n=8 PRE, n=6 PLA. Mixed design (split-plot) repeated measures ANOVA performed to determine main effect of week, treatment, and their interaction. *indicates p<0.05 between PRE and PLA at baseline.
Figure 5.3: Glycemic control indices in PRE and PLA at week 0, 12, 24 and 36. A) Serial glucose during the OGTTs, B) serial insulin during the OGTTs, C) glucose AUC, and D) insulin AUC. Data presented as mean ± SEM. n=8 PRE, n=5 PLA. A mixed design (split-plot), two way repeated measures ANOVA was used to determine main effects of min, week, treatment, and their interaction for glucose and insulin. Total AUC for glucose and insulin was analyzed with a mixed design (split-plot) one way repeated measures ANOVA to determine main effect of week, treatment, and their interaction.

5.4.4 Characterization of gut microbiota

*Bifidobacterium* increased by 60% with PRE whereas levels stayed consistent in PLA (week x treatment p=0.017) (Table 5.5, Figure 5.4). While levels of *Bifidobacterium* were the same in both groups at baseline, at week 24 *Bifidobacterium* was significantly elevated in PRE relative to PLA. There was an ~55% reduction in both *Clostridium* cluster XI (week x treatment p=0.030) and C. cluster I (week x treatment p=0.032) (Figure 5.4) with PRE whereas PLA resulted in an increase in both of these bacterial groups. Members of *C. coccoides* and *C. leptum* were not
affected by PRE. Interestingly, there was a significant positive correlation between delta C. cluster I and delta LPS concentrations ($r=0.609$, $p=0.021$). There was also a significant correlation between delta C. cluster I and C. cluster XI with delta leptin ($r=0.679$, $p=0.008$; $r=0.618$, $p=0.019$, respectively). *C. leptum* and *Faecalibacterium prausnitzii*, a prominent member of the *C. leptum* group(283), increased over the course of the 36 weeks in both PRE and PLA (week $p=0.017$). *Akkermansia muciniphila* was elevated in PLA relative to PRE, however this difference was already present at baseline (treatment $p=0.045$).
Table 5.5 Fecal gut microbiota profiles measured in PRE and PLA over 36 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>Week</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides/Prevotella</td>
<td>PRE</td>
<td>5.79 ± 0.56</td>
<td>5.85 ± 0.40</td>
<td>5.59 ± 0.55</td>
<td>5.68 ± 0.51</td>
<td>0.996</td>
<td>0.767</td>
<td>0.396</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>6.10 ± 0.17</td>
<td>6.26 ± 0.20</td>
<td>6.09 ± 0.14</td>
<td>6.23 ± 0.13</td>
<td>0.767</td>
<td>0.396</td>
<td>0.141</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>PRE</td>
<td>4.63 ± 0.56</td>
<td>5.19 ± 0.58</td>
<td>5.31 ± 0.61</td>
<td>5.08 ± 0.54</td>
<td>0.240</td>
<td>0.544</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>4.67 ± 0.48</td>
<td>4.51 ± 0.50</td>
<td>4.51 ± 0.52</td>
<td>4.59 ± 0.50</td>
<td>0.544</td>
<td>0.396</td>
<td>0.471</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>PRE</td>
<td>3.65 ± 0.48</td>
<td>3.86 ± 0.47</td>
<td>3.83 ± 0.42</td>
<td>3.30 ± 0.29</td>
<td>0.141</td>
<td>0.045</td>
<td>0.471</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>4.93 ± 0.32</td>
<td>4.90 ± 0.06</td>
<td>4.44 ± 0.22</td>
<td>4.46 ± 0.28</td>
<td>0.045</td>
<td>0.394</td>
<td>0.344</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>PRE</td>
<td>4.44 ± 0.25</td>
<td>4.33 ± 0.23</td>
<td>4.53 ± 0.32</td>
<td>4.09 ± 0.40</td>
<td>0.168</td>
<td>0.633</td>
<td>0.344</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>4.83 ± 0.52</td>
<td>4.67 ± 0.47</td>
<td>4.44 ± 0.51</td>
<td>4.49 ± 0.53</td>
<td>0.633</td>
<td>0.344</td>
<td>0.172</td>
</tr>
<tr>
<td>Methanobrevibacter</td>
<td>PRE</td>
<td>3.80 ± 0.15</td>
<td>3.66 ± 0.14</td>
<td>3.79 ± 0.14</td>
<td>3.64 ± 0.13</td>
<td>0.494</td>
<td>0.645</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>3.59 ± 0.15</td>
<td>3.71 ± 0.11</td>
<td>3.65 ± 0.09</td>
<td>3.61 ± 0.09</td>
<td>0.645</td>
<td>0.172</td>
<td>0.154</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>PRE</td>
<td>3.41 ± 0.47</td>
<td>3.35 ± 0.52</td>
<td>4.24 ± 0.48</td>
<td>3.31 ± 0.37</td>
<td>0.071</td>
<td>0.192</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>2.98 ± 0.30</td>
<td>2.73 ± 0.22</td>
<td>2.91 ± 0.26</td>
<td>2.83 ± 0.24</td>
<td>0.192</td>
<td>0.154</td>
<td>0.154</td>
</tr>
<tr>
<td>Clostridium cocoides</td>
<td>PRE</td>
<td>6.75 ± 0.14</td>
<td>6.90 ± 0.14</td>
<td>6.88 ± 0.07</td>
<td>6.60 ± 0.21</td>
<td>0.847</td>
<td>0.872</td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>6.98 ± 0.07</td>
<td>6.88 ± 0.08</td>
<td>6.82 ± 0.07</td>
<td>6.85 ± 0.08</td>
<td>0.872</td>
<td>0.440</td>
<td>0.315</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td>PRE</td>
<td>6.57 ± 0.35</td>
<td>6.97 ± 0.23</td>
<td>7.03 ± 0.25</td>
<td>6.61 ± 0.23</td>
<td>0.015</td>
<td>0.394</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>6.48 ± 0.41</td>
<td>6.61 ± 0.34</td>
<td>6.53 ± 0.42</td>
<td>6.64 ± 0.26</td>
<td>0.394</td>
<td>0.315</td>
<td>0.315</td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii</td>
<td>PRE</td>
<td>5.58 ± 0.40</td>
<td>6.15 ± 0.19</td>
<td>6.21 ± 0.21</td>
<td>5.77 ± 0.20</td>
<td>0.017</td>
<td>0.454</td>
<td>0.351</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>5.55 ± 0.47</td>
<td>5.56 ± 0.48</td>
<td>5.62 ± 0.46</td>
<td>5.78 ± 0.27</td>
<td>0.454</td>
<td>0.351</td>
<td>0.351</td>
</tr>
<tr>
<td>Clostridium cluster XI</td>
<td>PRE</td>
<td>4.33 ± 0.21</td>
<td>4.08 ± 0.27</td>
<td>4.38 ± 0.18</td>
<td>3.69 ± 0.31</td>
<td>0.951</td>
<td>0.386</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>3.64 ± 0.56</td>
<td>3.75 ± 0.49</td>
<td>3.32 ± 0.63</td>
<td>4.07 ± 0.52</td>
<td>0.386</td>
<td>0.030</td>
<td>0.032</td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>PRE</td>
<td>4.54 ± 0.11*</td>
<td>4.12 ± 0.15</td>
<td>4.06 ± 0.09</td>
<td>4.16 ± 0.12</td>
<td>0.097</td>
<td>0.063</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>3.99 ± 0.07</td>
<td>4.09 ± 0.12</td>
<td>3.99 ± 0.12</td>
<td>4.03 ± 0.11</td>
<td>0.063</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>Roseburia</td>
<td>PRE</td>
<td>5.80 ± 0.19</td>
<td>5.84 ± 0.13</td>
<td>5.81 ± 0.15</td>
<td>5.52 ± 0.16</td>
<td>0.639</td>
<td>0.699</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>5.87 ± 0.15</td>
<td>5.87 ± 0.13</td>
<td>5.82 ± 0.16</td>
<td>5.92 ± 0.14</td>
<td>0.699</td>
<td>0.452</td>
<td>0.452</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM Log10 16S rRNA gene copies/20ng total genomic DNA. n=8 PRE, n=6 PLA. Mixed design (split-plot) repeated measures ANOVA performed to determine main effect of week, treatment, and their interaction. *indicates p<0.05 between PRE and PLA at baseline; #indicates p<0.05 between PRE and PLA.
Figure 5.4: Fecal A) *Bifidobacterium*, B) *Clostridium* cluster I, and C) *C*. cluster XI 16S rRNA gene copies in PRE versus PLA groups over the course of 36 weeks. Data represented as mean ± SEM. n=8 PRE, n=6 PLA.

