Prebiotic Dietary Fibers Must Achieve a Threshold of Beneficial Gut Bacteria in Order to Prevent Adiposity and Fatty Liver in Rats Fed High Fat Diets.

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Dedication

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Abstract

Introduction

Prebiotic dietary fibers are dietary fibers that are highly fermented in the large intestine, produce beneficial changes in the gut microbiome, and impart a health benefit to the host. Using reactive extrusion, we have synthesized a novel dietary fiber that is an oligosaccharide of polymerized lactose, which we term polylactose. Here we report on two studies feeding polylactose to rats to determine its prebiotic potential.

Methods

In Exp. 1, the polylactose preparation contained 51% dietary fiber, 20% free lactose, 5% glucose, and 24% other materials. Rats were fed high fat diets containing 9% total dietary fiber, including cellulose (C, 9%), polylactose (PL, 6%), polydextrose (PD, 6%), and fructooligosaccharide (FOS, 6%). In Exp. 2, the polylactose preparation contained 75% dietary fiber, 9% lactose, 3% glucose, and 13% other materials. Rats were again fed high fat diets containing 9% total dietary fiber, including C (9%), polylactose (6% or 3%), PD (6%), and galactooligosaccharides (GOS, 6%). In both experiments, rats were fed for 10 weeks, then ceca (empty), cecal contents, livers, and epididymal fat pads were collected.

Results

In both experiments, final body weight and daily energy intake did not differ among the groups. In Exp. 1, feeding PL greatly increased cecum weight (an indicator of fermentation), cecal *Bifidobacterium* and *Lactobacillus* species abundance, increased cecal acetate and propionate, and reduced liver lipids and fat pad weight, compared to the HFC group. PD and FOS increased probiotic species and short chain fatty acids slightly

(compared to HFC), but not to the same extent as PL, and neither PD or FOS reduced fatty liver or adiposity. In Exp. 2, 6% PL increased cecum weight relative to 3% PL, PD and GOS, all of which were greater than HFC. The cecal microbiome was similar among PL (both 3 and 6%), PD, and GOS, all of which differed from HFC. Liver lipids, fat pad weight, and body composition did not differ among any of the groups in Exp. 2.

Conclusions

The prebiotic activity of polylactose differed depending on the preparation, for unknown reasons. However, our results suggest there is a threshold of probiotic bacteria abundance that must be attained before beneficial effects are imparted on the host by prebiotics.

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Chapter 1: Introduction

Obesity and its various comorbidities continue to be major public health issues on a global scale. According to the World Health Organization's 2018 Obesity and Overweight Report, it is estimated that obesity occurs in 39% of the global population [1]. The inflammation and insulin resistance that accompany increased adiposity can lead to many health issues, such as type 2 diabetes (T2D), cardiovascular disease, and non-alcoholic fatty liver disease (NAFLD). The incidence of NAFLD has been estimated to occurring in 25% of adults worldwide and 75-90% of adults who are overweight or obese [2, 3]. At the time, only a few effective treatments for NAFLD exist, those of which include loss of 10% of an individual's body weight [2, 3]. However, recent studies indicate an association between the abundance of specific large intestinal bacteria, obesity, and NAFLD. As diet is a major contributor to variation in the gut microbiome, dietary intervention is one potential method to predictably modify the gut microbiome in a beneficial way [4]. This raises the possibility that consumption of prebiotics, indigestible but fermentable dietary compounds that alter the gut microbiome in a beneficial way, may reduce NAFLD. Understanding which microbial profiles in the gut are beneficial for reduction of NAFLD, how those microbes communicate with the liver and other host tissues, and how to effectively promote those taxa is important for establishing long-term, novel treatments and preventive agents.

In the next chapter, this thesis will review important aspects of NAFLD and obesity, the role of the gut microbiome in health and disease, how the gut microbiome can be manipulated through diet, and evidence for prebiotic-gut microbiome-disease interactions. Chapter 3 reports on an animal study investigating the prebiotic potential of polylactose, a novel dietary fiber. Here, polylactose was investigated for fermentability, its ability to alter the gut microbiome in a beneficial way, and its effect on reducing obesity, fatty liver, and the development diabetes in rats fed high fat diets. Chapter 4 details a second animal study feeding polylactose and describes the effects of a different preparation of polylactose as well as a second, lower dose of the preparation. In chapter 5, a comparative analysis of the two studies is described, providing insights into the similarities, differences, and possible explanations for the divergence of results. This chapter includes suggestions for future directions into better understanding how polylactose acts a prebiotic dietary fiber. Lastly, chapter 6 summarizes the two studies and the potential impact of polylactose.

Chapter 2: Literature Review

Obesity & Non-Alcoholic Fatty Liver Disease

Overview & prevalence

Obesity continues to be a major public health issue worldwide. According to the World Health Organization's 2018 Obesity and Overweight Report, it is estimated that obesity occurs in 39% of the global population [1]. The inflammation and insulin resistance that accompany increased adiposity can lead to many comorbidities, such as type 2 diabetes (T2D), cardiovascular disease, and non-alcoholic fatty liver disease (NAFLD). Of particular interest, the incidence of NAFLD has been estimated at 75-90% of adults who are overweight or obese, occurring in 25% of adults worldwide [2, 3]. NAFLD, a largely asymptomatic disease, is defined as the accumulation of excess lipids in the liver [2, 3, 5]. Simple fatty liver (non-alcoholic fatty liver, NAFL) is the most common form of NAFLD and occurs with little to no inflammation or scarring. Non-alcoholic steatohepatitis (NASH) is the more severe form of NAFLD, and occurs with irreversible scarring, or cirrhosis, and fibrosis [2, 3, 5]. Continued liver lipid accumulation, inflammation, cirrhosis, and fibrosis in NASH lead to chronic liver disease, and, eventually, liver failure. At this time, there is no effective pharmacological treatment for NAFLD despite a large number of ongoing drug trials [2, 3, 5]. However, certain lifestyle interventions have been demonstrated to reduce liver lipid accumulation and prevent disease progression in human and animal models [2, 3, 5]. The overall approach of these interventions is weight loss, whether by improved diet and exercise, or by the more invasive approach of bariatric surgery [2, 3, 5]. In order to improve NAFLD by weight loss, it is recommended that an individual loses 10% of their body weight [6].

Given the difficulty and sustainability of weight loss for most individuals, an effective dietary agent to facilitate weight loss would be highly desirable. Understanding how NAFLD develops is important for understanding how to better treat or prevent NAFLD [7, 8]. As previously mentioned, NAFLD is common in individuals with high adiposity and T2D, therefore, understanding approaches to decreasing adiposity and its comorbidities, such as NAFLD, are key [7, 8].

Pathogenesis of NAFLD

The pathological characterization of NAFLD remains incomplete, but the "two-hit" and "multiple-hit" hypotheses are generally accepted [9-11]. The "two-hit" hypothesis states that lipid accumulation in the liver, due to high fat diet, sedentary lifestyle, and metabolic diseases, acts as a "first hit." This increases sensitivity of the liver, allowing for inflammation and other damage, the “second hit”, to initiate a cascade that leads to fibrosis [9-11]. This model was widely accepted until recently, when a more robust, "multiple-hit" hypothesis gained traction [10].

The "multiple-hit" hypothesis includes dietary patterns, obesity, adipocyte biology, insulin resistance and associated factors, and the gut microbiome [10]. Insulin resistance plays a key role in the accumulation of lipids in the liver. With insulin resistance, *de novo* hepatic lipogenesis increases, lipolysis in the adipose is inhibited, and adipokine secretion is increased [9, 10, 12]. Further, certain gut bacteria can result in disruption of large intestinal tight junctions, allowing uptake of lipopolysaccharide (LPS), which can lead to metabolic endotoxemia and increase liver inflammation [6]. These factors of increased liver lipid accumulation and inflammation, alongside genetic and environmental predispositions, lead to NAFLD and NASH in a "multi-hit" fashion.

Molecular mechanisms of NAFLD and obesity

It is clear that obesity, hypertriglyceridemia, insulin resistance, and T2D are associated with the development of NAFLD [7, 8]. A number of factors can result in the accumulation of lipids in the liver, including increased uptake of fatty acids from dietary fat and lipolysis of adipose tissue, increased *de novo* hepatic lipid synthesis, and a reduction in lipid oxidation and mobilization [5, 13, 14]. Many of these factors are dysregulated in obesity and hyperinsulinemia. During periods of hyperinsulinemia, many tissues, including the liver, become insulin resistant. With insulin resistance, hepatic lipid accumulation begins. This is largely attributable to the upregulation of lipogenic transcription factors, such as sterol regulatory element binding protein-1c (SREBP1c) [5, 13, 14], which results in an increased rate of lipid synthesis in the liver. Further, there is a reduction in lipid oxidation and mobilization. As the liver continues to accumulate lipids, low-grade inflammation develops that can eventually lead to NASH, fibrosis, and cirrhosis.

Due to the clear relationship between obesity and NAFLD, it is important to understand how increased adiposity contributes to NAFLD development. As adipose tissue accumulates lipid, macrophages infiltrate the tissue, leading to the increased release of pro-inflammatory cytokines such as leptin, resistin, tumor necrosis factor-α (TNFα), and monocyte chemotactic protein-1 (MCP-1) [15-19]. These activate NF-κB and other inflammatory mediators, resulting in systemic inflammation. Elevated concentrations of these cytokines appear to be driving forces that promote hepatic lipid accumulation and inflammation and are likely the primary connection between increased adiposity and NAFLD [5, 13, 14]. Additionally, the anti-inflammatory adipokine adiponectin is reduced in high adiposity states [20, 21]. Adiponectin activates AMP-activated protein kinase (AMPK), which promotes fatty acid oxidation and prevents lipid synthesis [20, 21]. A reduction in circulating adiponectin leads to reduced level of activated AMPK, thereby reducing fatty acid oxidation, allowing for further accumulation of lipids in the adipose and liver.

Gut Microbiome

Overview of the gut microbiome

The gut microbiome is defined as a population of microorganisms that resides within the gut of a host, predominately the large intestine. In the human gut, thousands of species make up tens of trillions of cells that are collectively considered the gut microbiome [22, 23]. This dynamic pseudo-organ is influenced by host genetics, dietary patterns, physical activity, environment, and other lifestyle factors [22]. Certain microbial profiles have also been correlated with health and disease, including obesity [24-35], cancer risk , and non-alcoholic fatty liver disease (NAFLD) [17, 36-41]. Understanding which microbial landscapes in the gut are beneficial for reduction of certain disease states, how those taxa communicate with the liver and other host tissues, and how to effectively promote those taxa is important for establishing appropriate recommendations to achieve specific health outcomes.

Dietary influence on the gut microbiome

A key contributor for shifts in microbiome composition in the gut is diet [4]. Studies have shown distinct changes in an individual's microbiome after dietary intervention, such as converting from plant- to animal-based diets and vice versa [42]. While studies observe similar changes between individuals of similar dietary patterns, their microbiomes remain distinct, indicating high interpersonal variation [43]. Rather, it is estimated that diet accounts for only 5-8% of the variation in gut microbiomes, despite being a major extrinsic modulator [4]. In a study by Johnson et al., 34 individuals collected stool samples and provided dietary records each day for 17 consecutive days.

They found that while microbiomes were highly personalized and did not tightly correlate with any single food group or nutrient, a model of exponential decay of dietary history leading up to a stool sample was the most predictive of that day's microbiome [4]. In this model, for each day leading up to a stool sample, the dietary data is weighted exponentially. Thus, the dietary data from 24 h before a stool sample would have the largest contribution, and each day prior has exponentially less. This predictive model demonstrates that even though the most consistent dietary patterns won't fully override the interpersonal variation of the microbial profile, the gut microbiome is incredibly dynamic and responds to changes in diet rapidly. Further, when picking out specific taxa, there are clear correlations with consumption of specific foods and nutrients. For example, Johnson et al. found that multiple individuals had inverse correlations between meat-based foods and fecal *Bacteroidaceae* [4]. This finding aligns with other studies that have identified specific taxa that are correlated with different types of diets [42, 43].

In studies of animal- vs plant-based diets, David and Wu have found that certain taxa are more responsive to dietary changes than others [42, 43]. For example, they found that *Prevotella* is among those dietary-responsive taxa [42]. David et al. found dietary fiber consumption for the year leading to baseline microbiome measures was predictive of *Prevotella* abundance in the gut microbiomes of the individuals [42]. *Prevotella* was reduced in response to dietary intervention with an animal-based diet, particularly in the long-term vegetarian individual. Furthermore, the animal-based diet, which was enriched for fat and protein but deficient in fiber, resulted in more robust changes in the gut microbiomes of the ten individuals when compared to the changes seen in the plant-based diet arm [42]. In addition to rapid reductions in *Prevotella* and increases in *Bacteroides,* the animal-based diet altered in the gut microbiome as soon as 24 h after switching from the individuals' normal, baseline diet [42]. This indicates that the gut microbiome can change rapidly in response to a dramatic dietary change. The authors also noted that the individual's microbiomes returned to baseline within two days of converting back to their habitual diets. This indicates strong dietary influence in addition to interpersonal variation.

In two experiments by Wu et al., dietary data and grouping was associated with some changes in the microbiome [43]. In a longitudinal feeding study, 10 participants were fed high fat/low fiber or low fat/high fiber diets while providing stool samples for 10 days were followed. They found that interpersonal variation was the biggest contributor to variation within the microbial profiles, as subjects clustered with themselves by Unweighted Unifrac, a measure of dissimilarity and beta-diversity [43]. They also found that even in a time of dietary stability, the individual microbiomes remained dynamic, similar to the findings of Johnson et al. [4, 43]. Further, instead of seeing similar patterns of microbial change between individuals in the same dietary group, they saw similarities in functional characteristics of the gut microbiomes that correlate to diet [43]. This indicates that shifts in the microbiome in response to diet is an adaptation of processing these constituents and less related to specific bacterial changes. Overall, this provides evidence that while interpersonal variability is a major contributor to microbiome profiles and stability, certain foods or nutrients also correlate to changes in specific taxa. These specific taxa can play a role in mediating host health benefits, therefore, in understanding specific correlations and how to manipulate the gut microbiome.

In another study from Wu et al., 98 individuals provided stool samples alongside short- and long-term dietary data (dietary recalls and food frequency questionnaires, respectively) [43]. Here, they found that certain dietary habits led to formation of microbiome enterotypes. One enterotype was driven by *Bacteroides*, which was associated with animal-based diets including protein, amino acids, and saturated fats [43]. Contrastingly, the other enterotype was dominated by *Prevotella*, which was associated with increased consumption of plant materials, including carbohydrates and simple sugars. This is similar to the findings of David et al., where the abundance of *Prevotella* was reduced during the animal-based diet study arm [42]. Notably, vegetarians and one self-proclaimed vegan all clustered into this *Prevotella* enterotype [43]. This indicates that despite large amounts of interpersonal variation, broad categories can be determined and associated with dietary patterns.

Related findings from O'Keefe et al. further indicate that the gut microbiome can be easily manipulated by dietary changes [44]. Past research has shown that native Africans and African Americans not only have distinctly different dietary patterns, but also have different gut microbiomes and colon cancer risk [44]. The typical diet of native Africans is high in dietary fiber and plant foods, whereas the Western diet consumed by African Americans is high in fat and protein while low in dietary fiber [44]. This results in a "Westernized" microbiome compared to the native "Africanized" microbiome. The "Westernized" microbiome, similar to the previously discussed animal-based microbiome profiles, are characterized by increased abundance of *Bacteroides* and reduced abundance of *Prevotella* [42-45]. The opposite is true for the "Africanized" microbiome, similar to what is seen on other plant-based diets [42-45]. O'Keefe et al. demonstrated that when native Africans are put on a Western diet, their microbiomes rapidly become Westernized, and vice versa [44]. They found that Westernized microbiomes have substantially less butyrogenic bacteria and less fecal butyrate [44]. Further, they saw an increase in colonic inflammation, as determined by colonocyte proliferation rates [44]. This, along with other biomarkers of colon cancer risk, indicate that the change in the gut microbiome but a dietary "swap" presents a change in disease risk, suggesting that the microbiome can be manipulated in order to modulate disease risk [44]. Increased understanding of other dietary interventions that can be used to predictably and sustainably alter the gut microbiome for reduction of disease risk is warranted.

The role of the gut microbiome in host health

While interpersonal variation dominates the variation of gut microbiomes, certain dietary interventions and correlations with changes in the gut microbiome remain important for understanding how to manipulate the microbial landscape, as these manipulations could provide host health benefits. Research indicates an influence of the gut microbiome on a myriad of disease states, including obesity and NAFLD [17, 38, 39, 41, 46-52]. This has been demonstrated in human and animal studies, where specific taxa in the gut microbiome have been correlated with various health outcomes.

One way to establish microbiome-disease correlations is by studying families. In a study by Turnbaugh et al., the microbiomes of 31 monozygotic and 23 dizygotic twin pairs and 46 mothers were assessed [32]. They found that the degree of similarity of the gut microbiomes between the monozygotic twin pairs was similar to that of the dizygotic twin pairs, indicating less similarity than expected. Furthermore, the mother's microbiomes were only slightly less similar to their children's than the twin pairs were to each other. This indicates considerable interpersonal variability in gut microbiomes, even under the same genomic conditions [32]. This interpersonal variability could be due to external factors, such as diet or the slight environmental differences of each individual. Turnbaugh et al. also failed to identify microbial profiles that directly correlate to adiposity or leanness in these pairs but did identify enriched microbial gene sets, similarly to the longitudinal study by Wu et al. [43]. They found that of the microbial genes enriched in obese individuals, 75% were attributed to Actinobacteria, whereas 0% of genes enriched in lean individuals were from Actinobacteria [32]. The remaining 25% of genes enriched in obese individuals were attributed to Firmicutes. Further, 42% of the enriched genes in lean individuals were attributed to Bacteroidetes [32]. Taken together, these results indicate that microbiomes vary by the origins of functional gene sets in an individual's microbial community. This interpersonal variation also gives rise to the need to understand specific taxonomical correlations with disease states, rather than the totality of the microbiome as an indicator [32].

While there is not a single microbial profile that is concordant with adiposity or leanness, differential abundances of specific taxa have been correlated with disease states including obesity and non-alcoholic fatty liver disease (NAFLD) [28, 31, 32]. Ley et al. found that familial connections were a major source of microbial similarity in a study of lean and obese mice [27]. Using C57BL/6J Ob/+ mothers (lean) and ob/ob (obese), ob/+ (lean), and +/+ (lean) progeny, they found that microbiome communities of mice within the same family had similar profiles, despite differences in genotype and phenotype. However, they did find that ob/ob mice had increased Firmicutes and reduced Bacteroidetes when compared to the other genotypes [27]. The increased efficiency of ob/ob mice at harvesting energy from food and releasing short chain fatty acids (SCFAs) could contribute to the increase in cecal Firmicutes compared to their lean littermates [27, 53, 54]. This relationship between genotype, Firmicutes, and phenotype could work in the reverse way, as Firmicutes ferment dietary fibers in the cecum, releasing energy sources that can be to be utilized by the host [27].