5.4.5 **Prebiotic improves histological parameters of NASH**

Of the 14 participants that started the study, all 14 completed a follow-up liver biopsy. One biopsy from the PLA group was excluded at the 36 week time point due to the pathologists’ indication of an inadequate sample for histology. Therefore, 5 subjects in the PLA group were considered in the final liver biopsy analysis. The group that received PRE had a significant within group decrease in hepatic steatosis whereas steatosis did not change significantly in PLA (Table 5.6). Overall when assessing the mean difference between PRE and PLA there was a significant between group decrease in steatosis. Interestingly, there was a significant correlation between the changes in steatosis and *C*. cluster I (r=0.565, p=0.044). Following a similar within-group trend, there was a decrease in lobular inflammation in PRE, although this finding did not reach statistical significance. Inflammation in PLA remained unchanged and there was no
between group difference in inflammation. While both PRE and PLA showed some trend towards a reduction in hepatocellular ballooning, there were no differences between groups. Overall, as a composite of steatosis, lobular inflammation, and hepatocyte ballooning, PRE significantly reduced within-group NAS relative to PLA, although the between group difference was not significant. There was a significant positive correlation between the change in NAS and the change in ALT (r=0.611, p=0.027). In contrast to the results observed with NAS, fibrosis scores were not affected by PRE and PLA. Similarly, stiffness values determined with a Fibroscan® did not reveal significant changes with PRE, although there was a trend towards reduced hepatic stiffness with PLA (Table 5.6). A total of 3 participants (1 PRE and 2 PLA) did not obtain a result for a pre or post Fibroscan® and thus a lower sample size was used for Fibroscan® analysis.
Table 5.6 Liver biopsy NAS and Fibroscan® scores before and after PRE and PLA diet intervention

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>PLA</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Within-group p-value</td>
<td>Change</td>
<td>Initial</td>
<td>Final</td>
<td>Within-group p-value</td>
<td>Change</td>
</tr>
<tr>
<td>Liver Biopsy¹</td>
<td>2.88 ± 0.13</td>
<td>2.13 ± 0.30</td>
<td><strong>0.034</strong></td>
<td>-0.75 ± 0.25</td>
<td>2.80 ± 0.20</td>
<td>3.00 ± 0.00</td>
<td>0.317</td>
<td>0.20 ± 0.20</td>
</tr>
<tr>
<td>Steatosis</td>
<td>1.63 ± 0.18</td>
<td>1.00 ± 0.19</td>
<td>0.059</td>
<td>-0.63 ± 0.26</td>
<td>1.40 ± 0.24</td>
<td>1.20 ± 0.20</td>
<td>0.564</td>
<td>-0.20 ± 0.37</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.38 ± 0.18</td>
<td>0.88 ± 0.23</td>
<td>0.102</td>
<td>-0.50 ± 0.27</td>
<td>1.20 ± 0.20</td>
<td>0.60 ± 0.24</td>
<td>0.083</td>
<td>-0.60 ± 0.24</td>
</tr>
<tr>
<td>Total NAS</td>
<td>5.88 ± 0.30</td>
<td>4.00 ± 0.60</td>
<td><strong>0.016</strong></td>
<td>-1.88 ± 0.61</td>
<td>5.40 ± 0.24</td>
<td>4.80 ± 0.37</td>
<td>0.180</td>
<td>-0.60 ± 0.40</td>
</tr>
<tr>
<td>Ballooning</td>
<td>1.38 ± 0.32</td>
<td>1.63 ± 0.50</td>
<td>0.589</td>
<td>0.25 ± 0.45</td>
<td>2.20 ± 0.37</td>
<td>2.20 ± 0.37</td>
<td>1.000</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>9.3 ± 41.7</td>
<td>10.3 ± 2.3</td>
<td>0.531</td>
<td>0.9 ± 2.5</td>
<td>17.3 ± 5.6</td>
<td>9.1 ± 1.3</td>
<td>0.063</td>
<td>-8.1 ± 4.8</td>
</tr>
</tbody>
</table>

Fibroscan²

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>PLA</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroscan, KPa</td>
<td>9.3 ± 41.7</td>
<td>10.3 ± 2.3</td>
<td>0.531</td>
<td>0.9 ± 2.5</td>
<td>17.3 ± 5.6</td>
<td>9.1 ± 1.3</td>
<td>0.063</td>
<td>-8.1 ± 4.8</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM. ¹n=8 PRE, n=5 PLA; ²n=7 PRE, n=4 PLA. Wilcoxon signed rank test was used to assess within group differences and a Mann Whitney U test was used to assess between group differences.
5.4.6 Food intake and physical activity

Energy and dietary fiber (excluding supplemental prebiotic fiber) intake were not different between PRE and PLA (Table 5.7). Using a modified version of GLTEQ to estimate physical activity, MET\textperiodcentered hours was lower in PRE relative to PLA at the start of the study. At study completion MET\textperiodcentered hours were equivalent between groups. The lack of an interaction between week and treatment suggests that changes in activity were not differentially affected over the course of the study in PRE and PLA.

5.4.7 Acceptability of treatment

There were no differences in VAS evaluation of treatment acceptability, convenience, tolerability, and hunger between PRE and PLA (Table 5.8). PRE and PLA participants agreed that supplement packages were convenient and indicated that they would continue to take the supplements if demonstrated to be effective for treating NASH. There was one participant in PRE and PLA that cited increased flatulence as a deterrent for consuming the supplements. Despite this concern, both participants remained amenable to continuing with supplement consumption post-study. Noteworthy, maltodextrin control has been reported to elicit side effects for patients from a previous study(130). Interestingly, although this finding did not reach statistical significance, there was a trend towards individuals in the PRE group having a higher degree of fullness throughout the day relative to PLA (p=0.059).
Table 5.7 Food Intake and physical activity patterns in the PRE and PLA groups over 36 weeks.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>16</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy intake and physical activity patterns</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake, kcal/day</td>
<td>PRE</td>
<td>2301 ± 364</td>
<td>1928 ± 236</td>
<td>2227 ± 376</td>
<td>1933 ± 237</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>1906 ± 240</td>
<td>1741 ± 299</td>
<td>1859 ± 275</td>
<td>1772 ± 238</td>
<td>0.474</td>
</tr>
<tr>
<td>Fiber intake, g/day</td>
<td>PRE</td>
<td>23.8 ± 6.5</td>
<td>18.2 ± 4.2</td>
<td>21.4 ± 4.2</td>
<td>18.8 ± 4.1</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>20.7 ± 3.4</td>
<td>17.3 ± 3.7</td>
<td>17.5 ± 2.8</td>
<td>15.8 ± 2.7</td>
<td>0.630</td>
</tr>
<tr>
<td>GLTEQ, MET•hours</td>
<td>PRE</td>
<td>12.0 ± 2.9*</td>
<td></td>
<td></td>
<td>14.9 ± 5.6</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>23.6 ± 3.9</td>
<td></td>
<td></td>
<td>20.6 ± 4.1</td>
<td>0.102</td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM. n=8 PRE, n=6 PLA. Mixed design (split-plot) repeated measures ANOVA performed to determine main effect of week, treatment, and their interaction. *indicates p<0.05 between PRE and PLA at baseline.
Table 5.8 Subjective visual analogue scale (VAS) evaluation of PRE vs PLA dietary intervention.

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>PLA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acceptability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Would you continue to take the supplements now that the study is complete? Not at all (0) - Yes, often (100)</td>
<td>86.9 ± 7.2</td>
<td>90.0 ± 5.6</td>
<td>0.950</td>
</tr>
<tr>
<td>If the supplements were demonstrated to improve your health, would you continue to take the supplements? Not at all (0) - Yes, often (100)</td>
<td>94.3 ± 2.4</td>
<td>94.5 ± 3.6</td>
<td>0.852</td>
</tr>
<tr>
<td><strong>Convenience</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>How convenient were the supplements? Not at all (0) – Extremely (100)</td>
<td>71.1 ± 11.7</td>
<td>78.8 ± 6.3</td>
<td>0.950</td>
</tr>
<tr>
<td>How difficult was it to adhere to taking supplement every day? Not difficult (0) - Very difficult (100)</td>
<td>12.6 ± 4.0</td>
<td>20.5 ± 7.6</td>
<td>0.491</td>
</tr>
<tr>
<td><strong>Tolerability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>How often did you experience negative side effects (flatulence, abdominal discomfort, etc.) that would prevent you from using the supplements? Never (0) – Often 100)</td>
<td>20.9 ± 9.4</td>
<td>31.8 ± 14.2</td>
<td>0.852</td>
</tr>
<tr>
<td>How often did you experience positive side effects that would encourage you to use the supplements? Never (0) – Often (100)</td>
<td>52.4 ± 10.8</td>
<td>24.8 ± 15.8</td>
<td>0.181</td>
</tr>
<tr>
<td><strong>Hunger</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did the supplements decrease cravings for other foods? Not at all (0) – Often (100)</td>
<td>20.3 ± 6.1</td>
<td>8.2 ± 4.6</td>
<td>0.142</td>
</tr>
<tr>
<td>Did the supplements enhance your feelings of fullness throughout the day? Not at all (0) – Often (100)</td>
<td>39.8 ± 9.4</td>
<td>13.0 ± 6.9</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM; n=8 PRE, n=6 PLA. Participants were asked mark an answer to the question along a 100mm continuum between the two provided opposing answers anchored at each end of the rating scale. Mann Whitney U test was used to assess between group differences.
5.5 Discussion

We investigated the therapeutic potential of oligofructose for improving histological parameters of NASH in a pilot randomized, placebo controlled clinical trial. Although changes in body weight and body composition were not observed, oligofructose supplementation resulted in a significant decrease in steatosis and overall NAS. Corresponding with these changes, oligofructose increased *Bifidobacterium* and decreased *C*. cluster I and XI. Individuals with NASH reported no concerns with tolerability to the supplement over the 36 week study period. While sample size was limited in this pilot study, this evidence supports oligofructose as a specific, safe, and adjunctive nutritional strategy for NASH management.

With evidence that gut microbiota impacts energy homeostasis and inflammation, mechanisms thought to underpin NASH pathogenesis, there has been significant interest in gut microbiota-based interventions in NASH(284). An early study by Daubioul et al. assessed the effects of 16g/day oligofructose for 8 weeks in a randomized, double-blind, cross-over study in 7 individuals with liver biopsy confirmed NASH(209). OFS was demonstrated to significantly reduce aspartate aminotransferase after 8 weeks, however follow-up histological measures were not assessed. In one of the few gut-microbiota based intervention studies to include a follow-up liver biopsy, a placebo-controlled trial by Malaguarnera et al. assessed the effects of a synbiotic containing *Bifidobacterium longum* (dose unspecified) with 2.5 g fructo-oligosaccharides (FOS) in combination with lifestyle management of NASH(211). After 24 weeks, *B. longum* and 2.5g FOS treatment enhanced reductions in liver steatosis, hepatocellular injury, parenchymal inflammation, fibrosis, and overall NASH activity as well as blood parameters AST, LDL, CRP, TNFα, HOMA-iR, and serum endotoxin to a greater extent than lifestyle management alone. Additional studies using both synbiotics(212) as well as probiotics(213,214,285) have been demonstrated to improve serum biomarkers of NASH. Here, using a liver biopsy to assess the histological markers of NASH, we observed a reduction in steatosis relative to placebo and an overall within-group reduction in NAS with oligofructose, although we did not observe any changes in serum lipids, inflammation, and liver function tests. The reason for the discrepancies between our clinical trial and the one conducted by Malaguarnera et al., in addition to the other probiotic studies, remains unclear. Unfortunately in the study conducted by Malaguarnera et al.,
gut microbiota was not profiled and thus it is not possible to ascertain the extent to which the gut microbiota was altered with this intervention. The 2.5g dose used by Malaguarnera et al. is a relatively low dose of fermentable carbohydrate compared to the 16g dose that we used in our study. Furthermore, although the dose of *B. longum* was not specified, synbiotic research in an animal model suggests that oligofructose elicits a greater modification of the gut microbiota than a probiotic alone(168). Although overall NAS was reduced in our study, these findings did not translate into a reduction in fibrosis. However, as shown previously, even with a significant reduction in NAS with an intensive weight loss and lifestyle intervention, fibrosis is not necessarily affected(193).

Despite altered hepatic histopathology in NASH with oligofructose, body weight and body composition did not change over the 9 month intervention. While our research group as well as others have demonstrated moderate weight loss with the prebiotics in otherwise healthy adults with overweight and obesity(98,286), this finding is not unanimous. A study by Daud et al. assessed the effects of 30g oligofructose versus cellulose for 8 weeks in individuals with overweight and obesity(287). Similar to our current observations, no changes in body weight or body composition were observed. Interestingly, in keeping with our results, lipid parameters and liver functions tests were not affected by oligofructose although the authors indicated that there was a trend towards reduced intrahepatocellular lipids, measured with magnetic resonance imaging. In another study, Dewulf et al., found that a 16g/day mixture of inulin/oligofructose did not affect body weight in women with obesity, although there was a trend toward a decrease in fat mass with oligofructose(130). In accordance with our study, oligofructose did not alter serum lipids TC, HDL, LDL, and TG. Given the histological improvement observed with oligofructose, it would appear that many of the benefits are weight independent. While it has been observed that weight loss and improvement of NASH are correlated(193), weight loss is not always necessary to facilitate improvement in NASH(288).

Highlighting the reputed role of gut microbiota in NASH development, numerous studies have identified that NAFLD and NASH are characterized by an altered gut microbiota(197,199,201,204,205), although there is not yet a consensus as to which organisms are specifically affected. Using 16S rRNA pyrosequencing, Zhu et al. demonstrated in a pediatric
population that NASH is characterized by a decreased number of Actinobacteria and *Bifidobacterium* (204). In contrast, in an adult population, differences in *Bifidobacterium* were not observed (199). Here we identified that oligofructose increased *Bifidobacterium* in NASH. Purported as a health promoting bacteria (38), a low abundance of *Bifidobacterium* is associated with obesity (79). Furthermore, an increase in bifidobacteria from oligofructose supplementation in obesity is inversely correlated with serum LPS, a marker of intestinal permeability (130). In addition to the changes we observed in *Bifidobacterium*, we identified that oligofructose supplementation resulted in a decrease in *C.* cluster I and XI, a finding that is consistent with inulin supplementation in the gut microbiota of humanized rats (289). While these bacterial groups have not been associated with NASH, they potentially have clinical significance. We observed that the changes in *C.* cluster I associated positively with the change in hepatic steatosis. While *C.* cluster I and XI both contain commensal bacterial, both yield medically relevant pathogenic species. *C.* cluster I includes *C. perfringens, C. tetanus; C. chauvoei,* and *C. botulinum,* whereas *C.* cluster XI contains *C. difficile* (290,291). While it is speculative to infer that oligofructose decreased pathogenic species in *C.* cluster I, *C. perfringens* has the ability to proteolytically degrade tight junction proteins and decrease transepithelial resistance (292). Interestingly, here we observed a positive association between changes in LPS and *C.* cluster I. Potentially, given the link between intestinal permeability and NASH, a decrease in this group of organisms could impact NASH pathogenesis. Furthermore, strains of bacteria in *C.* cluster XI are believed to contribute to increased production of the secondary bile acid deoxycholic acid (293). Yoshimoto et al. provides evidence that increased deoxycholic acid concentrations are involved in activation of hepatic stellate cells, the same cells believed culpable in mediating the progression of NAFLD to NASH (293). While the relation of these groups of bacteria to NASH requires further investigation, potentially a decrease in these organisms offers clinical benefit for offsetting the development of steatosis in NASH. Furthermore, oligofructose has in other studies been demonstrated to increase *F. prausnitzii* (130). While the increases that we observed with *F. prausnitzii* did not prove to be statistically significant, *F. prausnitzii* has anti-inflammatory properties (294) and has been found to be inversely associated with hepatic fat development (295). Potentially, with a larger sample size, oligofructose may have been demonstrated to elicit a
significant increase in this seemingly important *F. prausnitzii* species. Altogether, through increases in Actinobacteria and reciprocal influence on certain members of clostridia, oligofructose may contribute to improved metabolism in NASH.

With NASH, for any dietary intervention to be successful, it must be tolerable and adhered to long term. Individuals in both PRE and PLA indicated that the supplements were convenient and acceptable for long term use. Furthermore, only a small percentage remarked that flatulence, a common side effect of oligofructose(296), served as a deterrent. The dose of 16g/day oligofructose is in general accordance with limits of tolerance. While there is inter-individual difference in tolerance to oligofructose(297), research suggests that in a population with a BMI < 25 the dose after which GI symptoms develop with fructo-oligosaccharide is between 10-20g(298,299). It is not clear, however, whether individuals with obesity have an enhanced or reduced gastrointestinal response to fructo-oligosaccharide consumption. Daud et al. in their clinical trial used a 30g/day dose of oligofructose(287). While this dose was tolerated by the majority of participants, 4 participants had to temporarily decrease the dose to 20g/day. It would seem, based on the evidence collected in our study as well as the clinical trial conducted by Dewulf et al.(130), that a dose of 16g/day for treatment of NASH is an appropriate dose, posing no long term tolerability concerns.

This study was limited by several factors. Although liver biopsy is the gold standard for ranking and diagnosis of NASH, liver biopsies can be limited by sample error(300). Furthermore, due to the invasive nature of liver biopsies, obtaining a large number of participants in histologically-based studies proves difficult. With a sample size of 14, we had limited statistical power to discern differences between groups. Furthermore, with a low sample size, our randomization process did not evenly stratify treatment groups according to glycemic control indices. Although HOMA2-IR measures were not significantly different, fasting glycemia and predicted pancreatic β-cell function with the HOMA-%B formula were not the same at baseline between PRE and PLA. As another limiting factor, several participants, after receiving a NASH diagnosis, lost a significant amount of weight prior to randomization. As such, during the 36 week intervention phase, several participants experienced characteristic weight loss recidivism(194). The increased weight gain throughout the intervention period may have masked
potential effects that would have been observed if the participant was not recovering from a
period of negative energy balance. Furthermore, systematic error was introduced into our study
due to the fact that the blood samples and OGTTs were conducted at different labs across the city
in order to reduce participant burden for travelling long distances for blood work.

In summary, in a pilot clinical trial designed to examine the effectiveness of oligofructose for
treating NASH, we demonstrated that oligofructose supplementation improves histological
measures of steatosis and has a tendency to decrease hepatocellular inflammation. Overall,
prebiotic contributed to a decrease in overall NAS. Our results indicate the oligofructose
increases Bifidobacterium and decreases C. cluster I and XI in a group of individuals with
NASH. With a limited number of treatment options available for NASH, gut microbiota focused
interventions using prebiotics offer a safe and sustainable means to treat this exigent disease.
Larger scale clinical trials are warranted to further elucidate the potential of prebiotics to treat
NASH.