An increased abundance of the phylum Firmicutes and a reduction in Bacteroidetes (or increased Firmicutes:Bacteroidetes ratio) has been correlated with increased adiposity in other animal [55-57] and human [25, 26, 29, 30, 32, 34, 58, 59] studies. Additionally, human studies have shown correlations between lean subjects and increased abundances of *Bifidobacterium* [60], *Lactobacillus* [29, 59], and Bacteroidetes [32]. This provides evidence that the abundance of specific taxa has an influence on host health. In a weight loss intervention study of Spanish adolescents, Nadal et al. found that certain taxonomic abundances changed with caloric restriction-based weight loss [58]. In the group that lost more than 4 kg, a reduced post-intervention abundance in *C. histolyticum* was correlated with weight loss [58]. Additionally, an increase in *Bacteroides* was correlated with weight loss. These changes in taxonomic abundance were also associated with reduced fasting plasma glucose and total plasma cholesterol in this weight loss group [58]. Similar results were seen in another study of pre- and post-weight loss intervention and microbiomes in adolescents by Santacruz et al. [29]. They found that *Bacteroides* abundance was increased post-weight loss in the high weight loss group (> 4 kg). Additionally, they saw an increase in *Lactobacillus* post-intervention for the same group, but no differences in pre- and post-intervention in the low weight loss (< 2 kg) group [29]. Notably, the changes seen in the gut microbiome post-weight loss could be partly attributed to changes in dietary composition, rather than fully due to changes in body weight.

However, even in the presence of increased abundance of Bacteroidetes or *Bifidobacterium,* reduced adiposity is not always demonstrated [61-63]. In a study of 98 adults representing all three BMI categories, Schweirtz et al. found a reduced abundance of Bacteroidetes in the normal BMI group when compared to the overweight and obese groups [30]. They also saw a reduction in total SCFAs, and specifically propionate, in the fecal samples of the normal BMI group compared to others. These findings are not consistent with other studies in humans and animals, where increased SCFAs are correlated with increased Firmicutes, typically shown in the overweight and obese groups. Firmicutes, similar to Bacteroidetes, are strong fermenters producing SCFAs [27, 30]. Despite frequent touting of how higher concentrations or types of SCFAs in the large intestine are indicators of crosstalk and disease prevention, little is known about how exactly SCFA production and metabolism correlates to health status.

Additionally, taxonomical changes in the gut microbiome have been demonstrated in patients with varying degrees of NAFLD. In a cohort study by Boursier et al., liver biopsies and same day stool samples were collected [64]. When individuals were stratified by stages of liver fibrosis (F0/1 and F≥2) and presence of NASH, differences in large intestinal *Bacteroides*, *Prevotella*, and *Rumminococcus* were seen [64]. Specifically, in patients with NASH, the abundance of *Bacteroides* was increased and *Prevotella* was reduced when compared patients without NASH [64]. The same trend was true in patients with more severe fibrosis (F≥2 group), where *Bacteroides* and *Rumminococcus* were increased and *Prevotella* was decreased when compared to the gut microbiomes of patients with less severe (F0/1) fibrosis [64]. Similarly, Shen et al. compared the microbiomes of individuals with NAFLD (n=25) with healthy controls (n=22) [65]. They found that *Prevotella* was reduced in NAFLD patients when compared to healthy controls, where majority (n=21) of the patients had fibrosis scores under 2 (F0/1) [65]. This is consistent with Boursier et al., where the abundance of *Prevotella* was increased in F0/1 patients when compared to those with more severe steatosis (F≥2) [64]. Shen et al. further characterized the microbiome clusters of NAFLD patients vs controls and found the NAFLD patients also had increased abundance of *Fusobacteria* and *Proteobacteria* with reduced Bacteroidetes and *Faecalibacterium* [65]. When taken together, both studies indicate that low-grade NAFLD (F0/1) patients exhibit differential abundances of certain microbial taxa when compared to healthy individuals [65] or those with more severe fibrosis [64].

Proposed mechanisms of crosstalk between the gut microbiome and host tissues

To elicit host health benefits, the gut microbiome must communicate with other host tissues. This can be achieved through secretion and uptake of fermentation products or bacterial components, such as production of SCFAs, lipopolysaccharide (LPS), and recycling of bile acids.

Bile acids are amphipathic detergents that are synthesized from cholesterol and aid in lipid absorption and metabolism [66]. After synthesis and secretion by the liver, bile acids in the small intestine are either reabsorbed by active transport or utilized by gut bacteria in the large intestine [66, 67]. In the large intestine, bile acids can be biotransformed into secondary bile acids via 7α-dehydroxylation reactions. From here, secondary bile acids are either reabsorbed or excreted in the stool [68]. The 7α-dehydroxylation reaction can only be carried out by gut bacteria from the genus *Clostridium* [69, 70]. In addition to the biotransformation of bile acids by bacteria in the gut, bile acid concentrations in the large intestine can mediate other microbial populations. There is evidence that gram-positive bacteria are more sensitive to high concentrations of bile acids in the large intestine, allowing for the bile acid-tolerant gram-negative bacteria to persist [71]. Further, increased large intestinal cholic acid, a primary bile acid, is associated with increased Firmicutes and reduced Bacteroidetes, largely due to Firmicutes containing *Clostridium* species that are capable of 7α-dehydroxylation [72, 73].

Inflammation in the liver, as seen in NAFLD, has been shown to reduce bile acid pools, therefore reducing the amount of bile acids that reach the large intestine [73]. This can lead to a shift in microbial populations in the gut, increasing Bacteroidetes and reducing Firmicutes. Members of the phylum Bacteroidetes, specifically of the genus *Bacteroides,* secrete lipopolysaccharide (LPS), which can act to increase inflammation and exacerbate steatosis in the liver [73, 74]. This increase in inflammation via TNFα can further reduce bile acid synthesis through inhibition of CYP7A1, a key component of the classical bile acid synthesis pathway in the liver [73, 74].

In addition, it has been shown that lipopolysaccharide (LPS) released from gram negative bacteria in the intestine can disrupt tight junctions, which can lead to leaky gut and metabolic endotoxemia [24, 75]. The presence of LPS in portal circulation can lead to increased inflammation via activation of toll-like receptor-4 (TLR4), which increases TNFα production and release by adipose tissue [24, 75-79]. Increased systemic inflammation induced by LPS through TNFα contributes to NAFLD progression by promoting inflammation in the liver alongside steatosis, such as in NASH [6]. This is an example of metabolic endotoxemia.

Increasing the abundance of certain gut microbes results in decreased plasma LPS and metabolic endotoxemia. *Bifidobacterium* and *Lactobacillus* species are considered beneficial gut microbes, or probiotics. They are also gram-positive bacteria which do not contribute to plasma LPS. Further, an increased abundance of these bacteria results in a decreased abundance of other bacteria, such as the gram-negative phylum Bacteroidetes, due to competition for resources.

Short chain fatty acid (SCFA) production by the gut microbiome is thought to be a key determinant in regulating host health, including adiposity and fatty liver. An increased abundance of these bacteria in the large intestine and increased SCFA production have been correlated with several health benefits, such as reduced adiposity and improved glycemic control [37, 49, 52, 80-82]. Large intestinal bacteria are able to ferment dietary fibers and produce SCFAs as fermentation end products [37, 49, 52, 80-82]. However, SCFA production differs based on the gut microbiome. Specifically, bacteria that belong to the Firmicutes phylum are established fermenters of non-digestible carbohydrates, and will out compete other bacteria in the presence of fermentable substrates [37, 49, 52, 80-82]. Bacteroidetes are also SCFA producers [83], thus, having an increased abundance of this phylum results in increased SCFAs in the large intestine. Each SCFAs - butyrate, propionate, and acetate - serves a different purpose following their release by gut bacteria. When taken up into colonocytes, butyrate is primarily utilized for energy by these cells. Cellular uptake of SCFAs is primarily through the action of G-protein coupled receptors (GPRs 41 and 43), allowing the cells to utilize them for energy and metabolic regulation related to cell growth, adiposity, and NAFLD [15, 50, 84-86]. There is some evidence that GPCRs are upregulated in response to high levels of circulating SCFAs in order to increase cellular uptake [15, 50, 84-86]. Remaining SCFAs, including unused butyrate, are then transported into portal circulation and utilized by the liver or peripheral tissues [37]. Remaining butyrate is taken up by hepatocytes and used for energy. Acetate is primarily taken up by the liver, where it is utilized as a substrate for cholesterol, fatty-acid, glutamate, and glutamine synthesis [36, 37, 87]. The liver also utilizes the majority of circulating propionate as a gluconeogenic precursor [37, 88]. One mechanism by which SCFAs may regulate host metabolism is by activating AMPK, the central regulator of lipid metabolism in the liver [37, 49, 52, 80-82].

The activation of AMPK results in increased fatty acid oxidation and decreased *de novo* lipid synthesis and storage [37, 49, 52, 80-82]. A change in these lipid metabolic pathways leads to decreased lipid accumulation in the liver and adipose tissues. Understanding how changing the microflora may impact SCFA production and alter hepatic lipid metabolism may be important for developing effective therapeutics for NAFLD.

Prebiotic Dietary Fibers

Definition of prebiotic dietary fibers

Prebiotics are defined as indigestible substrates that are fermented in the large intestine, elicit a beneficial change in the gut microbiome, and provide a health benefit to the host [89]. Prebiotic dietary fibers are typically soluble dietary fibers that do not increase small intestinal viscosity. Established prebiotic dietary fibers include fructooligosaccharides (FOS), polydextrose (PD), and galactooligosaccharides (GOS). FOS is an oligosaccharide of fructose monomers, the truncated version of inulin, and GOS is an oligosaccharide of primarily galactose monomers. These oligosaccharides are fermented by large intestinal bacteria, producing fermentation byproducts that can be utilized by the host. Similarly, polydextrose is a polymer of glucose, with a degree of polymerization ranging from 3-100, with an average of 12.

Human and animal studies alike have demonstrated prebiotic activity with each of these commercial dietary fibers, however, their effects, dose-response relationships, and mechanisms of action vary. Upon intervention with prebiotic dietary fibers, many studies where a reduction in fatty liver and adiposity is seen demonstrate an increased abundance of *Bifidobacterium* and *Lactobacillus spp.* [55-57, 61, 62]. However, even in the presence of increased abundance of *Bifidobacterium,* reduced fatty liver and adiposity are not consistently demonstrated [61-63]. This suggests that a threshold of change in certain taxa needs to be achieved to provide these health benefits to the host. While a threshold effect of probiotic gut bacteria appears to exist, inconsistencies in data reporting pose a hurdle in identifying the effective abundance. Furthermore, identifying mechanisms of communication between the gut microbiome and host tissues is necessary for completely understanding how to manipulate the gut microbiome for therapeutic uses.

Established prebiotic dietary fibers & host health

Animal studies

Prebiotic dietary fibers, by definition, provide a host health benefit. Examples of such benefits include improved gut health or glycemic control or reduced fatty liver and adiposity [89]. The evidence supporting health benefits with respect to individual prebiotics varies.

In rodent studies, the dietary concentration of prebiotic has ranged from 2.5-21% [61-63, 90, 91]. Several studies have examined FOS feeding on body weight, fat mass, and circulating leptin. Reimer et al., using a FOS dietary concentration of 21%, found non-significant reductions in body weight and percent body fat but significantly lower serum leptin concentration in overnight-fasted rats [61]. In contrast, Cluny et al. fed FOS at 10% of the diet and found lower final body weight but no significant difference in fat mass or plasma leptin compared to the high fat control in non-fasted rats [63]. Notably, many studies using FOS or other prebiotics find reduced body weight in the prebiotic group compared to the high fat control but fail to find changes in body composition that would indicate reduced adiposity [61-63]. Frequently, this reduction in body weight is found in the presence of reduced food intake, making it difficult to separate reduced energy intake from changes in the microbiome as an explanation for reduced animal size [61].

Similarly, studies using polydextrose (PD) as a dietary intervention in animal models demonstrate that PD is effective in reducing adiposity in the context of a high fat diet. Raza et al. gavaged mice with a PD solution for 14 days while feeding a Westernized diet [56]. They found that the PD group had lower final body weight, plasma cholesterol, and circulating triacylglycerols (TAGs) compared to the non-PD control group [56]. They also saw an increase in cecal *Bifidobacterium* in the PD group. However, the PD group had reduced food intake compared to the control group, which likely explains the lower final body weights and reduced plasma lipids [56].

There is currently little evidence for reduced fatty liver by commercial prebiotics, particularly at physiologically attainable doses of under 10% wt/wt [61, 62, 90]. While other studies using prebiotics demonstrated reduced liver weights, only Nakamura et al. found a reduction in liver lipids [90], who fed the lowest dose of FOS (2.5%). In contrast, when FOS was fed at a higher dose (21%), liver weight and total liver cholesterol was reduced but liver lipids were not [61]. This suggests that the dose of dietary prebiotic alone is not the key mediator of reduced fatty liver. Rather, specific microbiome profiles are needed. Nakamura et al. further demonstrated that feeding FOS at 2.5% of the diet resulted in greater fermentation in the ceca of mice, as indicated by a reduction in cecal pH, increase in empty cecum weight, and increase in cecal short chain fatty acids when compared to the high fat control group. Similarly, Reimer et al. reported an increase in empty cecum weight with an increase in cecal *Bifidobacterium* [61], but no changes in liver lipids. Together, this indicates the need for a more comprehensive understanding for how FOS may mediate fatty liver and what doses are effective with respect to changing the gut microbiome.

Chen et al. demonstrated that GOS, when orally gavaged 800 mg/kg BW for 8 weeks in mice on high fat (HF) diets, reduced liver steatosis when compared to the HF control group [55]. They also found that the GOS group had increased cecal *Lactobacillus*, *Akkermansia muciniphila*, and Bacteroidetes when compared to the HF control group. Interestingly, the increase in the normal fat (NF) control group of both *Lactobacillus* and Bacteroidetes was similar to that of the GOS group [55]. When taken together, this suggests that both taxa are correlated with reduced liver steatosis. Similarly, Mistry et al. found that mice fed GOS at 7% wt/wt of the diet had increased cecal *Bifidobacterium, Akkermansia muciniphila,* and reduced Firmicutes [57]. They also demonstrated that epididymal fat pad weight, an indicator of total body adiposity, was reduced in GOS fed animals despite the HF diet. While not statistically significant, Mistry et al. found that the GOS group trended toward reduced liver lipids compared to the HF control group [57]. Thus, GOS increased beneficial gut bacteria while tending to reduce fatty liver in mice fed HF diets.

Overall, FOS, PD, and GOS do not consistently reduce host adiposity or fatty liver. This lack of effect appears partially independently of dose [55-57, 61-63, 90, 91]. More commonly, reduced adiposity and fatty liver correlates with the extent of change in certain taxa in the gut microbiome of these animals. Understanding if there is a threshold effect of these probiotic bacteria is key to understanding to how effectively manipulate the gut microbiome in order to establish novel therapeutics for these disease states.

Human studies

As with rodent studies, human studies of prebiotic intervention do not consistently demonstrate health benefits. In a cross-over study by Canfora et al., 49 overweight and obese, prediabetic individuals were supplemented with 7.04 g of Vivinal GOS powder (contains 69% GOS) for 12 weeks [92]. They found that while there were no changes in biometrics, markers of energy metabolism, or glucose tolerance, *Bifidobacterium* was significantly increased post-supplementation [92]. Additionally, short chain fatty acid concentrations did not differ in fecal or plasma samples between pre- and post-supplementation. This indicates that even a beneficial change in the gut microbiome does not necessarily result in health benefits.

Alternatively, Nicolucci et al. demonstrated that intervention with 8 g of oligofructose-enriched inulin (OI) per day in children with overweight and obesity was effective in reducing weight gain, percent body fat, and inflammation after 16 weeks when compared to the placebo group [93]. OI also increased Bifidobacterium abundance post-intervention when compared to baseline [93]. This aligns with animal studies of the anti-obesogenic effects of Bifidobacterium [55-57] but contrasts with some human studies that have demonstrated an increase in Bifidobacterium without reduced adiposity [92].

The relationship between prebiotic intervention, increased intestinal Bifidobacterium, and host health outcomes continues in studies of NAFLD and NASH. Bomhof et al. demonstrated that oligofructose (OF; also known as FOS) intervention was effective in reducing steatosis, inflammation, and overall non-alcoholic fatty liver activity score (NAS) [6]. In their study, 14 individuals had liver biopsies done at baseline and after 36 weeks of supplementation with 8-16 g OF (8 g OF per day for the first 12 weeks followed by 16 g OF per day for 24 weeks) or an isocaloric placebo (maltodextrin) [6]. Individuals also provided stool and blood samples at baseline and weeks 12, 24, and 36 for microbiome and biochemical analyses, respectively. In addition to the reduction in liver steatosis, inflammation, and total NAS, the OF group had increased intestinal *Bifidobacterium* throughout the intervention [6]. Blood biomarkers and other biometrics were not different between groups or over time. Together, this indicates that prebiotic supplementation can be a viable treatment for NAFLD and NASH, potentially mediated by changes in the gut microbiome. The human studies described indicate that supplementation with prebiotics and an increase in *Bifidobacterium* do not always result in reduced adiposity or body weight, similar to other studies in humans [6, 93].

Variation in host health benefits following increases in certain microbial taxa in the gut microbiome after prebiotic intervention could be due to a threshold effect of those gut bacteria. That is, a certain percent abundance of specific taxa, such as *Bifidobacterium*, must be achieved in order to elicit health benefits to the host, such as reduced adiposity. In order to fully understand this threshold effect and compare specific taxa in the gut microbiomes of individuals and animals with varying phenotypes, a standard of reporting needs to be established. In many nutrition-microbiome-disease studies, taxonomical abundances are reported as $\log_{10}$ signal intensity (microarray analysis) [92] or sequence reads/ 20 ng DNA (16S) [6, 61, 62] instead of corrected, percent abundance (using 16S rRNA other sequencing measures). Without appropriate data representation and normalization, it is difficult to compare microbial abundances among studies, making it impossible to ascertain whether a threshold of abundance of specific bacteria is needed to produce a health benefit.

Conclusions

When taken together, the literature indicates that 1) there is a need for novel interventions for NAFLD that are independent of weight loss and sustainable, 2) correlations between the gut microbiome and NAFLD could provide insights into how to exploit the gut microbiome for NAFLD treatment and prevention, and 3) the use of prebiotics present a potential solution for manipulating the gut microbiome as a means to prevent or treat NAFLD. Identifying strong correlations between the gut microbiome and NAFLD, along with potential mechanisms of disease prevention, with prebiotic intervention has potential to be a novel avenue of treatment and prevention.

At this time, there is evidence that differential abundances of certain taxa are correlated with NAFLD and obesity. Some mechanisms of crosstalk between the gut microbiome and host tissues have been proposed, such as signaling by short chain fatty acids and lipopolysaccharide, or recycling of bile acids. Additionally, there is substantial evidence that dietary intervention can be used to alter the gut microbiome in a somewhat predictable way. Further insights into how differences in the gut microbiome contribute to fatty liver and obesity are needed in order to understand how to utilize dietary constituents, such as prebiotics, to alleviate these disease states.

Chapter 3: Polylactose exhibits prebiotic activity and reduces adiposity and non-alcoholic fatty liver disease in rats fed a high fat diet.

Under review as: Abernathy BE, Schoenfuss TC, Bailey AS, Gallaher DD. Polylactose exhibits prebiotic activity and prevents adiposity and non-alcoholic fatty liver disease in rats fed a high fat diet.