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6.1 Introduction
Despite major public health campaigns to increase knowledge about healthy eating and the benefits of physical activity, obesity and associated comorbidities continue to rise. The persistence of obesity testifies to a complex biological system that regulates energy expenditure, appetite, and satiety in a manner that is heavily biased towards weight gain and defending against weight loss. Research to date suggests that for an obesity treatment to be effective, it is necessary for the treatment to target the underlying biological factors contributing to the disease.

One recently identified factor that plays a role in the pathogenesis of obesity is the gut microbiota. Being an ‘environmental’ and thus malleable component of the human body, the gut microbiota offers an intriguing intervention target. Two commonly utilized gut-microbiota manipulation strategies include the use of prebiotics and probiotics, both of which are safe and have been demonstrated to elicit beneficial metabolic effects in obesity(46).

The goal of this dissertation was to study both the mechanisms of prebiotic- and probiotic-mediated improvements in metabolism in obesity and to assess the clinical utility of treating NAFLD with a prebiotic. Three major experiments were completed to accomplish these research objectives. The first study assessed the combined and individual effects of oligofructose and the probiotic Bifidobacterium animalis ssp. lactis BB-12 on anthropometric and metabolic health in diet-induced obese rats. The second study examined the gut microbiota dependent and independent effects of oligofructose in diet-induced obese rats using antibiotics to selectively decontaminate the gut microbiota. The final study was a pilot clinical trial that assessed the effectiveness of 16g/day oligofructose supplementation versus placebo for improving histopathological parameters of non-alcoholic steatohepatitis (NASH).

6.2 General discussion
The major research findings from the studies completed for this thesis include:
1) Oligofructose, relative to the probiotic BB-12, provides a more effective means for improving adiposity, glycemia, and satiety hormones. Both oligofructose and BB-12 improve glycemia, although there is no synergism between the two treatments.

2) Oligofructose-mediated improvement on host adiposity and intestinal permeability are, in part, dependent upon the presence of specific microbial organisms, namely *Bifidobacterium* and *Lactobacillus*, within the gut.

3) Supplementation with oligofructose (8g/day for 12 weeks followed by 16g/day for 24 weeks) significantly reduced hepatic steatosis in individuals with NASH, an effect that was associated with increased *Bifidobacterium* and reductions in *C. cluster I and XI.*

### 6.2.1 Individual and combined effects of oligofructose and BB-12

Based on evidence that both prebiotics and probiotics elicit beneficial metabolic effects in obesity, we were interested in assessing the combined potential of the prebiotic oligofructose and the probiotic BB-12. While we hypothesized synergistic potential between oligofructose and BB-12, our findings revealed few synergistic effects. Rather, the major metabolic findings appeared to be induced by oligofructose or BB-12 alone with oligofructose decreasing body weight (Figure 3.1), adiposity (Table 3.2), and glycemia (Figure 3.2A, Table 3.3) and increasing GLP-1 concentrations (Figure 3.2H), while BB-12 significantly improved GLP-2 concentrations (Figure 3.3A) and glycemia (Figure 3.2A, Table 3.3). Interestingly, there was an additive effect for reduction in insulin concentrations with the combined treatment of OFS and BB-12 (Figure 3.2B). The predominantly independent effects of oligofructose and BB-12 suggest unique mechanisms of action for these agents.

While oligofructose had a significant influence on the gut microbiota, changing the majority of the bacterial groups measured, there were no significant effects of BB-12 for altering the gut microbiota (Table 3.4). To our surprise, we did not observe a significant independent effect of BB-12 for increasing *Bifidobacterium animalis* (Figure 3.4). We could only detect a slight increase when we measured *Bifidobacterium animalis* at the species level, comparing only BB-12 versus control. The finding that probiotics do not exert significant effects on the larger gut microbiota community is consistent with previous literature. Ishizuka et al. demonstrated that the
probiotic *Bifidobacterium animalis* subsp. *lactis* GCL2505, while increasing *B. animalis* subsp. *lactis*, failed to increase other endogenous bifidobacteria(231). Furthermore, Lievin-Le Moel and Servin et al. note that ingestion of probiotic strains of *Lactobacillus* fail to colonize the gut and disappear upon cessation of ingestion of the probiotic(301).

Despite minimal effects on the gut microbiota, probiotics still elicit metabolic improvement in the host. In a study by Poutahidis et al. it was demonstrated that 3 months of $3.5 \times 10^5$ *Lactobacillus reuteri* ATCC 6475 administration in genetically outbred Swiss mice protected against weight gain in response to a Westernized diet without changing the native gut microbiota. The mechanisms cited for eliciting these changes were enhanced anti-inflammatory cytokine IL-10 levels and induction of Foxp3$^+$ regulatory T cells(302). Furthermore, in a human clinical trial conducted by Simon et al. it was observed in a group of glucose-tolerant humans that $2 \times 10^{10}$ CFU/day *L. reuteri* SD5865 significantly improved GLP-1, GLP-2, and OGTT-induced insulin secretion despite no alterations to the gut microbiota(303). As low grade inflammation has been causally implicated in obesity-associated metabolic disease(304), an increase in GLP-2 may represent one mechanism by which probiotics mediate improvement in glycemic control. Being a gut trophic hormone, GLP-2 is purported to mediate improvements in tight junction proteins and intestinal permeability. Decreased paracellular transport limits the amount of bacterial components entering systemic circulation. LPS, a gram-negative cell-wall glycolipid, is a potent activator of TLR4 on innate immune cells. If low levels of LPS are permitted passage into systemic circulation, the chronic low-grade inflammatory state induced is believed to elicit deleterious effects on host glycemic control(122). Thus, by enhancing GLP-2 levels, probiotics and specifically BB-12 may assist in maintaining glycemia in obesity by targeting one of the physiological factors controlling insulin sensitivity.

Although the precise mechanisms by which probiotics mediate improvements in metabolism are not completely understood, it is believed that metabolic effects are species and strain dependent. As described by Cani and Van Hul(305), different strains of *Lactobacillus* and *Bifidobacterium* are not equal in terms of ability to impact body weight, fat mass, glucose metabolism, inflammatory markers, plasma and hepatic lipids, or cholesterol levels(305). As one example, in a study conducted in Sprague Dawley rats assessing the metabolic effects of four
different strains of bifidobacteria, it was demonstrated that only one strain *B. L66-5* was able to induce a significant improvement in body weight(152). The variable effects likely reflect unique putative mechanisms of action for each particular strain of organism. One mechanism believed to mediate probiotic effects on metabolism is enhanced bile salt hydrolase activity, affecting energy, glucose, and lipid metabolism through altered bile acid signalling(306). Other mechanisms include increased production of SCFAs, improvement in gut permeability via stimulation of mucin production, and production of anti-microbial peptides(305). Moreover, many of the strain-specific effects of probiotics are mediated via immunological mechanisms and interactions with both the innate and adaptive immune system(307). Frei et al. suggests that the immunological effects of probiotics are specific to probiotic strain or the metabolites produced(307). Thus, if one strain of probiotic elicits a particular metabolic effect, the findings cannot be extrapolated to another probiotic. Interestingly, while the discussion around strain-specific metabolic effects is focussed on probiotics, this observation is also true of prebiotics. Prebiotics that yield distinct gut microbial profiles and metabolites are likely to elicit specific metabolic effects as well(307).

While we originally hypothesized that some synergistic action would have occurred between oligofructose and BB-12, our results suggest oligofructose and BB-12 were complementary synbiotics as opposed to synergistic synbiotics(170). A synergistic synbiotic entails a “prebiotic substrate synergistically supporting the competitiveness, survival, or metabolic activity of a cognate probiotic strain in the gastrointestinal ecosystem”(170). Producing a truly synergistic effect with the combination of a prebiotic and probiotic may have been achieved via application of a novel technique proposed by Krumbeck et al.(308). The approach involves first identifying a candidate probiotic ‘responder’ organism that undergoes robust enrichment when exposed to a prebiotic. The ‘responder’ organism is then harvested, cultured, and packaged into a probiotic that can be consumed with the prebiotic. Provision of a synbiotic using this novel technique is much more effective at inducing synergistic changes in gut microbiota than previous synbiotic strategies(308). Had our synbiotic study been carried out using this novel *in vivo* selection technique, the results in our study may have been altered in a direction towards a potentiated or synergistic response in our outcome measures.
6.2.2 Oligofructose-mediated effects in adipose tissue

Both of our animal studies confirmed a unique adipose tissue targeting effect of oligofructose (Table 3.2, Figure 4.1B). As discussed in chapter 4, our results suggest that the decrease in body fat with oligofructose was not simply due to a decrease in energy intake. There are currently numerous theories about how prebiotics affect adipose tissue metabolism. The proposed mechanisms include increased production of SCFAs which play an active signalling role in adipose tissue via G-protein coupled receptor-43(110,115,261), reduced endocannabinoid tone(242), and reduced activation of PPARγ(116).

Given the prominent role of G-coupled protein receptors, notably GPR41 and GPR43, for mediating SCFA signalling in the gut(110,113), GPR43 is being actively investigated for its role in adipose tissue metabolism(115,261). Hong et al. demonstrated that incubation of 3T3-L1 cells directly with the SCFAs acetate and propionate increased adipocyte differentiation and GPR43 expression(261). Furthermore, Ge et al. demonstrated using an in vivo and in vitro model that SCFAs decrease lipolysis via a GPR43-driven mechanism(115). Our finding using oligofructose and not SCFAs per se showed that GPR43 expression was down-regulated with oligofructose suggesting that GPR43 signalling may not play a significant role in mediating oligofructose action in adipose tissue. Similar results of no effect or decreased GPR43 expression with other fermentable fibers provides further evidence that the SCFA produced in vivo from prebiotics/fibers is unlikely to impact adipose tissue metabolism via GPR43 signalling(116,262).

Although oligofructose does not appear to affect adipose tissue via GPR43, evidence suggests rather that oligofructose may mediate improvements in adipocyte metabolism through alteration of adipocyte differentiation and oxidative capacity(116,260). In a study by Dewulf et al. it was demonstrated that reductions in adiposity with oligofructose was associated with reduced activation of PPARγ, a prominent adipocyte differentiation factor(116). Furthermore, Alligier et al. and results discussed in chapter 4 suggest that oligofructose may increase oxidative capacity in adipose tissue(260). Although these noted metabolic effects are observed with oligofructose, it remains unclear if SCFAs are responsible for mediating these effects. In support of SCFAs playing some role in increased oxidative capacity, there is evidence to suggest that
SCFAs are capable of activating AMPK in liver and muscle tissue (309,310). Given that AMPK activation increases peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α expression, the master regulator of mitochondrial biogenesis, it is certainly possible that SCFA may play a role in stimulating oxidative capacity.

While SCFAs are a very likely candidate for mediating the benefits of oligofructose in adipose tissue, data from chapter 4 suggests that the benefits of oligofructose are maintained even when bacteria, and likely SCFA production, are substantially knocked-down with antibiotics. However, given that our antibiotic treatment did not eliminate all bacteria, we cannot exclude the possibility that some fermentation and production of SCFA still occurred. Measuring SCFA concentrations in stool and serum, although not possible for our studies, would have been very beneficial to elucidate these mechanisms.

Alternatively, studying the effects of oligofructose in a gnotobiotic or germ free animal would provide another means to ascertain the dependence of oligofructose on gut microbiota for mediating beneficial effects. In a germ free model, in the absence of any fermentation, one would suspect that oligofructose would not elicit any alterations in adiposity. Interestingly, Woting et al. showed that in a germ free mouse model, a high fat diet + oligofructose versus high fat diet alone resulted in significantly lower fat mass, lean mass, and energy intake (256). While this might suggest gut microbiota-independent effects of oligofructose for mediating changes in adiposity and weight, the authors speculate that lower energy intake was potentially due to increased osmotic pressure exerted by oligofructose. As there are no bacteria to break down oligofructose and mucin, there is an accumulation of these products in the cecum leading to an influx of additional water and swelling of the cecum. Given the enlarged cecum, it is possible that the decreased energy intake with oligofructose is secondary to increased satiety (256). As such, repeating the study in germ free animals using a pair feeding model would potentially eliminate this confounding variable. Overall, the germ free model provides a very unique opportunity to gain insight into the specific diet-microbe-host interactions that mediate health and disease. Certainly continued research in a germ free model to assess the mechanisms of prebiotics and the dependence of prebiotics on fermentation to mediate beneficial metabolic
effects is warranted. Such research will provide valuable information that will serve to inform future clinical research.

6.2.3 Satiety hormone response to bacterial manipulation

Two satiety hormones that are consistently increased with oligofructose include GLP-1 and PYY. We were interested in assessing the dependence of oligofructose on the gut microbiota for mediating increases in these satiety hormones. While the secretion of PYY and GLP-1 is influenced by many factors including macronutrients in the diet(311), SCFA production from prebiotic fermentation is also believed to stimulate the release of GLP-1 and PYY(110,113). In keeping with previous literature, we observed that oligofructose mediated increases in both GLP-1(Figure 3.2H, Figure 4.1E) and PYY (Figure 3.2G, Figure 4.1F). Unexpectedly, we also observed a significant effect of both ampicillin and ampicillin/neomycin for enhancing levels of PYY. The increase in PYY with both antibiotic treatments was higher than the increases we observed with oligofructose treatment. While it was a surprise to observe a potent effect of antibiotics in PYY induction, there is a growing body of research demonstrating that antibiotics influence a variety of satiety hormone levels(312-314).

It was demonstrated by Francois et al. that Helicobacter pylori elimination with a 14 day treatment of amoxicillin and clarithromycin contributed to elevated post-prandial ghrelin, leptin, and GLP-1(312). Furthermore, in a study conducted by Rajpal et al. it was observed in a diet-induced obese mouse model that ceftazidime (primarily anti-gram negative bacteria activity) led to significant increases in GLP-1 and PYY whereas vancomycin (mainly anti-gram positive bacteria activity) had no effect(314). Additionally, Wichmann et al. published a paper showing that antibiotic treatment, used to eradicate SCFA production, resulted in significant increases in GLP-1(313). Interestingly, the authors were able to mimic the experimental design using a germ free model. Similar to antibiotic treatment, germ free animals had increased GLP-1 levels. This is seemingly in contrast to the hypothesized role that SCFAs play in stimulating satiety hormone production(110), but the authors speculate that increased GLP-1 levels in a state of low energy availability in the colon (low SCFAs) may reflect an adaptive mechanism to slow gut motility in order to increase energy utilization(313).
While it was our goal to assess gut microbiota-dependent effects of oligofructose for mediating changes in satiety hormones, this collective evidence from studies using antibiotics and germ free animal models suggests that it would be very difficult to tease apart gut microbiota-dependent effects of oligofructose from other factors in the gut that exert effects on satiety hormone production in equal or greater magnitude to that observed with a prebiotic intervention.

6.2.4 Oligofructose-induced gut microbiota modification

Demonstrating the prominent bifidogenic effect of oligofructose, we observed an increase in *Bifidobacterium* in all three studies with oligofructose treatment. While *Lactobacillus* is another organism that is classically increased with oligofructose, it was only in our animal studies which utilized the higher dose of prebiotic that we observed an increase in *Lactobacillus*. Whereas oligofructose is purported to increase representation of the mucin-degrading bacterium *Akkermansia*, we did not observe an increase in *Akkermansia* with oligofructose. We observed consistent findings amongst our studies for reductions in Enterobacteriaceae and certain members of the genera *Clostridium*, although the changes in these bacterial groups proved more prominent in the animal studies. In all three studies we observed consistent decreases in *C.* cluster I and XI, two bacterial clusters known to contain medically relevant pathogenic bacteria(290,291). The observation that oligofructose elicits a decrease in clusters from which potentially pathogenic bacteria can arise is consistent with previous literature(289). It is believed that the lower colonic pH induced through the production of SCFAs from oligofructose fermentation inhibits the growth of these bacteria(310,315).

In recent years there has been significant interest in the bacterium *Akkermansia* given evidence that it protective in obesity(125,316,317). Whereas high fat feeding diminishes *Akkermansia*, oligofructose is reported to increase *Akkermansia* representation(125). We did not observe an increase in *Akkermansia* with oligofructose feeding in our rat antibiotic study or human clinical trial. Rather, while ampicillin led to increases in *Akkermansia*, when ampicillin and oligofructose were combined, oligofructose was associated with a decrease in *Akkermansia* (Table 4.4). Although *Akkermansia* appears to hold promise as another potential beneficial organism in obesity, some research findings are seemingly in contrast to an antiobesogenic role
of this organism. Qin et al. reports in a large metagenomic study that *Akkermansia muciniphila* is enriched in individuals with type 2 diabetes(318). Furthermore, given that *Akkermansia* and *Bifidobacterium* are reported to increase with oligofructose, it would be expected that these two organisms would be positively correlated. However, evidence from Krumbeck et al. demonstrated an inverse correlation between *Akkermansia* and *Bifidobacterium* in response to the bifidogenic prebiotic galacto-oligosaccharide(308). Moreover, similar to our observations, Van de Abbeele et al. reported that inulin does not support increases in *Akkermansia* in the cecum. However, in contrast to our findings (Figure 4.6E), Van de Abbeele et al. demonstrated that inulin stimulates *Akkermansia* growth in the distal colon(289). Altogether, while some research has demonstrated intriguing possibilities of *Akkermansia* for obesity treatment, further research is warranted to confirm the beneficial role of this organism and define prebiotic/probiotic strategies that effectively harness the potential of *Akkermansia* for mediating improvement in health in obesity.

In chapter 4, in addition to completing qPCR for microbiota analysis, we completed 16S rRNA gene sequencing. The added benefit of the 16S sequencing was insight into the diversity of the organisms represented within the gut microbiota of each treatment group. According to the Shannon and Simpson indices of alpha diversity, oligofructose reduced the diversity of the organisms represented within the gut microbiota. Although we generally consider prebiotics to mediate health improvements, decreased gut microbiota diversity is more consistent with ‘dysbiosis’ and detriments to metabolic health(29,33,238). It is possible that a prebiotic, by inducing only specific increases in select gut microbiota species, contributes to an overall lower diversity of organisms. Interestingly, other researchers have identified that the bifidogenic prebiotic galacto-oligosaccharide also lowers measures of alpha diversity(35).