Abstract

Background

Prebiotic dietary fibers are fibers that change the intestinal microbiome favorably and provide a health benefit to the host.

Objectives

Polylactose is a novel fiber, synthesized by extrusion of lactose. To evaluate it for prebiotic activity, we determined its fermentability, effect on the microbiome, and its effects on adiposity, fatty liver, and liver cholesterol in a diet-induced obesity animal model.

Methods

Male Wistar rats (n=12/group, initial body wt 75-99 g) were fed normal fat (NF, 25% fat by kcal) or high fat (HF, 51% fat by kcal) diets containing different fibers (6% fiber of interest and 3% cellulose, by weight); including cellulose (NFC and HFC, controls), polylactose (HFPL), lactose matched to the residual lactose in the HFPL diet (HFML), and two established prebiotic fibers, polydextrose (HFPD) and fructooligosaccharides (HFFOS). After 10 weeks on experimental diets, organs were harvested and cecal contents collected.

Results

HFPL animals had greater cecal weight (empty) and lower cecal pH when compared to all other groups, suggesting that polylactose is more fermentable than other prebiotic fibers ($p < 0.05$). HFPL animals also had an increase in taxonomical abundance of probiotic species, such as *Bifidobacterium* and *Lactobacillus*, in the cecum ($p < 0.05$).

Epididymal fat pad weight was significantly decreased in the HFPL animals compared to all other HF groups ($p < 0.05$) and did not differ from the NFC group. Liver lipids and cholesterol were reduced in HFPL animals when compared to HFC animals and were numerically lower than all other HF groups ($p < 0.05$).

Conclusions

Polylactose is a fermentable fiber that elicits a beneficial change in the gut microbiome as well as reduces adiposity and fatty liver in rats fed high fat diets. As these effects of polylactose were greater than those of two established prebiotics, fructooligosaccharides and polydextrose, this suggests that polylactose is a potent prebiotic.

Introduction

Obesity continues to be a major public health issue worldwide. The World Health Organization's Health Statistics Report of 2018 estimates that obesity occurs in 39% of the global population [94]. The inflammation and insulin resistance that accompany increased adiposity can lead to many health issues, such as type 2 diabetes (T2D), cardiovascular disease, and non-alcoholic fatty liver disease (NAFLD). NAFLD is of particular interest, as its incidence has been estimated at 75-90% of adults who are overweight or obese, occurring in 25% of adults worldwide [2, 3].

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of excess lipids in the liver [2, 3, 5]. Simple fatty liver (non-alcoholic fatty liver, NAFL) is the most common form of NAFLD and occurs with little to no inflammation or scarring. Non-alcoholic steatohepatitis (NASH) is the more severe form of NAFLD, and occurs with irreversible scarring, or cirrhosis, and fibrosis [2, 3, 5]. Continued liver lipid accumulation, inflammation, cirrhosis, and fibrosis in NASH lead to chronic liver disease, and, eventually, liver failure. Currently, there is no effective pharmacological treatment for NAFLD. However, weight loss, as a result of dietary changes, increased exercise, or bariatric surgery, has been shown to reverse the early stages of NAFLD [2, 3, 5]. Given the difficulty of achieving weight loss by diet or exercise, and the invasive nature of bariatric surgery, a dietary approach to reverse NAFLD that does not require weight loss would be highly desirable.

Little is known about the molecular mechanisms that lead to NAFLD [7, 8]. It is clear that obesity, hypertriglyceridemia, insulin resistance, and T2D are associated with the development of NAFLD [7, 8]. A number of factors can result in the accumulation of lipids in the liver, including increased uptake of triacylglycerols from dietary fat and fatty acids from lipolysis of adipose tissue, increased *de novo* hepatic lipid synthesis, and a reduction in lipid oxidation and mobilization [5, 13, 14]. Many of these factors are dysregulated in obesity and hyperinsulinemia. During periods of hyperinsulinemia, many tissues, including the liver, become insulin resistant, leading to hepatic lipid accumulation. This is largely attributable to the upregulation of lipogenic transcription factors, such as sterol regulatory element binding protein-1c (SREBP1c) [5, 13, 14], which results in increased lipid synthesis in the liver. Further, there is a reduction in lipid oxidation and mobilization. With continued lipid accumulation, low-grade inflammation develops that can eventually lead to NASH, fibrosis, and cirrhosis.

Recent studies have shown that there is a relationship between the gut microbiome, NAFLD, and obesity [17, 38, 39, 41, 46-52]. Increased large intestinal abundance of the phylum Firmicutes and a reduction in Bacteroidetes (or increased Firmicutes:Bacteroidetes ratio) has been correlated with increased adiposity in humans [25-27, 29, 30, 32, 34, 58, 59] and animals [55]. Similarly, human studies have shown correlations between lean subjects and increased abundances of *Bifidobacterium* [60] and/or *Lactobacillus* [29, 59]. Thus, changes in the large intestinal bacterial abundance of specific taxa appears to have an important influence on host health.

Increased production of short chain fatty acids (SCFAs) may mediate these host health benefits. Large intestinal bacteria ferment dietary fibers and other non-digestible carbohydrates to produce SCFAs, which can be taken up into circulation and utilized by host tissues [37, 49, 52, 80-82]. SCFA production differs based on the gut microbiome. Specifically, bacteria that belong to the Firmicutes phylum are established fermenters of non-digestible carbohydrates, and will outcompete other bacteria in the presence of fermentable substrates [37, 49, 52, 80-82]. An increased abundance of these bacteria in the colon has been correlated with increased SCFA production and with several health benefits, such as reduced adiposity and improved glycemic control [37, 49, 52, 80-82]. SCFAs, primarily composed of butyrate, propionate, and acetate, are utilized differently following their absorption in the large intestine. Butyrate is primarily utilized for energy in colonocytes. Remaining SCFAs, including unutilized butyrate, are transported through the portal circulation, with propionate and butyrate taken up by hepatocytes [36], where propionate is used as a gluconeogenic precursor [37, 88]. Acetate is primarily taken up by the adipose and skeletal muscle, where it is utilized as a substrate for cholesterol, fatty acid, glutamate, and glutamine synthesis [36, 37, 87]. Understanding how a non-digestible carbohydrate changes the microbiome, subsequently impacting SCFA production and altering hepatic lipid metabolism, will likely be important for developing effective dietary approaches to preventing or reversing NAFLD.

Prebiotics are a potential dietary intervention that can alter the gut microbiome by providing a substrate for SCFA production. Prebiotics are defined as indigestible substrates that are fermented in the large intestine, elicit a beneficial change in the gut microbiome, and provide a health benefit to the host [89]. The objective of the present study was to investigate the prebiotic potential of a novel dietary fiber, polylactose. Polylactose is produced by polymerizing lactose by reactive extrusion, using heat and citric acid as catalysts [95]. Purified polylactose analyzes as a dietary fiber [95]. It contains oligomers with a degree of polymerization (DP) of 3-11 monomeric sugars, with the bulk of the oligomers falling in the range of 3-5 DP. As several established prebiotic dietary fibers are oligomers, we hypothesized that this polylactose preparation might also perform as a prebiotic dietary fiber, as defined above. Therefore, we assessed its fermentability, its effect on the gut microbiome and on SCFA, and whether it produced host health benefits, focusing on its ability to reduce liver lipids and adiposity.

Materials and methods

Preparation of polylactose

Polylactose was produced on a twin-screw extruder as previously described [95, 96]. It was subsequently solidified, ground, rehydrated in distilled water, and purified on a mixed bed carbon and ion exchange column. The filtration column consisted of layers of diatomaceous earth, a 1:1 mixture of Ambersep 200 (H+) and Amberlite FPA 53 (OH-), and activated carbon (Norit GAC 1240 PLUS, Cabot Norit Americas, Inc). The eluate and 1L of rinse were collected and pooled prior to spray drying. This produced a white, almost tasteless powder. Polylactose fiber, lactose, glucose, and citric acid were measured in the final product using commercial kits (Integrated Total Dietary Fiber Assay Kit, Lactose/Sucrose/D-Glucose Assay Kit, Citric Acid Assay Kit, respectively, Megazyme, Bray, Ireland). The final product contained approximately 50% soluble polylactose fiber, 22% free lactose, residual glucose and citric acid, and other unidentified materials (**Table 3.1**).

Table 3.1 Composition of partially purified polylactose preparation

Dietary fiber (soluble), %	Lactose, %	Glucose, %	Citric acid, %
50.13 ± 1.16	21.92 ± 0.66	6.27 ± 0.22	0.05 ± 0.06

Number of determinations per analyte = 4. Values are means ± standard deviation.

Animals and treatments

Seventy-two male Wistar rats (initial body weight 75-99 g, 4-5 weeks of age) were purchased from Envigo Laboratories (Indianapolis, IN). Animals were singly housed in hanging wire cages and kept on a 12-h light/dark cycle. Food and water were provided to the animals ad libitum for the duration of the study. All animal use procedures were approved by the University of Minnesota Animal Care and Use Committee.

All animals were adapted to a purified rodent diet (modified AIN-93G diet) for two weeks prior to the start of experimental diets (**Supplemental Table 1**). At the start of week three, the animals were randomly assigned into six groups (12 rats per group), which were fed one of six experimental diets for 10 weeks. The six experimental diets were as follows: normal fat cellulose (NFC, modified AIN-93G diet), high fat cellulose (HFC, 9% cellulose), high fat polylactose (HFPL, 6% polylactose fiber, 3% cellulose), high fat matched lactose (HFML, 2.6% lactose, matched to amount of free lactose in the HFPL diet, 6.4% cellulose), high fat polydextrose (HFPD, 6% PD, 3% cellulose), and high fat fructooligosaccharide (HFFOS, 6% FOS, 3% cellulose). The prebiotic dose was chosen as it might be dietarily attainable to humans. The composition of the diets is shown in **Table 3.4**. Food intake and body weight were measured weekly. A 3-day fecal collection was conducted at week 8 of the experimental diets. Feces were lyophilized and stored at -20°C until further analysis.

After 10 weeks feeding the experimental diets, fasted animals were anesthetized with isoflurane and blood collected by cardiac puncture. Blood was centrifuged at 3,000 *g* for 20 min and plasma aliquots were stored at -80° C until use. Ceca were harvested, cecal pH was measured with a spear-tip electrode, and contents were expressed into sterile 15 mL conical tubes, frozen on dry ice, and stored at -80° C. Empty ceca were rinsed, weighed, and discarded. One epididymal fat pad from each animal was harvested, rinsed, weighed, frozen on dry ice, and stored at -80° C. Livers were excised, rinsed, weighed, and frozen on dry ice and stored at -80° C until further analysis.

Blood glucose control and gluconeogenesis potential.

Tolerance testing was performed during the final two weeks of feeding the experimental diets after overnight (8 h) fasts. Insulin (1 IU insulin/kg body weight) and pyruvate tolerance tests (60 mg sodium pyruvate/kg body weight) (ITT, PTT) were conducted during week 9, with each being administered intraperitoneally. Oral glucose (0.5 g glucose/kg body weight) tolerance testing (OGTT) was conducted during week 10. For all tolerance testing, blood glucose was measured using blood glucose test strips and glucometer (AlphaTrak, Zoetis Inc., Parsippany, NJ) at 0, 15, 30, 60, 90, and 120 min. Area under the curves were calculated using the trapezoidal method.

Liver lipids and cholesterol

Approximately 1 g of liver from each animal was homogenized and lipids extracted using chloroform:methanol (2:1) [97]. Extracted lipids were dried under N_2 gas and quantified gravimetrically. Lipid samples were reconstituted with 10 mL chloroform:methanol (2:1) and cholesterol was measured enzymatically using a previously described method [98].

Circulating adipokines and lipids

Circulating leptin and adiponectin were quantified by ELISA (Millipore Sigma EZRL-83K, abcam ab10874, respectively) using plasma samples from the time of harvest. Plasma triacylglycerols were measured spectrophotometrically using a commercial kit (T7532120, Pointe Scientific Inc, Canton, Michigan).

Microbiome sequencing

Cecal contents were thawed on ice and mixed prior to use. DNA was extracted from approximately 180-220 mg of contents using the QiAmp DNA Stool Mini kit (Qiagen, #51504) and DNA eluates quantified spectrophotometrically. DNA eluates were then submitted to the University of Minnesota Genomics Center for sequencing of the 16S ribosomal subunit with the V5/V6 region amplicon on an Illumina MiSeq using 2x300 bp paired end reads. Data was analyzed using DADA2 and phyloSeq in R.

Short chain fatty acids and bile acids

Short chain fatty acids (SCFAs) were extracted from cecal contents and measured by gas chromatography [99-101]. Bile acids were extracted from fecal samples, partially purified [102], and total bile acids quantified enzymatically [103].

Statistical analysis

All data except microbiome data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SAS (version 9.4, SAS Institute, Cary, NC). Duncan's multiple range test was used to conduct pairwise comparisons among all groups, reducing the change of making type II error. To examine for trends in differences, Student's t-tests were used to compare groups of interest. Correlation analyses were conducted in SAS for SCFAs and adiposity measures. Area under the curve was calculated in R and analyzed by ANOVA and Student's t-test in SAS. Microbiome data was analyzed in R [104], using DADA2 and phyloseq packages for beta diversity, taxonomical abundance, and statistical analyses.

Results

Polylactose reduced adiposity with no significant changes in energy intake or body weight

Daily average energy intake did not differ among the groups (**Table 3.2**). Although final body weight was lower in the NFC and HFPL groups, compared to the HFC group, this difference did not achieve statistical significance, and there were no significant differences in final body weight among the groups ($p = 0.3124$). The epididymal fat pads of the HFPL and NFC groups weighed less than the HFC group ($p = 0.034$, Table 3.2). Further, epididymal fat pad weight of the HFPL groups was significantly less than the HFML, HFPD, and HFFOS groups ($p < 0.05$). Plasma leptin concentration in the HFPL and NFC groups were less than in the HFML group. Plasma leptin concentration, which has been shown to correlate with total body fat [105, 106], was highly correlated with epididymal fat pad weight ($r = 0.69$, $p < 0.0001$). Plasma adiponectin concentration did not differ among the groups ($p = 0.8804$).

Table 3.2 Energy intake, final body weight, cecal pH, and tissue weights in rats fed polylactose, polydextrose, or fructooligosaccharides.

Variable	Basal	HFC	HFPL	HFML	HFPD	HFFOS
Energy intake, *kJ/day*	385.6 ± 14.3	405.7 ± 12.8	367.5 ± 16.0	367.5 ± 12.7	378.1 ± 13.5	381.4 ± 13.8
Final body weight, *g*	490.0 ± 11.8	534.8 ± 14.8	496.5 ± 12.5	517.8 ± 17.5	514.9 ± 19.6	523.0 ± 13.4
Empty cecum weight, *g*	0.58 ± 0.013[c]	0.59 ± 0.049[c]	1.60 ± 0.108[a]	0.53 ± 0.016[c]	0.79 ± 0.029[b]	0.79 ± 0.057[b]
Cecal pH	6.91 ± 0.66[a]	6.98 ± 0.06[a]	6.05 ± 0.08[c]	6.86 ± 0.06[a]	6.54 ±0.08[b]	6.98 ± 0.06[a]
Epididymal fat pad weight, *g*	5.31 ± 0.42[bc]	7.06 ± 0.39[a]	4.71 ± 0.35[c]	7.04 ± 0.65[a]	6.8 ± 0.63[a]	6.4 ± 0.45[ab]
Liver weight, *g*	15.02 ± 0.73[b]	18.47 ± 1.04[a]	15.95 ± 0.88[ab]	17.48 ± 1.00[ab]	16.19 ± 0.92[ab]	18.56 ± 0.70[a]

Values are means ± SEMs, n=12. Means with a row that do not share a common letter are statistically different, $p < 0.05$. NFC, normal fat cellulose control; HFC, high fat cellulose control; HFPL, high fat polylactose; HFML, high fat matched lactose; HFPD, high fat polydextrose; HFFOS, high fat fructooligosaccharides.

Table 3.3 Plasma glucose, hormones, and triacylglycerols concentrations, cecal total short chain fatty acid amount, and fecal bile acid excretion in rats fed polylactose, polydextrose, or fructooligosaccharides.

Variable	Basal	HFC	HFPL	HFML	HFPD	HFFOS
Fasting plasma glucose (OGTT 0), *mg/dL*	115.0 ± 4.2	115.5 ± 5.0	115.1 ± 7.3	118.1 ± 4.0	125.2 ± 4.2	127.7 ± 3.8
Plasma insulin, *ng/mL*	4.99 ± 0.57^{b}	6.44 ± 0.64^{ab}	6.58 ± 1.12^{ab}	7.03 ± 0.67^{ab}	8.45 ± 1.17^{a}	5.62 ± 0.50^{b}
Plasma leptin, *ng/mL*	9.18 ± 1.26^{c}	16.79 ± 1.94^{ab}	12.69 ± 1.92^{bc}	20.49 ± 3.13^{a}	15.05 ± 2.34^{abc}	14.18 ± 1.97^{abc}
Plasma adiponectin, *µg/mL*	19.41 ± 3.56	23.38 ± 3.52	23.33 ± 3.39	25.16 ± 3.85	20.01 ± 3.47	22.43 ± 3.68
Plasma triacylglycerols, *mmol/L*	1.77 ± 0.14^{a}	1.24 ± 0.12^{b}	0.91 ± 0.14^{bc}	1.02 ± 0.09^{bc}	1.01 ± 0.09^{bc}	0.87 ± 0.09^{c}
Total short chain fatty acids, *µmol/cecum*	95.32 ± 5.7^{c}	89.4 ± 8.6^{c}	286.0 ± 30.7^{a}	88.5 ± 9.3^{c}	149.2 ± 12.4^{b}	130.5 ± 16.6^{bc}
Daily fecal bile acid excretion, *µmol/d*	39.1 ± 2.5^{a}	32.6 ± 3.0^{ab}	26.6 ± 4.3^{ab}	27.1 ± 1.9^{b}	24.6 ± 2.3^{b}	33.4 ± 2.4^{ab}

Values are means ± SEMs, n=12, except daily fecal bile acid excretion, where n=7-12. Means with a row that do not share a common letter are statistically different, $p < 0.05$. Basal, normal fat cellulose control; HFC, high fat cellulose control; HFPL, high fat polylactose; HFML, high fat matched lactose; HFPD, high fat polydextrose; HFFOS, high fat fructooligosaccharides.

Polylactose is vigorously fermented in the large intestine

Most fermentation by the gut microbiome in the rat occurs in the first part of the large intestine, the cecum. Fermentation lowers the pH of the cecal contents and increases the mass of the cecal tissue [107]. Cecal pH was lower in the HFPL and HFPD animals compared to all other groups, but pH was lower in the HFPL group than in the HFPD group ($p < 0.0001$, Table 3.2). Empty cecum weight was dramatically greater in the HFPL group compared to all other groups ($p < 0.0001$). The HFPD and HFFOS groups also had a significantly greater empty cecum weight compared to the HFC and NFC groups. Total cecal SCFA amount was increased in the HFPL group compared to all other groups ($p < 0.0001$). This was due to greater amounts of acetate and propionate in the cecal contents of the HFPL group compared to all other groups ($p < 0.0001$) (**Fig. 3.1**). Total butyrate amount in the cecal contents did not differ among the groups. Total cecal contents SCFA was strongly correlated with empty cecal weight ($R^2 = 0.788$, $p < 0.0001$, for all animals). Total cecal acetate and propionate were also individually highly correlated with empty cecum weight ($R^2 = 0.792$ and 0.811, respectively, $p < 0.0001$), whereas the amount of cecal contents butyrate showed no correlation to empty cecum weight ($R^2 = -0.105$, $p = 0.40$).