New research suggests that a diet rich in dietary fiber is critical for maintaining a healthy, diverse microbiota(33,34). Comparison of the gut microbiota between Western populations and populations that consume a diet enriched with complex carbohydrates and dietary fiber reveals that a high fiber diet is associated with an increased gut microbiota diversity(35,236,237). Sonnenburg et al. demonstrated in an animal model that reduced diversity is a direct consequence of decreased intake of microbiota-accessible carbohydrates (MACs), which are
abundant in dietary fiber(34). The persistence of a low MAC diet across numerous generations creates a microbiota in which certain organisms become effectively ‘lost’ and are no longer recoverable even with reintroduction of MACs(34). While the precise importance of maintaining a highly diverse gut microbiota is not known, restoration of evolutionarily-linked gut microbiota may be necessary to mitigate pathophysiology associated with a gut microbiota of lower diversity. Certainly, prebiotic and probiotic strategies may play an important role in sustaining a diverse, eubiotic gut microbiota. However, in light of evidence of decreased diversity with singular prebiotics, it may be warranted to expand the prebiotic concept and explore prebiotics/dietary fibers that combine to ensure a diversified, health-promoting gut microbiome.

6.2.5 Improvement in hepatic steatosis with oligofructose in NASH
NAFLD is the most common liver disease in North America and is increasing in prevalence throughout the world. A large proportion of individuals with NAFLD will develop the more severe phenotype referred to as NASH, which is predicted to drive a dramatic increase in the need for liver transplant in future(175,275). Given the major health concerns associated with NASH, effective treatment strategies are critically needed. While our human clinical trial did not demonstrate a significant improvement in overall body fat, 16g/day oligofructose was observed to elicit a beneficial effect on ectopic fat storage, namely a reduction in hepatic steatosis. In contrast to our animal studies, where whole body adiposity was significantly reduced, our human clinical trial did not demonstrate an improvement in overall body fat composition. These disparate findings may relate to the fact that the dose of prebiotic consumed in animal studies (in relative terms) is higher than can be tolerated in human studies. A 10% wt/wt oligofructose diet was used in our animal studies. Humans can tolerate approximately 20g of oligofructose per day beyond which GI symptoms of flatulence and bloating are increasingly reported(298,299). In reference to an 1800kcal/day diet (50% CHO, 20% PRO, 30% Fat) a 20g dose of oligofructose would represent an ~5% wt/wt oligofructose diet. Considering that rats have a proportionally larger cecum than humans(319), it is possible that rats require and tolerate a higher dose of prebiotic to obtain the same relative proportion of fermentation achieved in humans with a smaller dose of prebiotic. However, the greater magnitude of adiposity changes observed in our animal studies compared to our human intervention study may suggest that a 5% wt/wt diet in an
animal model is more consistent with what we observe physiologically in humans. A previous study completed by Parnell et al. showed a 1kg decrease in fat mass with 21g/day oligofructose supplementation in otherwise healthy adults with obesity(98). Similarly, Dewulf et al. showed a trend towards a reduction in fat mass in a prebiotic trial in women with obesity when given 16 g/d of oligofructose and inulin(130).

Interestingly, although oligofructose did not affect overall body fat composition in our clinical study, there was a significant reduction in ectopic fat in the liver. This is potentially explained by the close proximity of the liver to the gut via the portal vein. Although the interaction between oligofructose and microbiota may not have been sufficient to change whole body adiposity, the signals appear to have been sufficient to affect steatosis in the liver. While it is difficult to speculate as to what factors might be mediating these changes, it is possible that bile acid or SCFA signalling may play have played a role.

In recent years there has been significant interest in the role that bile acids play in the regulation of energy, glucose, and lipid metabolism(132,320). The receptors purported to mediate the bile acid signalling effects include the farnesoid X receptor (FXR) and the transmembrane G protein-coupled receptor 5 (TGR5) receptor(321). Research conducted by Ryan et al. demonstrated that vertical sleeve gastrectomy surgery led to improvements in body weight and metabolic health in part through changes in gut microbiota and associated increases in bile acid circulation, resulting in elevated FXR activation(322). Furthermore, obeticholic acid, a first-in-class farnesoid X receptor (FXR) agonist, has been demonstrated to elicit significant improvement in histological parameters of NASH(189). As bile acids are susceptible to modification in the gut, which alters both bile acid reabsorption dynamics as well as the affinity of the bile acid derivatives for FXR and TGR5(321), alteration of bile acids by oligofructose-mediated gut microbiota modification may be one mechanism by which oligofructose decreases hepatic steatosis. As discussed in chapter 4, oligofructose upregulates expression of Cyp7α, a rate limiting enzyme in cholesterol and bile acid synthesis. Increased Cyp7α may reflect decreased FXR activation due to reduced enterohepatic circulation of bile acids, potentially the result of reduced bile acid reabsorption with oligofructose intake. Interestingly, a recent study by Degirolamo et al. demonstrated that the probiotic VSL#3, by increasing bile salt deconjugation
and fecal excretion of bile acids, repressed FXR activation which resulted in increased bile acid neosynthesis(323). Whether a similar mechanism is involved on oligofructose-mediated increases in Cyp7α remains to be determined. As we seek to understand the mechanisms of prebiotic-mediated improvement in metabolic health, future research into the effects of oligofructose on bile acid metabolism and signalling is warranted.

New research also suggests that SCFAs, particularly propionate, may mediate beneficial changes in steatosis in NASH. Chambers et al., using an inulin-propionate ester as a novel SCFA delivery system, investigated the potential for inulin-propionate to improve metabolic health in overweight and obesity. A dose of 10g/day inulin-propionate ester for 24 weeks significantly lowered intrahepatocellular lipid content (assessed with MR spectroscopy) and weight gain, and prevented deterioration of insulin sensitivity(210). While our 16g/day oligofructose diet was unlikely to produce a propionate concentration necessary to achieve the magnitude of the changes observed in the study conducted by Chambers et al. potentially there was some contribution of SCFA towards reduced hepatic steatosis in our clinical trial.

6.3 Strengths and limitations

6.3.1 Animal model
We selected the Sprague Dawley diet-induced obese rat as it effectively models common polygenic, human obesity. Approximately 50% of Sprague Dawley rats develop increased adiposity and metabolic perturbations when fed a high fat diet that are consistent with human obesity, whereas the remaining 50% are obesity resistant(324). Diet-induced obese Sprague Dawley rats have increased expression of neuro-peptide Y in the arcuate nucleus of the hypothalamus demonstrating that the homeostatic regulatory pathway is altered in a manner that is consistent with human obesity(325). Furthermore, in addition to developing dyslipidemia, hypertension, and insulin resistance, Sprague Dawley rats develop hepatic steatosis, which is consistent with the pattern of NAFLD development observed in humans(326). Given that we required a model in which we could elucidate the mechanisms of obesity treatment, we selected
the top 50th percentile of weight gainers as it best mimicked, in an animal model, the pathophysiology of obesity that we were targeting with our interventions.

One potential limiting factor with our animal studies as it relates to treating adult human obesity is that animals used in our study had not necessarily reached the ‘adult’ stage of development. Using the general guidelines for assigning human years to rat age(327), the animals in our study were nearing the completion of the growth stage of life (~16-18yrs in human years). As such, all animals continued to gain weight throughout the course of the intervention phase of the study. Therefore, treatments that affected weight did not necessarily decrease body weight, rather treatment decreased the rate at which animals accrued adipose tissue mass and body weight, thereby allowing them to achieve a significantly lower final body weight compared to control. Although the age of the rats used in our study was more consistent with adolescence than the adult stage of life, this is not likely to have a significant effect on the generalizability of the results to human adult obesity. For one reason, the gut microbiota transitions into an adult-like microbiome by the age of 3(328). Additionally, evidence from both adult and pediatric studies assessing the effects of inulin-type fructans demonstrate similar physiological effects in both adults and children(329). Thus, the relative age difference between the animals used and the adult obesity population is not likely to impact the generalizability of the findings.

6.3.2 Clinical participants

The pilot clinical trial we completed was the first study to assess the effect of a prebiotic alone for treatment of liver biopsy confirmed NASH. Given that liver biopsies are currently the gold standard for assessing NASH, a major strength of this study was the inclusion of baseline and post-intervention liver biopsy. Part of the goal of the pilot trial was to assess feasibility related to study design and outcome measures which we in fact determined was highly dependent on factors associated with the biopsy. Specifically, recruitment was dependent on confirmation of biopsy-confirmed NASH but many individuals were apprehensive about undergoing a follow-up liver biopsy and declined participation in the study based on this requirement. A liver biopsy carries some risk and is limited by its lack of representation of the liver as a whole(330),
representing only about 1/50,000th of the liver. Considering that histopathological parameters of NASH are not necessarily homogenous throughout the liver, with such a small representation of the liver, misdiagnoses and staging inaccuracies in NASH are possible(300). Given these limitations, there has been a move away from invasive tests to diagnose NASH (i.e. liver biopsy) and many clinicians are now using a combination of less invasive tests, including blood biomarkers, vibration-controlled transient elastography (FibroScan®), and magnetic resonance imaging (MRI) and associated magnetic resonance elastrography (MRE) to assess hepatic steatosis and fibrosis in patients(175,331). Research has demonstrated that the FibroScan® has a high sensitivity for diagnosis of advanced liver fibrosis(175,332,333). Although costly and not routinely accessible, the MRI can detect fat in microscopic quantities and measure hepatic steatosis greater than 5.56% with very high accuracy. Moreover, MRE can predict level of fibrosis with a high degree of accuracy(175,334). Despite the advancements in non-invasive strategies to diagnose NASH, the liver biopsy remains the only way to definitively distinguish NAFLD from NASH. However, considering the invasive nature of liver biopsies and the concerns it poses for patient recruitment, utilization of a combination of these non-invasive diagnostic techniques is an important consideration for future large-scale clinical trials.

6.3.3 Diet interventions
For the animal interventions we used a 10% wt/wt oligofructose diet. Experimental diets were formulated based on the recommendations set forth by the American Institute of Nutrition and used a base AIN-93 purified diet to adjust the fat and sugar content for the high energy diet and/or supplement with oligofructose(335). Providing a purified diet permits comparison of our results with other studies that have used the same diet formulation(336). The 10% wt/wt is commonly utilized in animal studies(72,125,337). One issue that arises with the selected diet is the slight energy dilution that results when the diet is formulated with oligofructose. Given that animals receiving an oligofructose diet have reduced energy intake, the question arises as to whether the effects observed with oligofructose are simply due to an energy dilution effect. Evidence, however, suggests that decreased energy intake is, in part, due to unique satiety promoting properties of oligofructose. Other fiber sources, such as cellulose, while also diluting
the energy in the diet, do not contribute to a reduction in the energy intake as animals increase food intake to compensate for the reduced energy content in the diet\(^{(229)}\). Considering that we observed significant increases in GLP-1 and PYY in association with oligofructose intake, it is likely that increased satiety contributed to the reduced energy intake. Furthermore, evidence from chapter 4 demonstrated that oligofructose reduced adiposity to a greater extent than groups that received ampicillin, despite ampicillin reducing energy intake to the same degree as oligofructose.

The intervention utilized in the pilot clinical trial was initiated slowly with an 8g/day oligofructose supplement for the first 12 weeks and 16g/day oligofructose for the remaining 24 weeks. As discussed in chapter 5, 16g of oligofructose represents a dose that is tolerated and acceptable. Everyone in our study was able to adhere to the prescribed dose of prebiotic, with only a few individuals indicating increased flatulence and abdominal discomfort. Interestingly, there is no research to date exploring tolerance of inulin-type fructans in people with obesity. Given that some individuals experience no adverse side effects of oligofructose, potentially a larger dose of prebiotic would provide additional benefit to those who could tolerate a larger dose. As a possibility for future studies, study participants could be provided with doses of prebiotics that increase slowly in a step-wise manner until an appropriate tolerable limit is reached. Alternatively, provision of prebiotics on a per kg body weight basis may have a similar effect. Providing prebiotics in this way may serve to maximize the metabolic benefit achieved by each person.

### 6.3.4 Gut microbiota analysis

Characterization of the gut microbiota was completed via qPCR for all three studies and via 16S rRNA amplicon Illumina sequencing for the antibiotic study discussed in chapter 4. Although next generation sequencing is now the gold standard for gut microbiota taxonomic analysis to the genera level\(^{(338)}\), qPCR and 16S amplicon sequencing are complementary. qPCR permits quantitative measurement of specific microbial groups from the phyla to the species level whereas 16S sequencing offers the advantage of characterizing the entire gut microbiota community, permitting discovery of unknown bacteria that may be affected by a dietary
intervention and assessment of the overall diversity of the gut microbiota (338). While 16S sequencing can only measure proportional representation, qPCR offers the advantage of being able to quantify the total number of bacteria present in the microbiota samples. In our antibiotic study, this was particularly relevant as it allowed us to quantify the relative effects of ampicillin versus ampicillin/neomycin on total bacteria numbers. Another advantage of 16S rRNA sequencing is that it now permits researchers to predict functional composition of the metagenome using the newly developed bioinformatics software, phylogenetic investigation of communities by reconstruction of unobserved states (PiCRUSSt) (339). PiCRUSSt, by assigning specific gene profiles to 16S marker gene sequences, provides an accurate assessment of gut microbiota functional capacity. However, due to high computational demands associated with this type of analysis, we were not able to complete this analysis in our studies.

One potential limitation associated with our gut microbiota analysis stems from the fact that all of the fecal matter samples that we collected were frozen prior to analysis. Although the evidence is not clear, there is some indication that freezing the samples prior to analysis may artificially elevate the Firmicutes/Bacteroidetes ratio (340). However, it has also been demonstrated, using 16S rRNA Illumina sequencing analysis, that freezing does not affect phylum or family differences, although freezing may alter representation of the genera Faecalibacterium and Leuconostoc (341). Moreover, freezing does not appear to affect the diversity of the gut microbiota. Regardless of whether sample composition is affected by freezing, all samples in our study were subject to the same freezing protocol, so any changes would have likely altered all the samples uniformly.

6.3.5 Glycemic and satiety hormone testing

Blood collected during the oral glucose tolerance tests was utilized for both glycemic and satiety hormone analysis. While measuring the satiety hormones provides valuable insight into how hormone-regulating homeostatic appetite control might be affected by treatment, the repeated blood collection over the course of the OGTT may have adversely impacted the glycemic response for the animals, particularly in the antibiotic study. Whereas glucose levels are expected to rise initially and diminish over the course of the OGTT, we observed that blood
glucose levels initially increased and plateaued in all treatment groups. As one explanation, we hypothesize that the repeated blood draws may have contributed to an elevated stress response that persisted over the 2hr OGTT. Reducing the stress associated with the initial gavage and serial blood collections may permit a more accurate assessment of dietary treatment effects on glycemic homeostasis. Future studies may consider removing the serial blood draws at time 15, 30, 60, 90, and 120 minutes. This would still permit measurement of baseline satiety hormones but would potentially mitigate stress-induced hyperglycemia over the course of the 2hr OGTT.

One disadvantage of the human clinical trial was the exclusion of satiety hormone analysis. Given that the participants in our study had blood drawn at any of the numerous Calgary Laboratory Services locations throughout Calgary for the sake of participant convenience, we were unable to include the specific inhibitors required for satiety hormone analysis in our collection protocol. Research suggests that GLP-1 secretion is reduced in individuals with NAFLD(342). With increased satiety hormone production often being cited as a mechanism by which prebiotics mediate metabolic and anthropometric changes(97,98), it would be worthwhile to investigate whether GLP-1 enhancement mediates beneficial effects of oligofructose in NASH. Interestingly, a probiotic clinical trial conducted by Alisi et al. showed that VSL#3-mediated improvement in BMI and NASH in children may be due to increased GLP-1(215). With the majority of prebiotic research in humans assessing satiety hormones, the analysis of GLP-1 and PYY would have allowed us to contrast our findings with other literature. Future prebiotic human clinical trials in NASH would benefit from the analysis of these important satiety hormones.

### 6.4 Future directions and perspective

Individuals with NASH have increased risk of mortality from liver disease as well as an increased risk of mortality from cardiovascular disease and malignancy(175). Treatment strategies that assist individuals with maintenance of long term weight loss and mitigating risks associated with NASH are urgently required. The most recent 2012 Practice Guidelines according to American Association for the Study of Liver Diseases recommend that treatment of NAFLD focus on comorbidities of NAFLD including obesity, hyperlipidemia, insulin resistance,
and hypertension(175,343). Upon progression to NASH, more aggressive treatment of the liver disease is recommended. Given strong evidence for histological improvement of NASH with weight loss(193,343), first line NASH treatment is focused on weight loss through lifestyle intervention. Unfortunately, for reasons described in this thesis, weight loss accomplished through lifestyle intervention is difficult to maintain(194). Apart from weight loss, other treatment options include 800 IU/day vitamin E (α-tocopherol), pioglitazone, and obeticholic acid; however, effects sizes with each of these treatments is modest and safety concerns have been raised with each of these treatment options(175,343). Furthermore, none of these treatments have received approval from US Food and Drug Administration(175). With few effective treatment strategies available for individuals with NASH, new strategies that attenuate the pathogenesis of NASH are needed.

The research findings from our pilot clinical trial highlight the potential of the prebiotic oligofructose to improve histopathological markers of NASH. With these promising results, a large scale, adequately powered, randomized control trial is warranted to assess whether the observations from our pilot trial hold true in a clinical population with NASH. If demonstrated that prebiotics are beneficial in a large scale prebiotic trial, this evidence would provide physicians with specific nutritional, evidence-based dietary guidelines to offer patients with NASH. Even if there were only relatively small benefits observed with prebiotic treatment, given the low risk profile associated with prebiotic consumption, prebiotics would potentially complement other treatment strategies that are in development. Moreover, given that all patients are recommended to consume 25g fiber per day for an adult female and 38g/day for an adult male(344), prebiotic supplementation would assist individuals in meeting the Adequate Intake dietary fiber guidelines. As increased dietary fiber intake is associated with increased maintenance of weight loss(345) and prebiotics contribute to reduced postprandial glucose and insulin levels(99), incorporation of prebiotics into clinical guidelines may be warranted given that NAFLD and NASH treatment is targeted at weight loss and controlling NAFLD comorbidities.