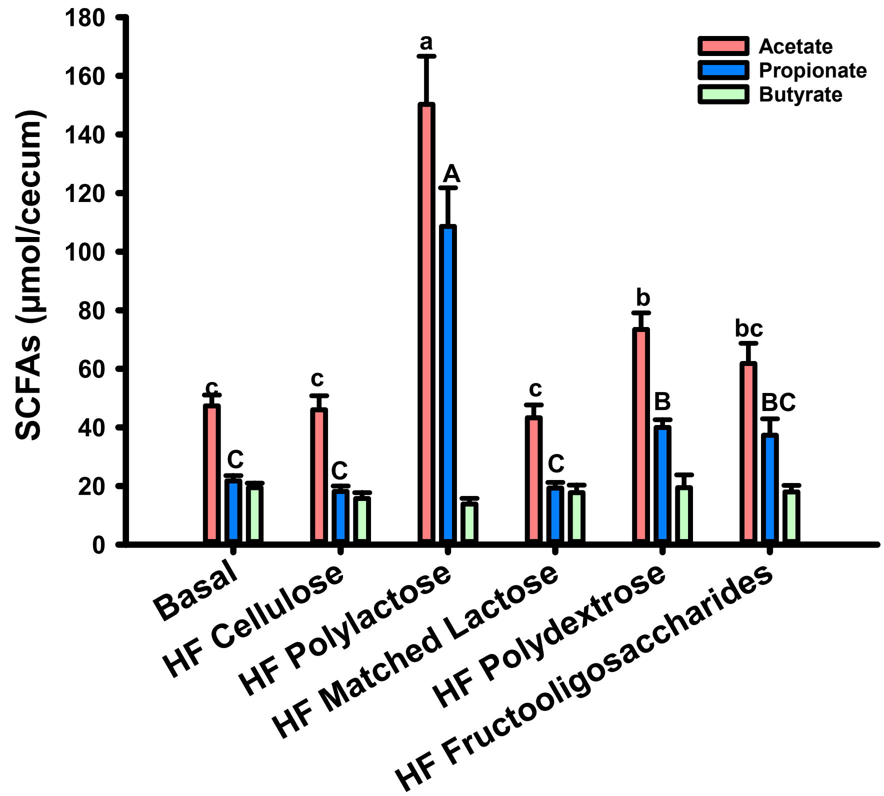

Figure 3.1 Amount of individual short chain fatty acids in cecal contents by dietary group.

Values represent mean ± SEM, n=8-12. Values not sharing a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Polylactose reduced fatty liver in animals fed high fat diets

Liver weights were numerically lower in the HFPL and HFPD groups than the remaining HF-fed groups ($p = 0.0304$ for all groups, Table 3.2). The HFPL group had less liver lipid than the HFC group ($p = 0.0035$), and numerically less than the HFML, HFPD, and HFFOS groups (**Fig. 3.2**). Liver cholesterol was also decreased in the HFPL group compared to the HFC group ($p = 0.0028$) and was similar to the NFC group (Fig.

3.2). Plasma triacylglycerol concentration was lower in all high fat diet groups compared to the NFC group ($p < 0.0001$, **Table 3.3**). Among the high fat diet groups, plasma triacylglycerol concentration was lower in the HFFOS than the HFC group ($p < 0.0001$). Daily fecal total bile acid excretion was not different among the high fat-fed groups, but excretion was less in the HFML and HFPD groups compared to the NFC group (Table 3.3).

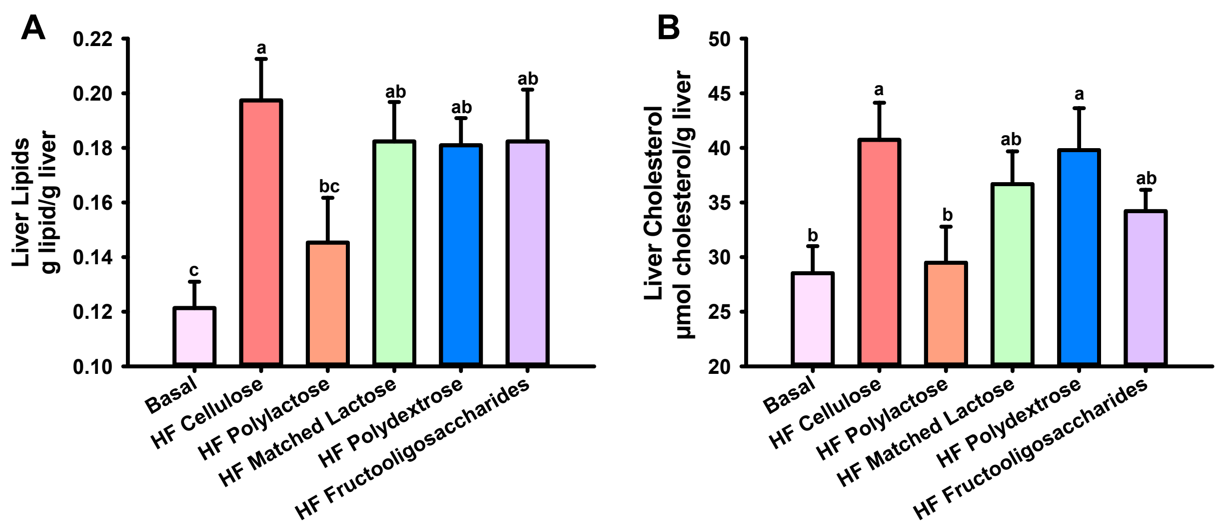

Figure 3.2. Liver lipids and cholesterol in animals fed polylactose, polydextrose, or fructooligosaccharides.

(A) Liver lipid concertation measured gravimetrically. (B) Concentration of cholesterol from lipids extracted from 1 g of liver. Values represent mean ± SEM, n=12. Values not sharing a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Polylactose fed animals trended towards improved glycemic control

There were no significant differences among the groups in fasting plasma glucose (Table 3). Fasting plasma insulin trended towards being greater in the HFC group relative to the NFC group (p = 0.0755). Plasma insulin was significantly greater in the HFPD group than the HFFOS and NFC groups, but the HFC, HFPL, HFML groups did not differ from the HFPD or HFFOS groups (p < 0.05). Pyruvate and insulin tolerance tests showed no differences in glucose response (p = 0.9431, data not shown). However, the HFPL group showed a trend towards a smaller area under the curve for the oral glucose tolerance test than the HFC group (p = 0.056 when compared by Student's t-test, **Fig. 3.3**).

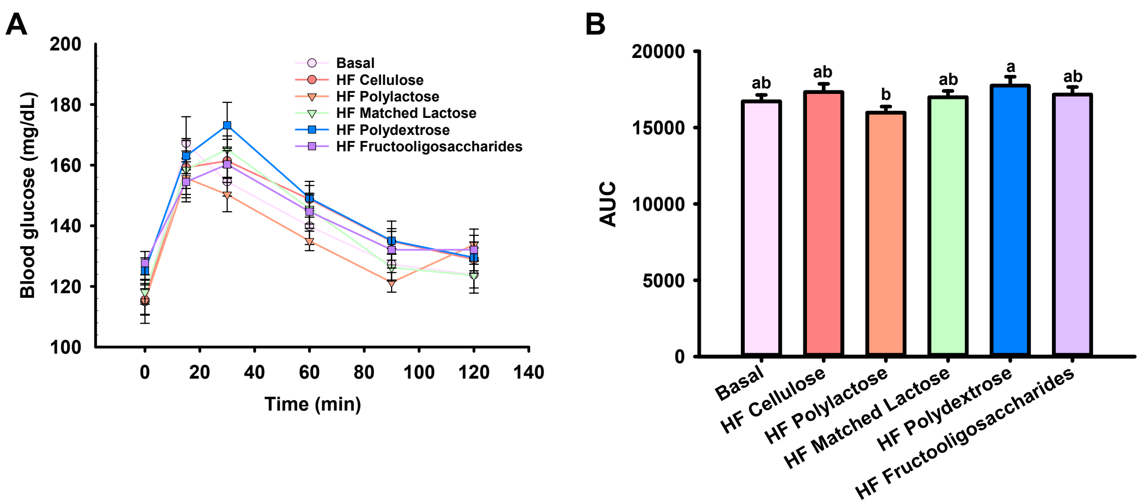

Figure 3.3 Oral glucose tolerance test in animals fed polylactose, polydextrose, or fructooligosaccharides.

(A) Mean OGTT curve by diet group. (B) Area Under the Curve (AUC) for the OGTT. Values represent mean ± SEM, n=12. Values not sharing a common letter are statistically different, p < 0.05, by ANOVA followed by Duncan's Multiple Range Test. For HFC vs HFPL, p=0.056 by Student's t-test.

Polylactose elicits a beneficial change in the gut microbiome

Beta-diversity analysis of the gut microbiome revealed three distinct clusters: control groups (NFC, HFC, and HFML), commercial prebiotics (HFPD, HFFOS), and polylactose (HFPL). The HFPL cluster was most dissimilar from the control groups and in line with, yet separated from, the prebiotic cluster. This indicates greater change in the gut microbiome when compared to the prebiotic groups, as was shown by the Bray Curtis dissimilarity measure of beta-diversity ($p < 0.001$ by diet, **Fig. 3.4**). Taxonomic analysis (**Fig. 3.5**) revealed that the HFPL contained greater *Bifidobacterium* species and Gammaproteobacteria when compared to all other HF groups ($p < 0.0001$). *Lactobacillus* species were also increased numerically in the HFPL group compared to the HFC group. *Akkermansia muciniphila* was increased in the HFPL group when compared to all other HF groups ($p < 0.0001$), and increased in the HFFOS group when compared to the control groups (NFC, HFC, and HFML, $p < 0.0001$) Lastly, the ratio of *Firmicutes* to *Bacteroidetes* (F/B) was reduced in the HFPL group compared to all other groups ($p < 0.0001$, Fig. 3.5). The F/B correlated significantly but inversely with cecal total SCFA amount ($r=-0.377$; $p=0.0021$) and with cecal acetate and propionate amount ($r=-0.399$; $p=0.0012$ and $r=-0.383$, $p=0.0018$, respectively) but not with cecal butyrate amount ($r=0.176$, $p=0.164$).

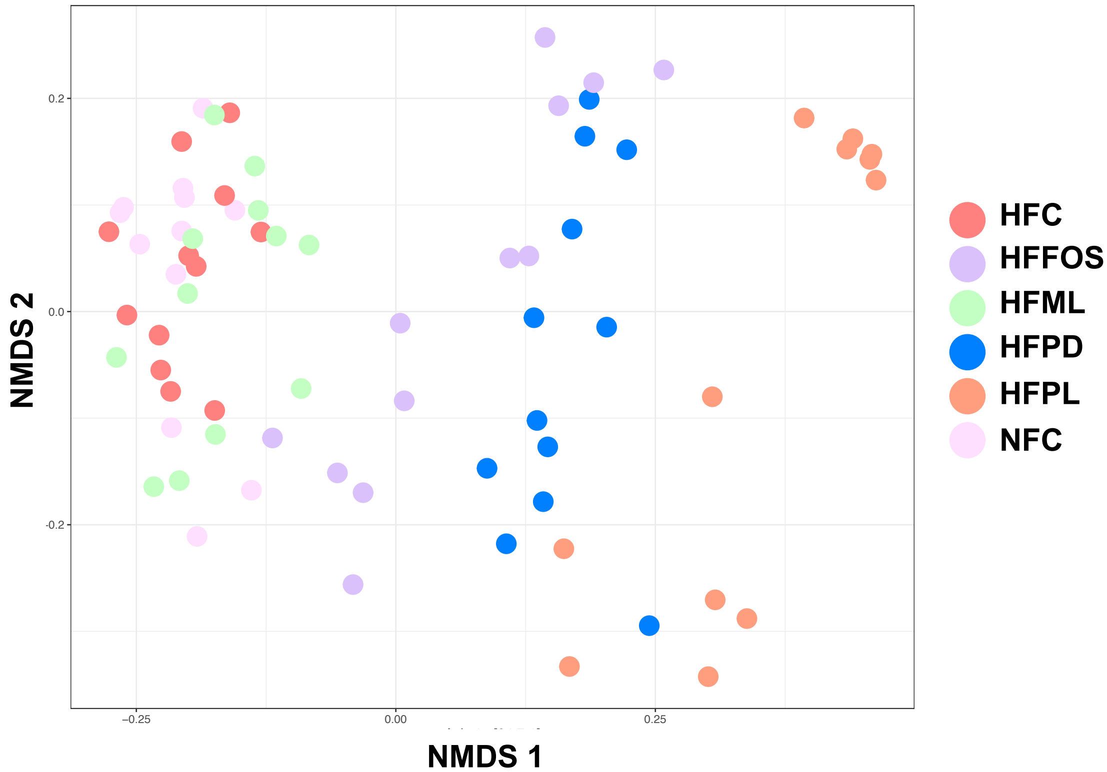

Figure 3.4 Bray Curtis principles coordinate analysis for beta-diversity.

Each point represents an animal, colored by diet. Groups are statistically different by diet, by adonis test.

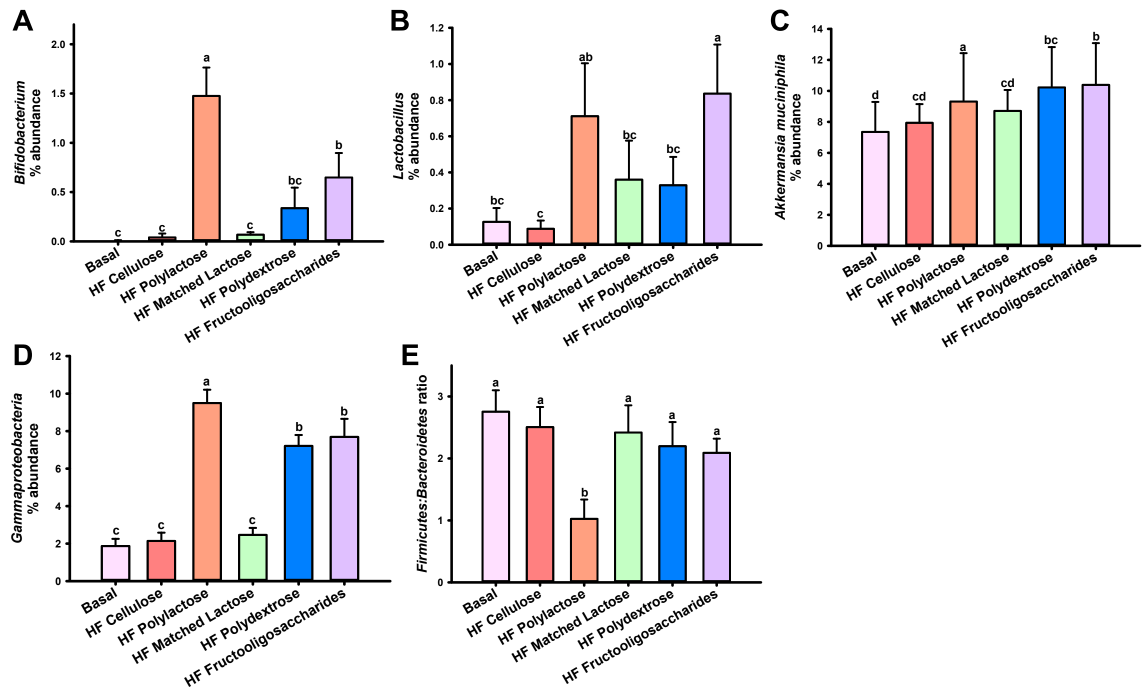

Figure 3.5 Select taxonomy in cecal contents

(A) *Bifidobacterium* (genus), (B) *Lactobacillus* (genus), (C) *Akkermansia muciniphila*, (D) *Gammaproteobacteria* (class), (E) Firmicutes:Bacteroidetes ratio (phyla). Abundance represented as percent (proportion of taxa sequence reads/total reads per sample). Values represent mean ± SEM, n=11-12. Values not sharing a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Discussion

Here we present the first study of prebiotic activity of polylactose. Prebiotic dietary fibers are fibers that produce beneficial changes in the gut microbiome and that provide a health benefit to the host [108]. Studies of commercially available prebiotic dietary fibers, such as polydextrose (PD) and fructooligosaccharides (FOS), have demonstrated their ability to reduce adiposity [56, 61, 63, 90], improve glycemic control [61], and reduce NAFLD outcomes [56, 61, 90] in tandem with altering the gut microbiome [56, 61-63, 90, 91]. Here, we show that polylactose, a novel, synthetic dietary fiber, fulfills the criteria for a prebiotic, in that it elicited a beneficial change in the gut microbiome in a diet-induced obesity animal model, and provided several health benefits, specifically reducing adiposity and fatty liver, and trending towards improvement of glycemic control. In contrast, PD and FOS fed animals, who presented only a modest change in the microbiome, showed no change in adiposity, liver lipids, or improvement in glycemic control at the dietary concentration of 6% used in the present study. Results of other animal studies feeding PD and FOS, at dietary concentrations between 2.5%-21%, have been inconsistent in terms of health benefits, independent of the dose fed [61-63, 90, 91].

In most studies feeding PD and FOS, a change in the gut microbiome has been demonstrated [56, 61-63, 90, 91]. While sometimes only indicated by fermentation in the cecum [63, 91], this still indicates utilization of the fibers by bacteria in the large intestine. This is consistent with our findings that both PD and FOS were fermented in the large intestine, as indicated by a reduction in cecal pH and increase in empty cecum weight and a change in the gut microbiome. However, these differences were to a much lesser degree than with polylactose, which was vigorously fermented and produced dramatic changes in the microbiome. This suggests that polylactose is a better substrate for certain taxa in the gut, such as *Bifidobacterium* and *Lactobacillus* species, leading to greater fermentation. This is further corroborated by the greater amount of total and individual SCFAs in the cecal contents of polylactose-fed animals, specifically an increase in acetate and propionate, with a similar but much more modest trend in the PD and FOS groups. In studies that measured SCFAs in PD and FOS groups, a similar increase in total SCFAs and propionate were demonstrated when compared to the high fat control group [90, 91]. In several studies a high fat diet suppressed SCFA production, relative to a normal fat diet, but FOS or other highly fermentable fibers fed as part of a high fat diet restored SCFA levels to levels found in the normal fat diet group [90, 91]. However, in the present study, there was no difference in cecal contents SCFA amount between the normal fat and high fat groups.

Rodent studies feeding dietary concentrations of PD or FOS similar to that used in the present study, between 2.5-10%, have found variable effects on physiological endpoints. While adiposity was sometimes reduced [56, 61, 63, 90], final body weight typically was reduced as well, suggesting that the prebiotic fibers reduced body size but not necessarily body composition. In the present study, we found that while all groups had statistically similar final body weights, rats fed polylactose had greatly reduced epididymal fat pad weights compared to all other high fat groups. Although there was a numeric reduction in fat pad weight in the FOS group, it did not differ statistically from the HF control. Thus, animals consuming polylactose had a change in body composition, with reduced adiposity. Leptin is an adipokine whose plasma concentration correlates directly with adiposity [105, 106]. Several studies have examined FOS feeding on body weight, fat mass, and circulating leptin. Reimer et al., using a FOS dietary concentration of 21%, found non-significant reductions in body weight and % body fat but significantly lower serum leptin concentration in overnight-fasted rats [61]. In contrast, Cluny et al. fed FOS at 10% of the diet and found lower final body weight but no significant difference in fat mass or plasma leptin compared to the high fat control in non-fasted rats [63]. We found that both FOS and PD groups had plasma leptin concentrations numerically similar to the high fat cellulose group, and not statistically different from any other group. However, plasma leptin trended lower in polylactose fed animals compared to all other HF fed groups and was significantly lower than one of the HF control groups (HFML). This finding is consistent with the finding of lower fat pad mass in the polylactose group but not in the PD and FOS groups.

A common feature of the diet-induced obesity animal model is impaired glycemic control. The AUC of the OGTT of the polylactose fed animals was similar to the normal fat control group (NFC), and was reduced compared to the HFC group. FOS at 6% of the diet did not reduce either fasting plasma glucose or the AUC of the OGTT, although there was a tendency for a reduction in fasting insulin in this group compared to all other HF fed groups. This suggests that polylactose prevented the impairment in glycemic control experienced by rats fed a high fat diet, whereas the commercial prebiotics PD and FOS did not. Consistent with our finding, FOS fed at 5% on a high fat background did not reduce OGTT AUC, fasting glucose, or fasting insulin [91]. Similarly, no reduction in fasting insulin and fasting glucose in rats fed 10% FOS on a high fat background was found by Cluny et al. [63]. In contrast, FOS fed at a higher dose of 21% was reported to reduce the OGTT AUC and ITT AUC when compared to a HF control group [62]. This suggests that a high dietary concentration of FOS is needed to produce an improvement in glycemic control in animals fed high fat diets, a dietary concentration that is perhaps unattainable in a human diet.

The high fat diet-induced obesity model also presents with increased hepatic lipid accumulation [109, 110]. Fatty liver is the first step in the disease etiology of non-alcoholic fatty liver disease (NAFLD), a serious condition with few effective treatments [2, 3, 5, 10]. Simple fatty liver is considered a point for reversal of the disease by dietary- or exercise-based interventions [2, 3, 5, 109]. Presently, there is little evidence for reduced fatty liver by commercial prebiotics, particularly at physiologically attainable doses [61, 62, 90]. While these studies demonstrated reduced liver weights, only Nakamura et al. found a reduction in liver lipids [90]. In contrast, when FOS was fed at a higher dose (21%), liver weight and total liver cholesterol was reduced but liver lipids were not [61]. In the present study, FOS fed animals did not exhibit a statistically significant reduction in liver weight, liver lipid, or liver cholesterol concentration. However, polylactose feeding led to lower liver lipid accumulation. Liver cholesterol concentration was lower in both the polylactose and PD groups compared to the HF cellulose group, indicating reduced cholesterol storage in the liver. For both liver lipids and cholesterol, the polylactose group was similar to that of the NF cellulose group.

Changes in the large intestinal microbiome are a likely explanation for the decrease in adiposity and liver lipids and modest improvement in glycemic control found in the group fed polylactose. This group had greatly increased taxonomical abundance of *Bifidobacterium* and *Lactobacillus* species and Gammaproteobacteria in the cecal contents, all of which are galactose fermenters [111, 112], one of the two monosaccharides composing lactose, which was used to synthesize polylactose. *Bifidobacterium* and *Lactobacillus* are established probiotic organisms. An increase in their abundance has been correlated with reduced fatty liver [17, 24, 39, 40, 113, 114]. Interestingly, fecal Gammaproteobacteria abundance increases after bariatric surgery [115], which is known to reduce fatty liver [116], yet Gammaproteobacteria abundance was slightly but significantly greater in obese children with NAFLD than healthy, normal weight children [117]. Thus, the influence of Gammaproteobacteria on fatty liver warrants further investigation.

These galactose fermenters also produce large amounts of acetate and propionate, typically the two most abundant SCFAs [48, 118, 119], which were in much greater abundance in the cecal contents of the polylactose group relative to all other groups. After uptake into the portal circulation, acetate and propionate are used by the liver for energy metabolism and also regulate lipid synthesis [36, 37, 87, 88]. This is largely via activation (phosphorylation) of AMPK by acetate, increasing lipid oxidation in the liver [36, 37, 87, 88]. Additionally, polylactose, PD, and FOS fed animals all exhibited an increase in *Akkermansia muciniphila* when compared to the non-prebiotic control animals. *Akkermansia muciniphila* is suggested as a beneficial gut bacteria [120] and has been associated with reduced adiposity and improved glycemic control in animal and human studies [39, 46, 51, 121]. While *Akkermansia muciniphila* was increased in abundance in all three prebiotic groups, polylactose fed animals had a statistically significant greater abundance than the other prebiotic groups.

The mechanism by which polylactose may reduce fatty liver and adiposity is uncertain. Viscous dietary fibers are effective at reducing fatty liver and adiposity in animal models, independent of their fermentability [122, 123]. However, polylactose consumption does not increase small intestinal viscosity (data not shown). The *Firmicutes* to *Bacteroidetes* (F/B) ratio shows a modest correlation with BMI in humans [124] and is positively associated with increased adiposity in human and animal studies [125, 126]. Only the polylactose group reduced the *Firmicutes* to *Bacteroidetes* ratio (F/B) relative to the normal fat and high fat cellulose groups, consistent with this association. How changes in the F/B might mediate changes in fatty liver and adiposity is unknown. The correlation in the present study of the F/B with the amount of acetate and propionate in the cecum suggests that SCFA may be the mediator of the effect of F/B on adiposity and fatty liver. Feeding acetate, propionate, or butyrate to mice as part of a high fat diet has been found to attenuate the accumulation of adipose tissue relative to the high fat diet alone [127]. However, our results suggest there is a threshold of SCFA production needed to reduce adiposity and fatty liver, as only polylactose, which produced much greater amounts of cecal SCFA than polydextrose or fructooligosaccharide, reduced adiposity and fatty liver.

In conclusion, our findings demonstrate that polylactose, a novel dietary fiber, behaves as a prebiotic in rats fed a high fat diet. That is, polylactose analyzes as dietary fiber, it changes the large intestinal microbiome in a manner considered to be beneficial, and it provides health benefits to the host, specifically reduced adiposity and liver lipids, thus fulfilling the definition of a prebiotic dietary fiber [108]. Further, polylactose produced these effects at a dietary concentration at which other prebiotic dietary fibers did not provide health benefits. Additional studies of the potential benefits of polylactose consumption seem warranted.

Table 3.4 Diet composition of normal fat cellulose, high fat cellulose, high fat polylactose, high fat matched lactose, high fat polydextrose, and high fat fructooligosaccharides diets.

Constituent (g/kg)	NF cellulose (NFC)	HF cellulose (HFC)	HF polylactose (HFPL)	HF matched lactose (HFML)	HF polydextrose (HFPD)	HF fructooligosaccharides (HFFOS)
Cornstarch	337.45	202.47	176.58*	202.47	202.47	202.47
Casein	200	200	200	200	200	200
Dextrinized Cornstarch	132	132	132	132	132	132
Sucrose	100	50	42.495*	50	50	50
Soybean Oil	28.89	66.66	66.66	66.66	66.66	66.66
Lard	86.66	200	200	200	200	200
Mineral mix	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
TBHQ	0.01	0.01	0.01	0.01	0.01	0.01
Cholesterol	1	1	1	1	1	1
Cellulose	90	90	3.76*	63.76	30	30

Polylactose mixture	0	0	119.69*	0	0	0
Lactose	0	0	0	26.24	0	0
Polydextrose	0	0	0	0	60**	0
Fructooligosaccharides	0	0	0	0	0	60***
TOTAL	1025.51	991.64	991.70	991.64	991.64	991.64
% kcal, Carbohydrate	55.16	32.38	32.38	32.38	32.38	32.38
% kcal, Protein	19.66	17.10	17.10	17.10	17.10	17.10
% kcal, Fat	25.18	50.53	50.53	50.53	50.53	50.53
% wt, Dietary fiber	9	9	9	9	9	9

*HFPL diet was adjusted for cornstarch, sucrose, and cellulose to account for other constituents of the polylactose mixture. Cornstarch was removed to account for remaining "unknown" products, sucrose was removed to account for free glucose, and cellulose was removed to account for the non-fermented, non-digestible free lactose.
**Polydextrose (Litesse Polydextrose, Danisco USA, Terre Haute, IN) contained 93.6% PD fiber, 5.6% glucoses, 1.6% sorbitol.
***Fructooligosaccharides mixture (NutraFlora L95-S, Ingredion, Westchester, IL) contained 95% FOS fiber, 5% sugars.
Diets are modified AIN-93G, where 0.1% cholesterol is added to all diets and fat is increased for the HF diets. Vitamin and mineral mixes are from Dyets Inc. and formulated based on AIN-93G.

Chapter 4: Prebiotic activity of polylactose fed at two different dietary concentrations.

Introduction

Obesity afflicts approximately 39% of the global population and is accompanied by several comorbidities [1]. One of these comorbidities, non-alcoholic fatty liver disease (NAFLD), affects approximately 25% of adults worldwide, with increasing prevalence in developed countries [2, 3]. While obesity has established treatments, most successfully through diet and lifestyle changes, NAFLD does not. Currently, the only treatment for NAFLD is weight loss, by whatever means possible [2, 3].

Recent studies in both humans and animals indicate an influence of the gut microbiome on both of these disease states [17, 38, 39, 41, 46-52]. Differential abundances of specific taxa have been correlated with obesity and non-alcoholic fatty liver disease (NAFLD) [28, 31, 32]. Increased abundance of the phylum Firmicutes and/or a reduction in Bacteroidetes (or increased Firmicutes:Bacteroidetes ratio) has been correlated with increased adiposity in humans [25-27, 29, 30, 32, 34, 58, 59] and animals [55]. Similarly, human studies have shown correlations between lean subjects and increased abundances of *Bifidobacterium* [60] and/or *Lactobacillus* [29, 59]. This provides evidence that the abundance of specific taxa has an influence on host health.

One method to alter the gut microbiome somewhat predictably is through the use of prebiotics. Prebiotics are defined as indigestible substrates that are fermented in the large intestine, elicit a beneficial change in the gut microbiome, and provide a health benefit to the host [89]. In a previous study, we demonstrated that polylactose, a novel dietary fiber, behaved as a potent prebiotic in rats fed high fat diets. Compared to the established prebiotics polydextrose (PD) and fructooligosaccharides (FOS), polylactose was more vigorously fermented, elicited a more dramatic change in the gut microbiome, reduced fatty liver, and prevented obesity in rats fed high fat diets [**Chapter 3**]. This finding is consistent with the literature, where PD and FOS do not consistently provide health benefits to the host, partially independent of dose [55, 56, 61-63, 90, 91].

In the present study, we aimed to reproduce the results from the first polylactose feeding study [**Chapter 3**], to continue comparisons to established prebiotics (PD and galactooligosaccharides, GOS), to explore a dose-response response relationship for polylactose, and to better understand mechanisms of crosstalk between the gut microbiome and host tissues.

Materials and Methods

Polylactose preparation

Polylactose was produced as previously reported [95], modified as described in Chapter 3 of this thesis. Due to increased extruder and purification efficiency, this preparation contained 72.5% soluble polylactose fiber, 9% residual lactose, 3% glucose, and 15% other materials (**Table 4.1**).

Table 4.1 Composition of partially purified polylactose.

Dietary fiber (soluble), %	Lactose, %	Glucose, %	Citric acid, %
72.45 ± 0.35	9.25 ± 1.23	2.83 ± 0.21	0.04 ± 0.03

Number of determinations per analyte = 4. Values are means ± standard deviation.

Animals and experimental design

Seventy-two male Wistar rats (75-99 g, 4-5 weeks of age, Envigo Labs, Indianapolis, IN) were fed experimental diets for 10 weeks after 2 weeks of adaptation to the basal diet. All animals were randomly assigned to individual, hanging wire cages and kept on a 12-h light/dark cycle. Food and water were provided ad-libitum for the duration of the study, with food intakes monitored weekly. All animal use and procedures were approved by the University of Minnesota Animal Care and Use Committee (animal use protocol #1901-36703A).

The animals were divided into 6 dietary groups, with 12 animals per group. The groups include normal fat cellulose (basal, modified AIN-93G diet), high fat cellulose (HFC, 9% cellulose), high fat polylactose 6% (HFPL, 6% polylactose fiber, 3% cellulose), high fat polylactose 3% (HFPL3%, 3% polylactose fiber, 6% cellulose), high fat polydextrose (HFPD, 6% PD, 3% cellulose), and high fat galactooligosaccharides (HFGOS, 6% GOS, 3% cellulose). The composition of the diets is shown in **Table 4.3**

After 8 weeks on the experimental diets, the animals were transported by the University of Minnesota Research Animal Resources to the Minneapolis Veterans Medical Center in Minneapolis, MN where body composition was determined by magnetic resonance imaging following an overnight fast.

At the end of the 10th week on the experimental diets, animals were anesthetized with isoflurane and blood collected by cardiac puncture. Blood was centrifuged at 3,000 *g* for 20 minutes and serum aliquots were stored at -80° C until use. Ceca were harvested, cecal contents pH was measured with a spear-tip electrode, and contents were expressed into sterile 15 mL conical tubes, frozen on dry ice, and stored at -80° C. Empty ceca were rinsed, weighed, and discarded. One epididymal fat pad from each animal was harvested, rinsed, weighed, frozen on dry ice, and stored at -80° C. Livers were excised, rinsed, weighed, frozen on dry ice, and stored at -80° C until further analysis.

Blood glucose, insulin, and adipokines

Fasting blood glucose at time of harvest was measured spectrophotometrically by an enzymatic method using serum [128]. Fasting insulin was also measured enzymatically (Invitrogen, #ERINS) using serum taken at time of harvest. Circulating leptin was quantified by ELISA (Invitrogen, #KRC2281) using serum samples from the time of harvest.

Liver lipids and cholesterol

Approximately 1 g of liver from each animal was homogenized and lipids extracted using chloroform:methanol (2:1) [97]. Extracted lipids were dried under N_2 gas and quantified gravimetrically. Lipid samples were reconstituted with 10 mL chloroform:methanol (2:1) and cholesterol was measured enzymatically using a previously described method [98].

Adipocyte and lipid droplet size distribution

Adipocyte and lipid droplet size distribution were quantified by flow cytometry. Adipocytes were isolated by collagenase digestion as described by Majka et al. [129]. Adipocyte and lipid droplet size distribution determination by flow cytometry was carried out by a modification of the method of Majka et al. [129]. Briefly, isolated adipocytes were stained with LipidTox deep red (LTX; LifeTechnologies, #H34477) and DyeCycle

Violet (DCV; LifeTechnologies, #V35003) and analyzed at the University Flow Cytometry Resource (Minneapolis, MN). Laser voltage parameters on the BD LSRII H4760 flow cytometer (BD Biosciences) were set to 494 forward scatter (FSC), 255 side scatter (SSC), 333 LipidTox (633 nm), 300 DyeCycle Violet (407 nm). Cells were gated for size (FSC-A x SSC-A) and roundness, followed by singularity (DCV-A x DCV-H), and lipid events (LTX-A). Single, lipid containing cells were further gated into three horizontal tertiles for cell size (FSC-A x LTX-A) and three vertical tertiles for lipid droplet size (LipidTox intensity).

Microbiome sequencing

Cecal contents were thawed on ice and mixed prior to use. DNA was extracted from approximately 180-220 mg of contents using the QiAmp DNA Stool Mini kit (Qiagen, #51504) and DNA eluates quantified on a DU 730 Life Science UV/Vis Spec (Beckman Coulter). DNA eluates were then submitted to University of Minnesota Genomics Center for sequencing of the 16S ribosomal subunit with the V5/V6 region amplicon on an Illumina MiSeq using 2x300 bp paired end reads. Data was analyzed using DADA2 and phyloseq in R.

Short chain fatty acids

Short chain fatty acids (SCFAs) were extracted from cecal contents with diethyl ether acidified with sulfuric acid and measured by gas chromatography on a Supelco Omegawax capillary column [99-101]. Un-derivatized SCFAs were quantified using 2-ethyl butyrate as an internal standard.

Statistical analysis

All data except microbiome data was analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SAS (version 9.4, SAS Institute, Cary, NC). Microbiome data was analyzed in R [104] using DADA2 and phyloseq packages for determination of beta diversity, taxonomical abundance, and dissimilarity by adonis. The data for bifidobacterial abundance was semi-continuous, as there were values of zero. Thus, this parameter was evaluated with a zero-inflated gamma model using PROC FMM in SAS, employing the maximum likelihood estimation method.

Results

Polylactose did not influence body weight or food intake

Daily average food intake was less in the high fat-fed groups than the basal group but did not differ among the high fat groups (**Table 4.2**). Similarly, the basal group had a statistically significant lower final body weight compared to all HF groups, but there were no differences in final body weight or change in body weight among the high fat groups (**Fig. 4.1A**).

Table 4.2. Energy intake, change in body weight, cecal pH, cecum weight, liver weight, and blood biomarkers in rats fed polylactose, polydextrose, or galactooligosaccharides.

Variable	Basal	HFC	HFPL	HFPL3%	HFPD	HFGOS
Energy intake, *kJ/day*	339.0 ± 14.5	363.4 ± 10.1	347.5 ± 13.3	341.3 ± 12.8	341.9 ± 11.4	338.2 ± 11.6
Change in body weight, *g*	289.4 ± 9.1^{b}	333.9 ± 11.7^{a}	347.4 ± 14.0^{a}	339.9 ± 10.5^{a}	336.5 ± 8.1^{a}	334.0 ± 9.7^{a}
Empty cecum weight, *g*	0.59 ± 0.05^{d}	0.59 ± 0.02^{d}	1.32 ± 0.07^{a}	0.86 ± 0.03^{c}	1.01 ± 0.05^{b}	0.93 ± 0.04^{bc}
Cecal pH	6.71 ± 0.09^{a}	6.63 ± 0.09^{ab}	6.31 ± 0.07^{cd}	6.45 ± 0.07^{bc}	6.15 ± 0.05^{d}	6.76 ± 0.06^{bc}
Liver weight, *g*	12.08 ± 0.49^{b}	14.28 ± 0.67^{a}	13.90 ± 0.82^{a}	14.12 ± 0.63^{a}	14.32 ± 0.46^{a}	14.94 ± 0.61^{a}
Leptin, *ng/mL*	33.47 ± 4.96	47.89 ± 8.31	48.91 ± 11.14	58.72 ± 7.50	43.67 ± 7.85	46.32 ± 8.27
Serum insulin, *μIU/mL*	6.33 ± 0.40	5.37 ± 0.68	5.44 ± 0.59	5.29 ± 0.45	6.02 ± 0.70	4.92 ± 0.66
Fasted serum glucose, *mg/dL*	89.28 ± 2.63	99.79 ± 3.37	91.26 ± 4.49	89.63 ± 2.52	93.98 ± 4.31	94.91 ± 2.99
Total short chain fatty acids, *μmol*	252.3 ± 23.36^{b}	194.8 ± 11.23^{b}	376.7 ± 46.15^{a}	221.6 ± 19.65^{b}	272.6 ± 40.87^{b}	269.2 ± 24.35^{b}

Values are means ± SEMs, n=8-12. Means with a row that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test. Basal, normal fat cellulose control; HFC, high fat cellulose control; HFPL, high fat polylactose 6%; HFPL3%, high fat polylactose 3%; HFPD, high fat polydextrose; HFGOS, high fat galactooligosaccharides.