Although the definition of prebiotic has evolved since prebiotics were originally defined in the mid 1990s, the current definition of prebiotic proposed by Gibson et al. in 2010 is
“selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health(36)”. The two primary dietary agents that are assigned prebiotic status include fructo-oligosaccharides and galacto-oligosaccharides, principally because they are ‘selectively’ fermented and result in ‘specific’ increases in Bifidobacterium and Lactobacillus(38). At present, more broadly categorized dietary ingredients such as dietary fiber, resistant starch, and pectins, although interacting with the gut microbiota and producing metabolic by-products that are beneficial for metabolic health, are not considered prebiotics by the current definition. However, with the emergence of new high throughput, next-generation sequencing technologies, we now understand from community-wide gut microbiota analysis that a diverse gut microbiota, stimulated by the provision of a high fiber diet is important for maintaining health(34). Thus, a high fiber diet is, in essence, a prebiotic diet. However, the current definition of prebiotic does not permit this distinction. Given the limitations of the current prebiotic definition, Bindels et al. has proposed a new prebiotic definition. The suggested definition is “a prebiotic is a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host(346).” Such a definition removes the current restriction of dietary agents being “selective” and “specific” to be classified as a prebiotic. Interestingly, in 2008 the Food and Agriculture Organization of the United Nations (FAO) also proposed a definition of prebiotic that removed the ‘selectivity’ criterion, defining a prebiotic as a “non-viable food component that confers a health benefit on the host associated with modulation of gut microbiota”(346,347). The FAO prebiotic definition, however, has not received widespread acceptance. Ashighlighted by Bindels et al., this may be due to the failure of the FAO definition to differentiate between substances that alter the gut microbiota through inhibitory mechanisms alone(346). Furthermore, the FAO definition states that a prebiotic only needs to be associated with a modulation of the gut microbiota, whereas other definitions imply that gut microbiota manipulation is a causal factor in mediating metabolic benefits in the host. Despite the differences between the Bindels et al. and FAO prebiotic definitions, the consensus for the removal of the ‘specificity’ criterion paves the way for an expansion of the prebiotic concept. As
researchers gain an increased understanding of how different dietary agents affect gut microbiota and how communities of microbes affect host health, these new definitions permit for novel prebiotic strategies that can take advantage of the vast array of metabolic potential available in the gut microbiome. Novel prebiotic strategies could include combinations of dietary prebiotic agents that collectively stimulate a diverse gut microbiota which has an enhanced ability to facilitate intestinal homeostasis and improved metabolic health.

A growing body of research now attributes the success of bariatric surgery to the appetite-reducing metabolic alterations that it induces(348). Thus, rather than being solely a malabsorptive or restrictive procedure, bariatric surgery is regarded by some as a metabolic surgery(348,349). This has prompted the question as to whether the beneficial effects of bariatric surgery can be induced through alternative metabolic manipulation without having to subject individuals to an invasive, risky surgery(25). As bariatric surgery affects multiple physiological variables that impact the very complex neurohormonal homeostatic and hedonic appetite regulatory pathways, a multi-metabolic intervention approach would also be required to mimic the effects of bariatric surgery. Although many centrally targeted pharmaceutical interventions must be avoided due to harmful psychological and cardiovascular side effects(12,24), present day pharmaceutical (e.g. liraglutide, orlistat) and dietary obesity treatment strategies affect the same pathways that are impacted in bariatric surgery(350), however the magnitude of change is small in comparison to bariatric surgery. Prebiotics, for instance, while impacting homeostatic appetite regulation, are not sufficient to induce clinically significant weight loss by themselves. While the impact of some obesity interventions may be underwhelming as an individual treatment, potentially the combination of multiple intervention strategies, each impacting one specific variable involved in the complex pathophysiology of obesity, could yield clinically relevant weight loss and improvement in metabolic health in similar magnitude to that of bariatric surgery. Currently one intervention strategy that is being investigated in obesity treatment is fecal microbiota transplantation. Already proven to be highly effective for treatment of recurrent Clostridium difficile infections(351), fecal microbiota transplantation may offer a viable means to correct gut microbiota dysbiosis in obesity. An initial 6 week clinical trial completed by Vrieze et al. in adult males with metabolic syndrome demonstrated the potential
for this novel treatment strategy to improve insulin sensitivity(73). Building on this, there are currently numerous clinical trials underway assessing the intriguing potential of this treatment strategy in obesity and NAFLD (https://clinicaltrials.gov/). As a complement to fecal microbiota transplantation, prebiotics and probiotics could be utilized as type of ‘fertilizer’ for newly transplanted microbiota to ensure that eubiosis is maintained. Creating a ‘metabolic’ strategy by combining complimentary obesity treatments offers a very intriguing possibility for future obesity treatment, one that would be cost effective, have a lower risk profile, and be much more accessible for individuals with obesity compared to limited treatment options that are currently available.

Diet and exercise are complimentary in regards to weight control and maintenance of metabolic health. Although exercise interventions themselves have proven to be minimally effective for weight loss, the US National Weight Control registry, a large prospective investigation into the factors that are involved in successful weight loss, indicates that maintenance of weight loss is achieved by most individuals through a reduction in energy intake as well as consistent exercise(352). While the vast majority of research has focussed on the effects of diet-gut microbiota interactions, there is now some research demonstrating that exercise and physical activity affect gut microbiota in a manner that appears to increase gut microbiota diversity(353). Interestingly, there is also evidence suggesting that exercise, while credited as being a factor that increases energy output, also impacts appetite control(354). Demonstrating that acute physical activity influences homeostatic appetite regulation, a study completed by Deighton et al. showed that an exercise-induced energy deficit was associated with decreased subjective appetite and increased PYY concentrations relative to a food restriction-induced energy deficit(355). Furthermore, research from McNeil et al. demonstrated that resistance exercise decreases measures of the hedonic drive for high fat foods(356). Collectively, this evidence shows that exercise impacts some of the same physiological parameters in appetite regulation that are influenced by prebiotics and probiotics. The ability of prebiotic strategies and exercise to impact the same appetite-regulating pathways could help to explain the complementary nature of these weight loss strategies. Future research to assess the mechanisms by which exercise impacts these pathways is an exciting area of future research and could shed
light on new intervention strategies that work alongside diet to facilitate improved and sustained weight loss for people with obesity.

6.5 Conclusion and significance
Overall, the basic and clinical research presented in this thesis provides evidence for the benefit of the prebiotic oligofructose and to some extent probiotics for improvement of metabolic health in obesity. The results from this research provide useful information about nutritional strategies to support improved health, particularly in regards to NAFLD. Furthermore, the results will serve to inform future research efforts that seek to understand the complex interactions between gut microbiota and host and research that aims to develop increasingly effective microbiota-based obesity treatments. There remains a significant portion of the metabolic potential contained within the microbiome that has yet to be discovered or harnessed. As the science and technology advances within the microbiota field and new discoveries are made into the unique function of individual bacteria and the interactions between microbe, diet, and host, the opportunities for the development of novel prebiotic and probiotic strategies for treatment of obesity and other gut-microbiota based diseases are abundant. Although the ultimate cure for obesity will likely require a multifactorial approach given the complex nature of the disease, the optimization of gut microbiota for improvement of energy balance and metabolic health using minimally-invasive prebiotic and probiotic strategies offers great potential for assisting individuals with this pervasive disease.
References


(54) O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep 2006 Jul;7(7):688-693.


(121) Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine 2008 May;42(2):145-151.


(172) Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. Gastroenterology 2014 Mar;146(3):726-735.


(175) Rinella ME. Nonalcoholic Fatty Liver Disease. JAMA 2015;313(22):2263.


(178) Khan RS, Newsome PN. Non-alcoholic fatty liver disease and liver transplantation. Metabolism 2016 Mar 3.


(221) Lesniewska V, Rowland I, Cani PD, Neyrinck AM, Delzenne NM, Naughton PJ. Effect on components of the intestinal microflora and plasma neuropeptide levels of feeding Lactobacillus delbrueckii, Bifidobacterium lactis, and inulin to adult and elderly rats. Appl Environ Microbiol 2006 Oct;72(10):6533-6538.


(316) Schneeberger M, Everard A, Gomez-Valades AG, Matamoros S, Ramirez S, Delzenne NM, et al. Akkermansia muciniphila inversely correlates with the onset of inflammation, altered


(321) Yuan L, Bambha K. Bile acid receptors and nonalcoholic fatty liver disease. World J Hepatol 2015 Dec 8;7(28):2811-2818.


(334) Singh D, Das CJ, Baruah MP. Imaging of non alcoholic fatty liver disease: A road less travelled. Indian J Endocrinol Metab 2013 Nov;17(6):990-995.


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Kevin

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> > Thank you,
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> > Marc
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