Polylactose did not reduce adiposity in rats fed high fat diets

Magnetic resonance imaging revealed no significant differences in body composition among any of the groups. There was a trend for a lower percent body fat in the HFPL group compared to the HFPL3% group (**Fig. 4.1C**). Additionally, the HFGOS group had significantly greater percent lean mass when compared to the HFPL3% group only (**Fig. 4.1D**). Epididymal fat pad weights were statistically similar across all HF groups, with a numerically lower epididymal fat pad weight in the basal group ($p < 0.05$ compared to HFPL3% only) (**Fig.4.1B**). Additionally, circulating leptin concentration, which correlates with total body fat [43, 44], did not differ among the groups (**Table 4.2**).

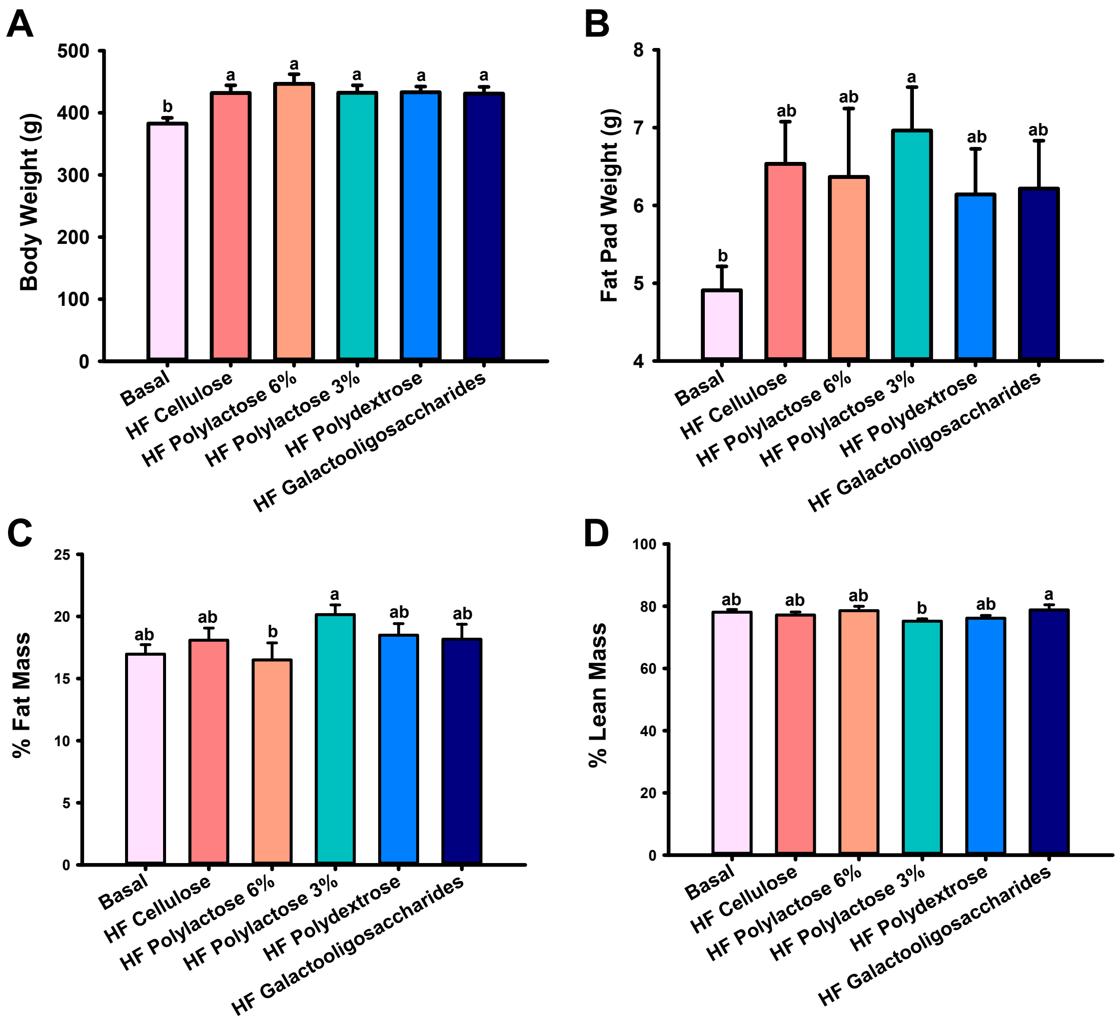

Figure 4.1 Body weight and adiposity in rats fed polylactose, polydextrose, or galactooligosaccharides.

(A) Final body weight (B) Epididymal fat pad weight (C) Percent fat mass by MRI. (D) Percent lean mass by MRI. Values represent mean ± SEM, n=12. Values not sharing a common letter are statistically different, $p < 0.05$.

Lipid droplet size distribution varied in the adipocytes from polylactose fed animals

Adipocyte and lipid droplet size distribution was analyzed in the basal, HFC, HFPL, HFPD groups by flow cytometry. Adipocyte size distribution differed only among the large cells in the four groups analyzed, where the HFPL group had significantly greater proportion of large cells than the HFPD group (**Fig. 4.2A**). Lipid droplet size distribution also differed among the groups. The HFPL group had a greater proportion of small lipid droplets compared to the basal group, and a smaller proportion of large lipid droplets in adipocytes compared to the HFC and HFPD groups (**Fig. 4.2B**).

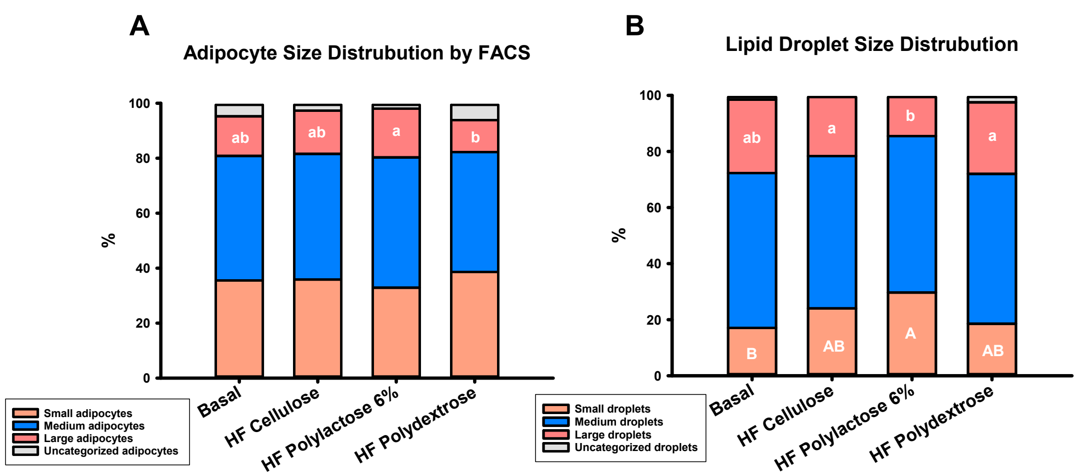

Figure 4.2 Adipocyte and lipid droplet size distribution by flow cytometry.

(A) Adipocyte size distribution determined by forward scatter, expressed as percent of adipocytes. (B) Lipid droplet size distribution determined by LipidTox intensity, expressed as percent of adipocytes. Values represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Polylactose fiber is vigorously fermented in the large intestine

Most fermentation by the gut microbiome in the rat occurs in the first part of the large intestine, the cecum. Greater fermentation is indicated by an increase in cecal tissue weight and decrease in cecal contents pH [45]. Cecal contents pH was lowest in the HFPL and HFPD groups compared to all other groups (**Table 4.2**). The HFPL3% group cecal contents pH tended to be lower than the basal, HFC, and HFGOS groups, but did not differ from them statistically. Further, the HFPL group had the heaviest ceca (empty) and was significantly different from all other groups (**Table 4.2**). The HFPD, HFGOS, and HFPL3% groups all had heavier ceca than the basal and HFC groups.

Cecal contents short chain fatty acid (SCFA) analysis revealed greater amounts of acetate and propionate in the cecal contents of the HFPL group compared to all other groups ($p < 0.0001$, **Figure 4.3**). Acetate amount in the cecal contents did not differ among the other groups, but there was a statistically significantly greater amount of propionate in the HFPD and HFGOS groups compared to the HFC group. Total cecal SCFA amount was greater in the HFPL group compared to all other groups ($p < 0.0001$), which did not differ from each other.

Total cecal SCFAs were strongly correlated with empty cecal weight ($R^2 = 0.509$, $p < 0.0001$, for all animals). Total cecal contents acetate and propionate were also individually highly correlated with empty cecum weight ($R^2 = 0.535$ and 0.609, respectively, $p < 0.0001$). The F/B ratio was inversely correlated with the amount of SCFAs in the cecal contents ($R^2 = -0.322$, $p < 0.0155$) and positively correlated with the concentration of cecal contents SCFAs ($R^2 = 0.719$, $p < 0.0001$).

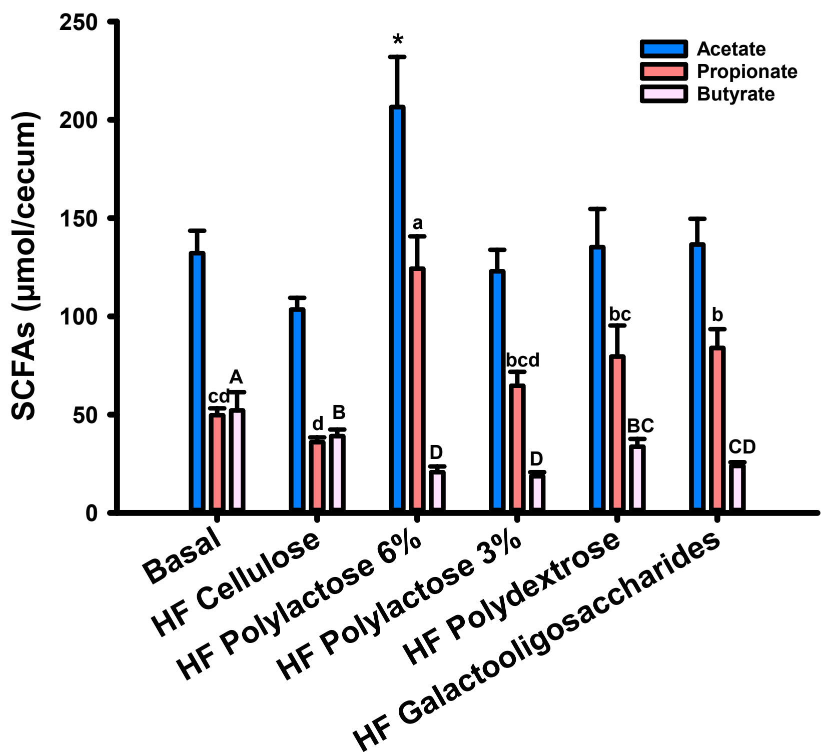

Figure 4.3 Amount of individual short chain fatty acids in cecal contents by dietary group.

Values represent mean ± SEM, n=8-12. Values not sharing a common letter are statistically different, $p < 0.05$. * indicates significantly different from all other groups for acetate.

Changes in the gut microbiome were similar across prebiotics

Beta-diversity analysis of the gut microbiome, as shown by a Bray Curtis plot, revealed two clusters (**Fig. 4.4**). Both polylactose groups (HFPL, HFPL3%) were clustered with the commercial prebiotic groups (HFPD, HFGOS), while the controls (basal, HFC) were clustered separately. This suggests a similar gut microbiome among all prebiotic groups.

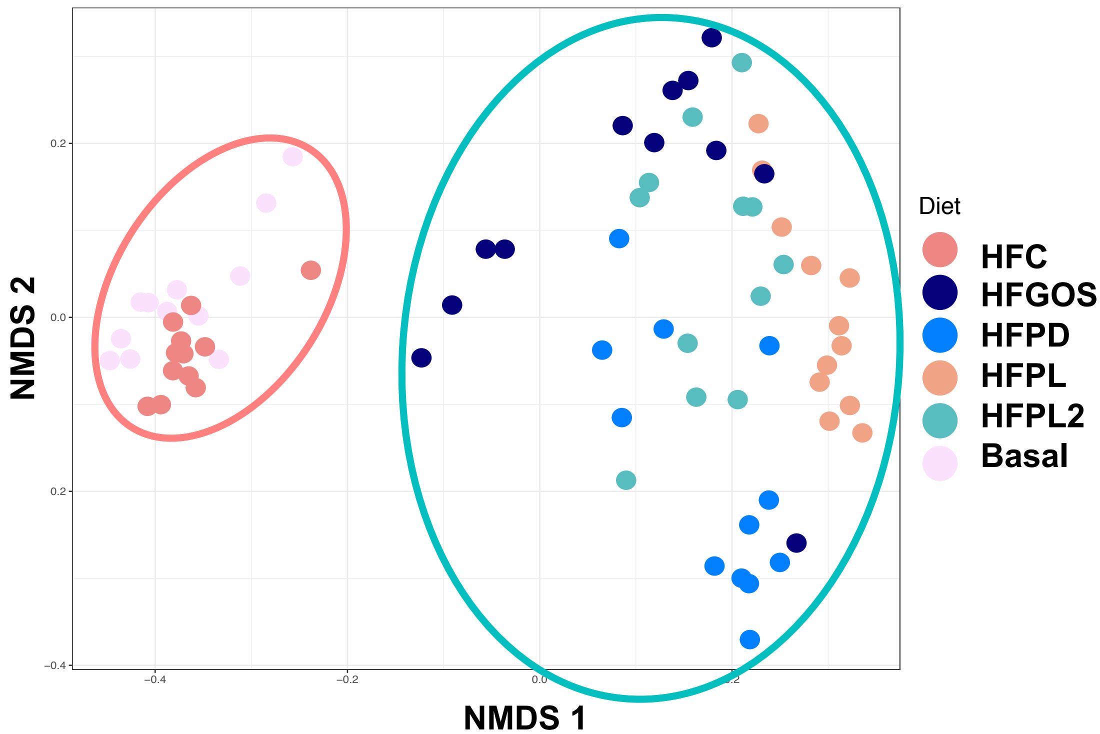

Figure 4.4 Bray Curtis principles coordinate analysis for beta-diversity.

Each point represents an animal, colored coded by diet. Clusters are statistically different by diet, by adonis test.

Taxonomic analysis (**Figure 4.5**) revealed a trend towards greater *Bifidobacterium spp.* abundance in the HFGOS group compared to all other groups ($p=0.068$). *Lactobacillus spp.* abundance was greater in the HFC ($p=0.018$) and HFPL ($p=0.027$) groups relative to the basal group. Both HFGOS and HFPD showed a trend towards a greater abundance than the basal group ($p=0.054$ and $p=0.058$, respectively). The Firmicutes:Bacteroidetes (F:B) ratio was reduced in all prebiotic groups (HFPL, HFPL3%, HFPD, HFGOS) compared to the control groups (basal, HFC).

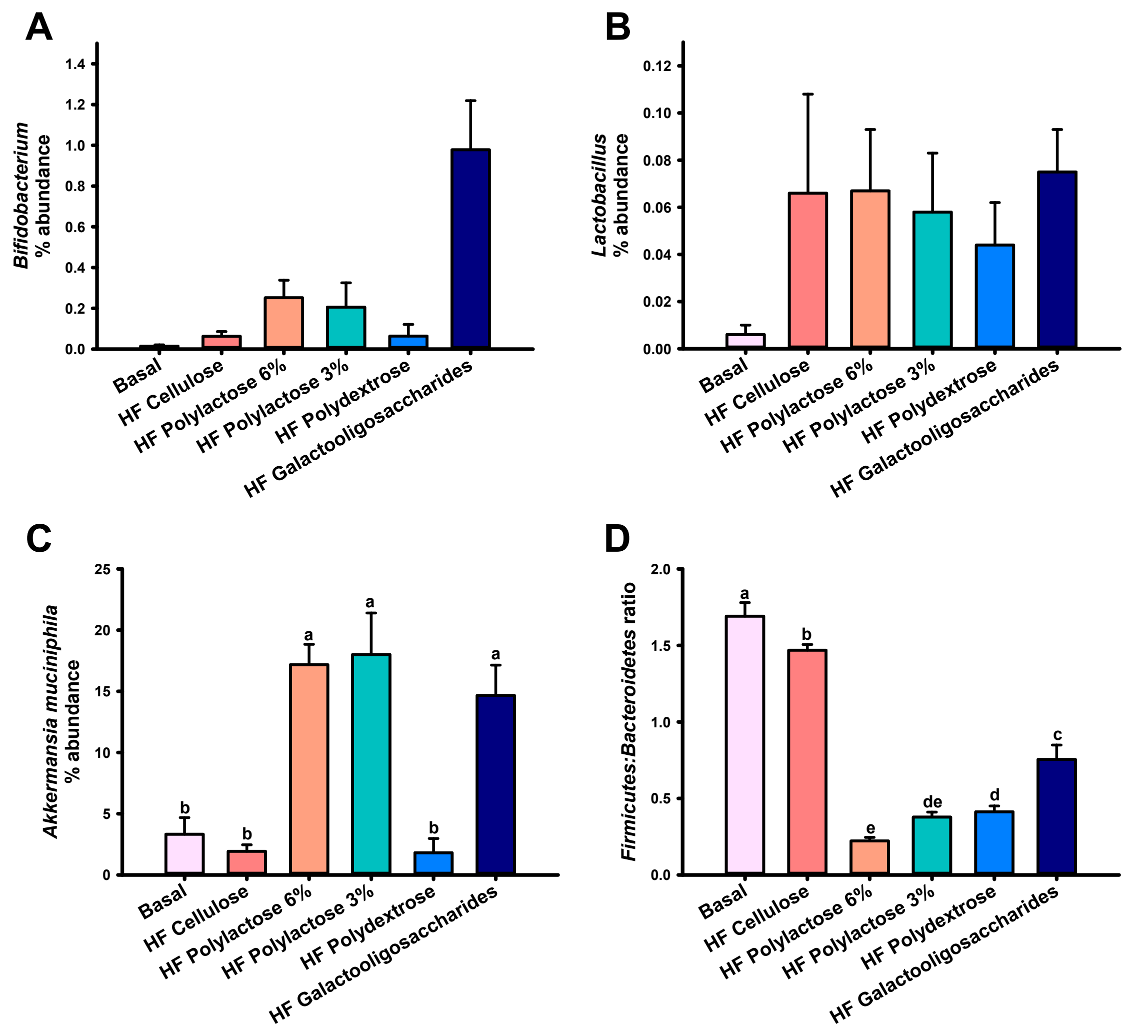

Figure 4.5 Select taxonomy in cecal contents

(A) *Bifidobacterium* (genus), (B) *Lactobacillus* (genus), (C) *Akkermansia muciniphila*, (D) Firmicutes:Bacteroidetes ratio (phyla). Abundance represented as percent (proportion of taxa sequence reads/total reads per sample). Values represent mean ± SEM, n=11-12. Values not sharing a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test or zero-inflated gamma model for *Bifidobacterium.*

Polylactose did not reduce liver lipids

There were no differences in liver weight among the HF groups (**Figure 4.6**), which were all heavier than the basal group. Liver total lipids and liver cholesterol concentration also did not differ among the HF groups, which were all greater than the basal group.

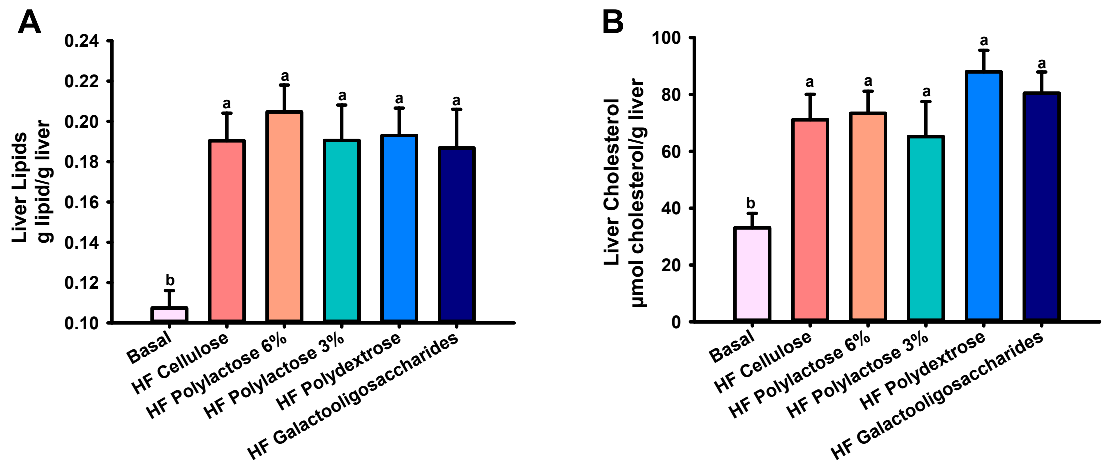

Figure 4.6 Liver lipids and cholesterol in animals fed polylactose, polydextrose, or galactooligosaccharides.

(A) Liver lipid concertation measured gravimetrically. (B) Concentration of cholesterol from lipids extracted from 1 g of liver. Values represent mean ± SEM, n=12. Values not sharing a common letter are statistically different, $p < 0.05$.

Polylactose did not improve glycemic control

There were no differences in fasted blood glucose among any of the groups (**Table 4.2**). Likewise, there were no differences in fasting circulating insulin among the groups (**Table 4.2**).

Discussion

In a previous study, we demonstrated that polylactose was vigorously fermented in the large intestine, elicited a beneficial change in the gut microbiome, and provided multiple health benefits to the host, including reducing adiposity and fatty liver, in rats fed high fat diets. These results indicated that the preparation of polylactose used in the previous study exhibited prebiotic activity. After concentrating soluble polylactose fiber and reducing other materials in the polylactose preparation in the present study, the preparation no longer provided the health benefits seen in the previous study. This suggests that the two polylactose preparations differed in an important way. Interestingly, the polylactose preparation used in the present study, regardless of dose (6% or 3%), behaved similarly to each of the established prebiotic tested at 6% in the diet. The similarities in the gut microbiomes of these groups, along with the lack of health benefits seen in this study, suggests that a threshold of beneficial, probiotic bacteria in the gut must be achieved in order to manifest those effects.

Results of studies feeding FOS, GOS, and PD vary considerably and often fail to provide health benefits, even after taking dose into account. However, taxonomic abundances in the gut microbiomes could provide some insight into these inconsistencies [56, 61-63]. Specifically, *Bifidobacterium* and *Lactobacillus spp.* are commonly reported at increased abundance in prebiotic fed groups where a reduction in fatty liver and adiposity is seen [55-57]. However, even in the presence of increased abundance of *Bifidobacterium,* reduced fatty liver and adiposity are not always demonstrated [61-63]. Reimer et al. reported an increase in empty cecum weight with an increase in cecal

Bifidobacterium after feeding FOS at a dietary concentration of 21% [61]. They also found non-significant reductions in body weight and fat mass but significantly lower serum leptin concentration in overnight-fasted rats [61]. In a further analysis of the same animal study, no differences in liver lipids were found among any of the groups [62]. Thus, even at this high dietary concentration of prebiotics, and despite an increase in *Bifidobacterium,* only modest health benefits were achieved. In contrast, Mistry et al. found that GOS-fed mice had increased cecal *Bifidobacterium* and *Akkermansia muciniphila,* and reduced Firmicutes [57]. They also demonstrated that epididymal fat pad weight, which strongly correlates with total body adiposity, was reduced in GOS-fed animals. Further, Mistry et al. found that the GOS group trended toward a reduction in liver lipids compared to the HF control group [57]. This indicates that GOS, when fed at 7% wt/wt of the diet, can increase beneficial gut bacteria and possibly reduce fatty liver in mice fed HF diets. This is also in contrast to the present study, where feeding 6% GOS had modest effects on the gut microbiome but no impact on host physiology. When taken together, this supports the concept that a certain level of increase in probiotic gut bacteria is needed in order to see host health benefits.

In the absence of dietary interventions with prebiotics, correlations between taxonomic abundances and adiposity have been demonstrated. In animal studies, differential abundances of specific taxa have been correlated with disease states including obesity and non-alcoholic fatty liver disease (NAFLD) [28, 31, 32]. Similarly, in humans, increased abundances of *Bifidobacterium* [60, 92] and/or *Lactobacillus* [29, 59] have been correlated with reduced adiposity. Further, increased adiposity has been correlated with increased abundances of the phylum Firmicutes, and a reduction in Bacteroidetes (or, an increase in the Firmicutes:Bacteroidetes ratio) [25-27, 29, 30, 32, 34, 58, 59]. In this study, there was a reduction in F/B ratio in prebiotic fed animals, despite no reductions in adiposity. This suggests that prebiotic feeding reduces the F/B ratio but is not the driving force for reduced adiposity. Others have also reported that a reduced F/B ratio is not consistently associated with reduced adiposity [27, 29, 30, 32]. In early microbiome studies, Ley et al. demonstrated that a feature of the ob/ob mouse enterotype was an increased F/B ratio compared to that of their ob/+ and +/+ lean siblings [27]. Alternatively, Schwiertz et al. and two reports by Duncan and colleagues found the opposite, that increased F/B ratios were found in individuals with normal BMIs compared to those with overweight or obesity [30, 130, 131]. The inconsistencies in the literature about the correlations of the F/B ratio with adiposity align with the present findings, where a reduced F/B ratio is not correlated with reduced adiposity.

With a reduction in the F/B ratio, an increase in total cecal SCFAs is expected, as Bacteroidetes are excellent SCFA producers [83]. SCFA production by the gut microbiome is thought to be a key determinant in regulating host health, including adiposity and fatty liver [37, 49, 52, 80-82]. Here, we demonstrate that an increased abundance of SCFAs in the cecal contents did not correspond to physiological benefits. They do, however, have an inverse correlation with the Firmicutes:Bacteroidetes ratio. Bacteroidetes are known SCFA producers [83], thus, having an increased abundance of this phylum would be expected to result in increased SCFAs in the ceca, as was seen in the present study. Aside from that, physiological differences (fatty liver, adiposity) had no correlation to SCFA abundance or concentration in the ceca. Together, these data question whether SCFAs are the driving microbial products for crosstalk between the gut microbiome and host tissues (specifically adipose and liver). It also questions whether changes in the F/B ratio, even the large changes found in the present study, drive decreases in adiposity or fatty liver.

Other molecules secreted by gut bacteria, such as lipopolysaccharide (LPS), may influence host physiology. LPS, which was not measured in the present study due to interference of blood components with the assay, is secreted by gram negative bacteria, such as Bacteroidetes [24, 75]. It has been shown that LPS released from gram negative bacteria can disrupt tight junctions in the intestine, which can lead to a leaky gut and metabolic endotoxemia [24, 75]. The presence of LPS in the circulation can lead to increased inflammation via activation of toll-like receptor-4 (TLR4), which increases TNFα production and release by adipose tissue [24, 75-79]. Increased systemic inflammation induced by LPS through TNFα contributes to NAFLD progression by promoting inflammation in the liver alongside steatosis, which occurs in NASH [6]. Establishing a better method for measuring LPS is needed in order to establish its involvement in gut-liver crosstalk relevant to this research.

Overall, our study suggests that differences in the preparation of polylactose, leading to differences in the other constituents in the mixture, is important for eliciting specific changes in the gut microbiome and host health effects. It also provides evidence that a certain threshold of beneficial gut bacteria needs to be achieved to see reductions in fatty liver and adiposity in rats fed high fat diets. Lastly, our findings challenge the role of SCFAs as the primary mechanism of crosstalk between the gut microbiome and host tissues, although there may exist a threshold of SCFA production that will lead to beneficial physiological effects.

Table 4.3 Diet ingredients of basal, high fat cellulose, high fat polylactose, high fat polylactose 3%, high fat polydextrose, and high fat galactooligosaccharides diets.

Constituent (g/kg)	Basal	HF cellulose (HFC)	HF polylactose (HFPL)	HF polylactose 3% (HFPL3%)	HF polydextrose (HFPD)	HF galactooligosaccharides (HFGOS)
Cornstarch	312	210	210	210	210	210
Casein	200	200	200	200	200	200
Dextrinized Cornstarch	132	132	132	132	132	29.7***
Sucrose	100	50	40*	45*	48**	8.4***
Soybean Oil	30	66.66	66.66	66.66	66.66	66.66
Lard	85	200	200	200	200	200
Mineral mix	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
TBHQ	0.014	0.01	0.01	0.01	0.01	0.01
Cholesterol	1	1	1	1	1	1

Cellulose	90	90	17.24*	53.62*	30	30
Polylactose	0	0	82.75*	41.38*	0	0
Polydextrose	0	0	0	0	62**	0
GOS	0	0	0	0	0	211***
TOTAL	1000.5	1000.2	1000.2	1000.2	1000.3	1007.3
% kcal, Carbohydrate	54.09	32.80	32.24	32.52	32.80	32.80
% kcal, Protein	20.18	16.99	17.13	17.06	16.99	16.99
% kcal, Fat	25.73	50.21	50.63	50.42	50.21	50.21
% wt, Dietary fiber	9	9	9	9	9	9

*HFPL, HFPL3%, HFPD, and HFGOS diets were adjusted for dextrinized cornstarch, sucrose, and cellulose to account for other constituents of the prebiotic fiber mixtures. Prebiotics were added at a weight to equal 6% wt/wt of the diets (except for HFPL3%, which contained 3% polylactose fiber). Dextrinized cornstarch was removed to account for maltodextrin; sucrose was removed to account for free glucose, galactose, and lactose; and cellulose was removed to account for "other" materials.

**Polydextrose (Litesse Polydextrose, Danisco USA, Terre Haute, IN) contained 93.6% PD fiber, 5.6% glucoses, 1.6% sorbitol.
***Galactooligosaccharides mixture (Vivinal GOS, FrieslandCampina, The Netherlands) contained 28.5% GOS fiber, 48.5% maltodextrin, 10.1% lactose, and 9.7% free sugars.
Diets are modified AIN-93G, where 0.1% cholesterol is added to all diets and fat is increased for the HF diets. Vitamin and mineral mixes are from Dyets Inc. and formulated based on AIN-93G.

Chapter 5: Prebiotics must achieve a threshold of increase of gut probiotics to impart beneficial health effects on the host

Abstract

Introduction

Prebiotic dietary fibers are dietary fibers that are highly fermented in the large intestine, produce beneficial changes in the gut microbiome, and impart a health benefit to the host. Using reactive extrusion, we have synthesized a novel dietary fiber that is an oligosaccharide of polymerized lactose, which we term polylactose. Here we report on two studies feeding polylactose to rats to determine its prebiotic potential.

Methods

In Exp. 1, the polylactose preparation contained 51% dietary fiber, 20% free lactose, 5% glucose, and 24% other materials. Rats were fed high fat diets containing 9% total dietary fiber, including cellulose (C, 9%), polylactose (PL, 6%), polydextrose (PD, 6%), and fructooligosaccharide (FOS, 6%). In Exp. 2, the polylactose preparation contained 75% dietary fiber, 9% lactose, 3% glucose, and 13% other materials. Rats were again fed high fat diets containing 9% total dietary fiber, including C (9%), polylactose (6% or 3%), PD (6%), and galactooligosaccharides (GOS, 6%). In both experiments, rats were fed for 10 weeks, then ceca (empty), cecal contents, livers, and epididymal fat pads were collected.

Results

In both experiments, final body weight and daily energy intake did not differ among the groups. In Exp. 1, feeding PL greatly increased cecum weight (an indicator of fermentation), cecal *Bifidobacterium* and *Lactobacillus* species abundance, increased cecal acetate and propionate, and reduced liver lipids and fat pad weight, compared to the HFC group. PD and FOS increased probiotic species and short chain fatty acids slightly

(compared to HFC), but not to the same extent as PL, and neither PD or FOS reduced fatty liver or adiposity. In Exp. 2, 6% PL increased cecum weight relative to 3% PL, PD and GOS, all of which were greater than HFC. The cecal microbiome was similar among PL (both 3 and 6%), PD, and GOS, all of which differed from HFC. Liver lipids, fat pad weight, and body composition did not differ among any of the groups in Exp. 2.

Conclusions

The prebiotic activity of polylactose differed depending on the preparation, for unknown reasons. However, our results suggest there is a threshold of probiotic bacteria abundance that must be attained before beneficial effects are imparted on the host by prebiotics.

Introduction

Two studies were conducted to evaluate the prebiotic activity of a novel dietary fiber, polylactose. In total, 144 male Wistar rats were fed experimental diets for 10 weeks. With the exception of the negative control groups, all diets were on a high fat background (51% kcal from fat) containing 9% fiber, 6% of which was soluble prebiotic fiber for those groups. In the first study, we found that polylactose behaves as a potent prebiotic, in that it is vigorously fermented in the large intestine, leads to beneficial changes in the gut microbiome, prevented fatty liver, and reduced adiposity in rats fed high fat diets. These effects occurred to a much greater extent with polylactose than with two commercial prebiotics - polydextrose (PD) and fructooligosaccharides (FOS). In the second study, we failed to reproduce health benefits and to see a dissimilarity in the gut microbiome of polylactose from the commercial prebiotics, despite similarities in fermentation. The differences in results between these two studies is likely attributed to differences in the polylactose mixture used in each study. In the first, the partially purified polylactose mixture contained 51% soluble polylactose fiber, 20% free lactose, and residual glucose and other materials. In the second preparation, the dietary fiber concentration was 75%, while free lactose, glucose, and other materials were reduced (**Fig. 5.1**).

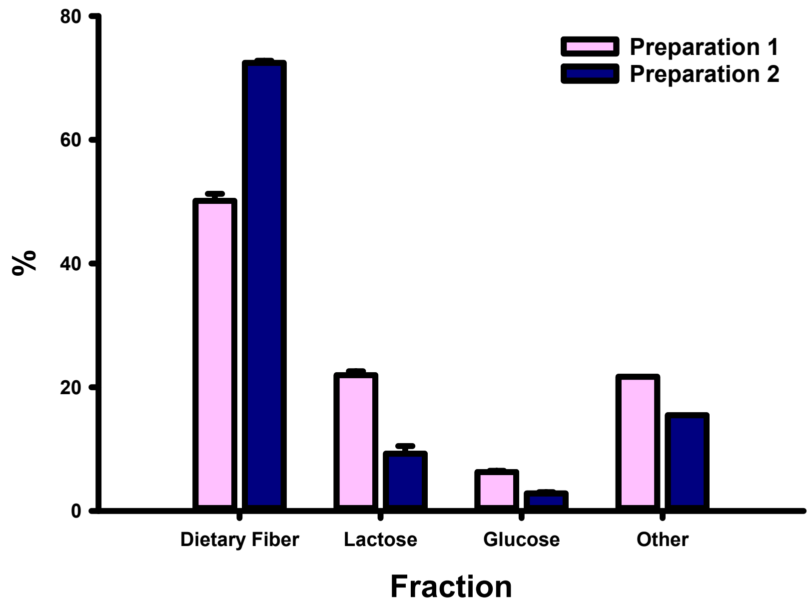

Figure 5.1 Preparations of polylactose in each study.

The purpose of this chapter is to compare the results of the two studies and to explore possible explanations for the differential results. This chapter will also propose experiments that could elucidate the reason(s) for the differences in prebiotic activity between the two preparations of polylactose and confirm the presented hypotheses.

Discussion

In Exp. 1, we demonstrated that polylactose was vigorously fermented in the large intestine, elicited a beneficial change in the gut microbiome, and provided multiple health benefits to the host, including reducing adiposity and fatty liver in rats fed high fat diets. Together, this data indicates that the preparation of polylactose used in Exp. 1 exhibited prebiotic activity. After concentrating soluble polylactose fiber and reducing other materials in the mixture in Exp. 2, polylactose no longer imparted health benefits. This indicates that composition of the preparation is key for the potent prebiotic effect of polylactose. When comparing the two studies, we first see that, despite no differences among groups in either study, the final body weights in Exp. 2 were lower in all groups compared to those in Exp. 1. This is due to a reduction in the study duration and difference in age of the rats at the start of the study. In the first study, the adaption period was 16 days, whereas in the second study the adaption period was only 9 days. This means that the animals were 7 days younger at the end of Exp. 2, accounting for some of the differences in final body weight between the two studies. However, the change in body weight (initial to week 10) was similar for all group between the two studies, with the exception of the polylactose group in Exp. 2 which had an increase in change in body weight compared to that of Exp. 1 (**Fig. 5.2A**). Given the differences in animal size between the two studies, we would expect to see physiological differences that follow the same pattern. However, epididymal fat pad weights revealed a similar trend between the two studies for most groups, with the exception of the polylactose groups (**Fig. 5.2B**). The fat pad weights in the HFPL group in Exp. 2 were larger when compared to the same group in Exp. 1. They are also similar in weight to the HFC group in Exp. 2, indicating that we failed to reproduce the reduced adiposity that we saw in Exp. 1 for the polylactose group.

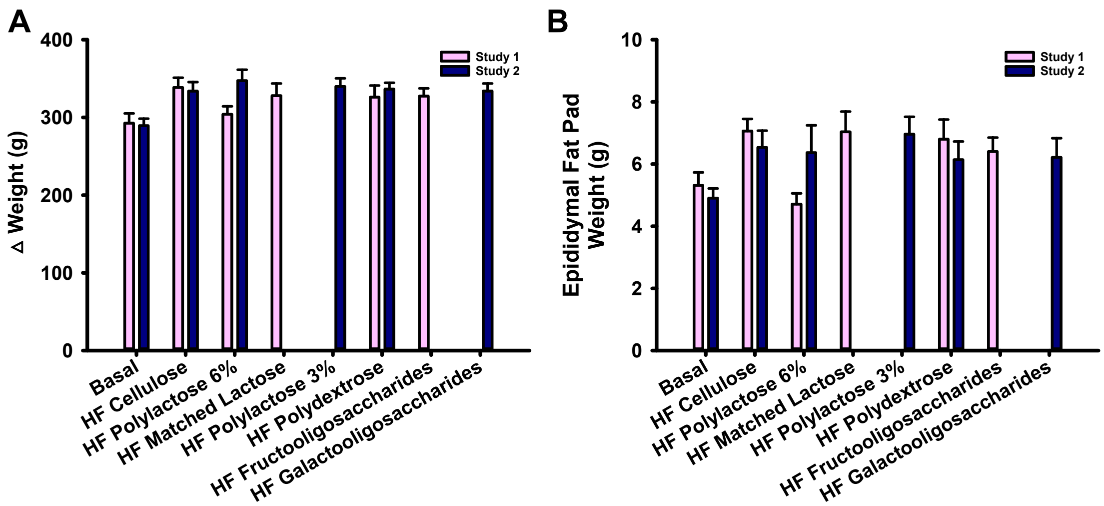

Figure 5.2 Body weight and adiposity in two studies feeding polylactose to rats.

(A) Final body weight of rats. (B) Epididymal fat pad weights. Values represent mean ± SEM, n=12. Values not sharing a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Similar trends are found in the of liver lipids and cholesterol (**Fig. 5.3**). In the first study, liver lipids were significantly lower in the polylactose fed group compared to the high fat controls, approaching the level of the basal group. In the second study, this effect is lost. Instead, the polylactose group has a similar amount of liver lipids compared with all other HF groups. When comparing the two studies, liver lipids are similar in each paired group, with the exception of the polylactose group in the second study, which is similar to the remaining HF groups.

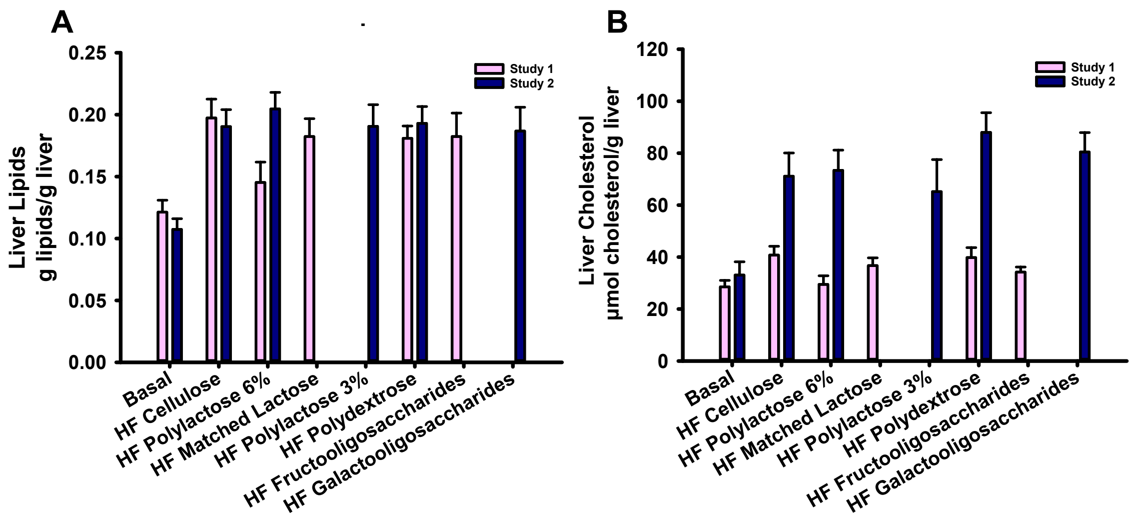

Figure 5.3 Liver lipids and cholesterol in rats fed high fat diets.

(A) Liver total lipid concentration. (B) Liver cholesterol concentration. Values represent mean ± SEM, n=12.

Interestingly, polylactose from Exp. 2, regardless of dose (6% or 3%), behaved similarly to each established prebiotic tested at 6% in the diet. The similarities in the gut microbiomes of the prebiotic groups in Exp. 2 and the PD and FOS groups in Exp. 1 (that is, groups HFPL-2, HFPL2-2, HFPD-1, HFPD-2, HFFOS-1, HFGOS-2), along with the lack of health benefits seen in those groups, suggests that a threshold of beneficial, probiotic bacteria in the gut must be met in order to achieve those effects. The key differences in taxonomic abundance between these groups and the polylactose fed animals in Exp. 1 (HFPL-1) include *Bifidobacterium spp.* and *Lactobacillus spp*.

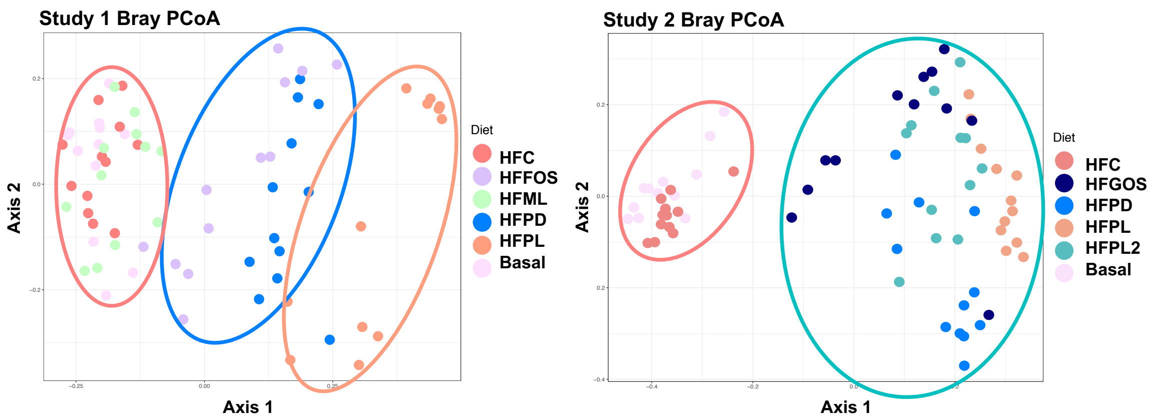

Figure 5.4 Bray Curtis principle coordinates analysis for beta-diversity.

Each point represents an animal, color coded by diet. Groupings (indicated by circles) are statistically different, by the adonis test.

In Exp. 1, there was a significant increase in Bifidobacterium and Lactobacillus in the polylactose and FOS fed groups when compared to the controls. In Exp. 2, we saw an increase in each of these in the GOS group only, but to a lesser extent than in the polylactose group in Exp. 1. There was also a lack of reduction in adiposity and fatty liver in Exp. 2 by GOS, or any prebiotic fiber, suggesting that there is a threshold effect of probiotic gut bacteria that must be achieved in order to provide these health benefits to the host.

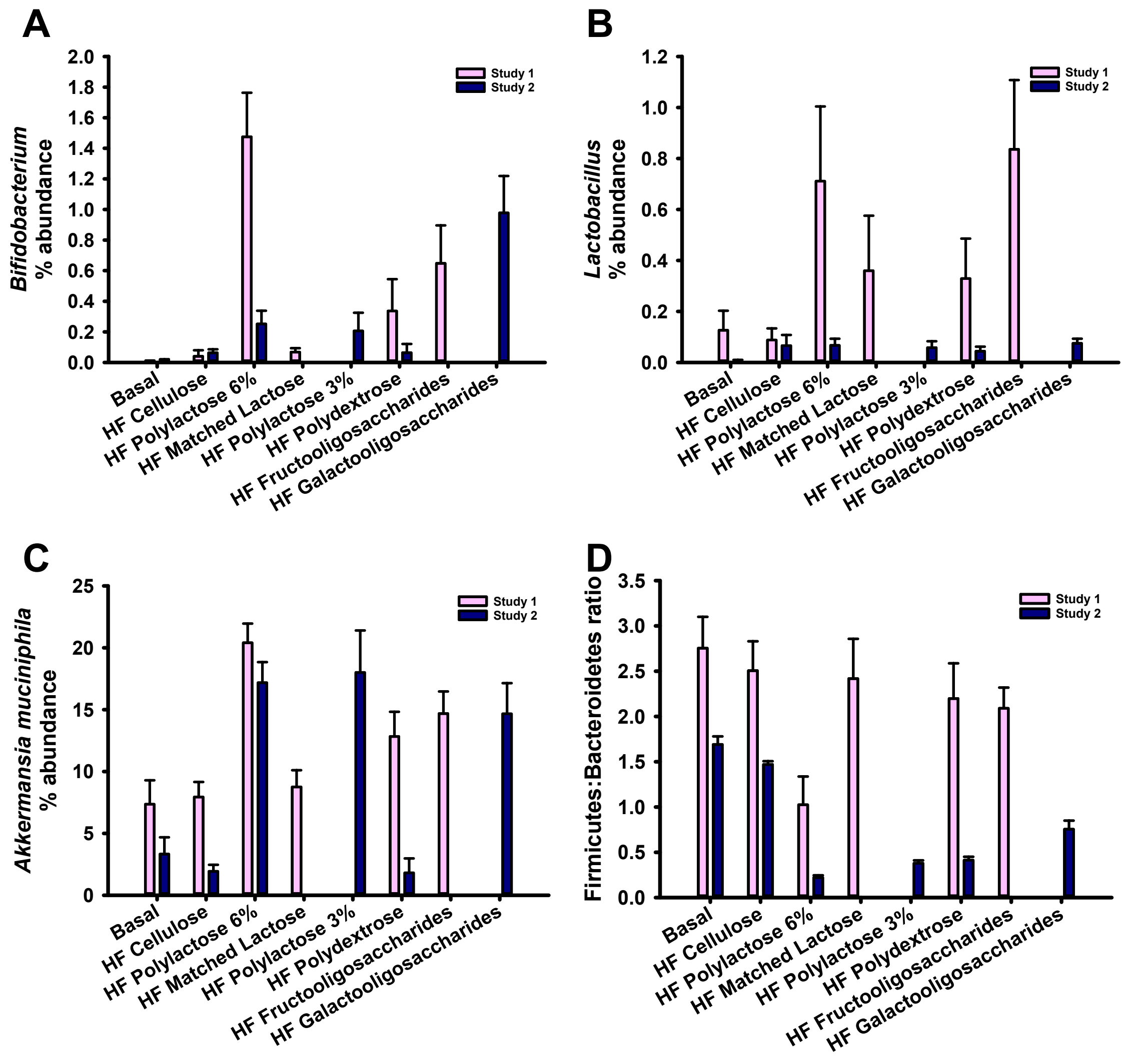

Figure 5.5 Select taxonomical abundance in cecal contents.

(A) *Bifidobacterium* (genus), (B) *Lactobacillus* (genus), (C) *Akkermansia muciniphila*, (D) Firmicutes:Bacteroidetes ratio (phyla). Abundance represented as percent (proportion of taxa sequence reads/total reads per sample). Values represent mean ± SEM, n=11-12.

While results of studies on the effect of FOS, GOS, and PD vary considerably and fail to consistently provide health benefits, even after taking dietary concentration into account, taxonomic abundances in the gut microbiomes could provide some explanations [56, 61-63]. Specifically, *Bifidobacterium* and *Lactobacillus spp.* are commonly reported at greater abundance in prebiotic fed groups where a reduction in fatty liver and adiposity is seen [55, 56]. However, even in the presence of increased *Bifidobacterium* abundance*,* reduced fatty liver and adiposity are not always demonstrated [61-63]. In rodent studies, the dietary concentration of prebiotic typically ranges from 2.5-21% [61-63, 90, 91, 132]. Several studies have examined prebiotic feeding on adiposity and fatty liver. Saha and Reimer, using FOS at a dietary concentration of 21%, found non-significant reductions in body weight and percent body fat but significantly lower serum leptin concentration in overnight-fasted rats [61, 62]. They also reported an increase in empty cecum weight with an increase in cecal *Bifidobacterium* [62], but no changes in liver lipids. While they did not report taxonomical abundances as corrected percentages, calculations can be made using their reported total reads and reads for *Bifidobacterium*. By calculating percent abundance, *Bifidobacterium* was present at 0.7% in the ceca of rats fed high FOS diets. This is similar to what we saw in the Exp. 1, where FOS fed animals (at 6% wt/wt) had 0.7% *Bifidobacterium.* Similarly to Saha and Reimer, we did not see differences in liver lipids in the FOS group of Exp. 1. Further, in the polylactose group of Exp. 1, we found an abundance of 1.5% *Bifidobacterium* along with a reduction in fatty liver. Mistry et al. found that mice fed GOS at 7% wt/wt of the diet had increased cecal *Bifidobacterium* and *Akkermansia muciniphila,* and reduced Firmicutes [132]. They also demonstrated that epididymal fat pad weight, an indicator of total body adiposity, was reduced in GOS fed animals despite the HF diet. While not statistically significant, Mistry et al. found that the GOS group trended toward reduced liver lipids compared to the HF control group [132]. Despite both studies reporting increased *Bifidobacterium,* only Mistry et al. found reductions in adiposity and fatty liver [132]. This is true for other prebiotic feeding studies in humans and rodents, where there are inconsistencies in health benefits despite increases in beneficial gut bacteria, independent of prebiotic dose [61-63, 90, 91, 132].

In order to fully understand a potential threshold effect and compare specific taxa in the gut microbiomes of individuals and animals with varying phenotypes, a standard of reporting needs to be established. In many nutrition-microbiome-disease studies, taxonomical abundances are reported as $\log_{10}$ signal intensity (microarray analysis) [92] or sequence reads/ 20 ng DNA (16S) [6, 61, 62] instead of corrected, percent abundance (16S, other sequencing measures). Without appropriate data presentation, normalization, and reporting, it is difficult to compare microbial abundances among studies, making it impossible to ascertain whether a threshold of abundance of specific bacteria is needed to produce a health benefit, and what that threshold might be.

Additionally, increased adiposity has been correlated with increased abundances of the phylum Firmicutes, and a reduction in Bacteroidetes (or, an increase in the Firmicutes:Bacteroidetes ratio) [25-27, 29, 30, 32, 34, 58, 59]. This was also shown in Exp. 1, where the Firmicutes:Bacteroidetes (F:B) ratio was reduced in polylactose fed animals compared to all other groups. This group also displayed less adiposity than the other high fat-fed groups, but so did the basal group in the absence of a reduced F:B ratio. However, we still saw a reduction in F:B ratio in prebiotic fed animals in Exp. 2, despite no reductions in adiposity. This indicates that prebiotic feeding reduces the F:B ratio but is not the driving force for reduced adiposity.

SCFA production by the gut microbiome is thought to be a key determinant in regulating host health, including adiposity and fatty liver. Here, we demonstrate that the abundance of SCFAs in the cecal contents do not correspond to physiological response. They do, however, have a modest inverse correlation with the Firmicutes:Bacteroidetes ratio ($r^2 = -0.32199$, $p = 0.0155$, Exp. 2). Bacteroidetes are known SCFA producers [83], thus, having an increased abundance of this phylum results in increased SCFAs in the ceca. Aside from that, physiological changes (fatty liver, adiposity) showed no correlation to SCFA abundance or concentration in the ceca. Together, these data question whether SCFAs are the driving microbial products for crosstalk between the gut microbiome and host tissues (specifically adipose and liver) and whether the F:B ratio is the primary taxonomic change needed for the reduction of adiposity and fatty liver.

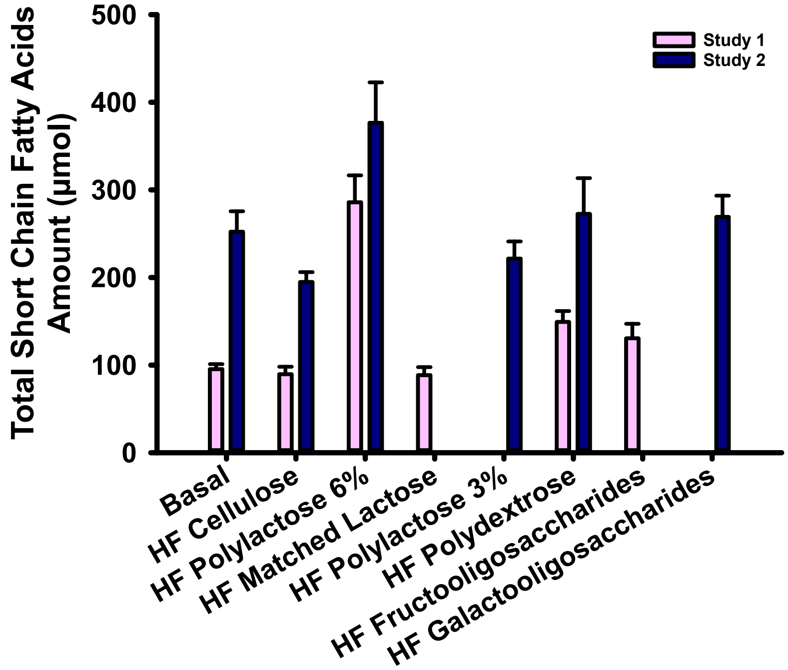

Figure 5.6 Total short chain fatty acids in cecal contents.

Values represent mean ± SEM, n=11-12.

One possible explanation for the more modest change in the microbiome in Exp. 2 compared to Exp. 1 is a cross-feeding effect in the gut microbiome. Cross-feeding refers to the preliminary breakdown of polysaccharides into shorter chains by certain bacteria, such that other microbes can utilize the degradation products [133]. For example, the breakdown of inulin into oligofructose by some gut microbes allows fermentation of the oligofructose by Bifidobacterium [133]. This has been demonstrated *in vitro* by Falony et al., who found that *Bifidobacterium* species grew better on inulin-like fructans when co-cultured with *Bacteroides thetaiotaomicron* than they did in a single culture. This is due to the presence of certain carbohydrate metabolizing genes in the *B. thetaiotaomicron* genome that allows longer chain fructans to be fermented into smaller parts, such that it can be used by *Bifidobacterium*. Cross-feeding can also occur when the fermentation end products, such as SCFAs and other organic acids, from one type of bacteria are used by another to facilitate growth, as demonstrated between *Lactobacillus* species and after fermentation by *Bifidobacterium* [133, 134]. Both *Lactobacillus* and *Bifidobacterium* produce acetate and lactate as fermentation end products, both of which can be used by other genera, including some *Rosburia* and *Lactobacillus* species [133, 134]. In particular, *Rosburia* species are butyrate producers, which is why a butyrogenic effect is often seen in tandem with bifidogenicity as a product of cross-feeding [133, 135]. In a study by Moens et al., co-cultures of *Bifidobacterium longum* and *Lactobacillus paracasei* revealed quick fermentation of short chain inulin by *B. longum* followed by degradation of long chain inulin by *L. paracasei* [135]. The short chain inulin that was produced by *L. paracasei* was then utilized by *B. longum* [135]. This results in acetate and lactate production by both bacterial strains [135]. Together, this chain of events demonstrates a symbiotic relationship between members of the *Bifidobacterium* and *Lactobacillus* genera. This type of cross-feeding is a potential explanation for the differences that we see in the gut microbiomes of the polylactose groups in Exp. 1 vs Exp. 2. In Exp. 1, there was an increase in *Lactobacillus* species in the HFPL, HFML, and HFFOS groups compared to the remaining groups, where the abundance was similar in the HFPL and HFML groups. As both diets had equivalent amounts of free lactose (2.6% wt/wt), this indicates fermentation of lactose by *Lactobacillus* species in the large intestine, resulting in an increased abundance of *Lactobacillus* species in the ceca of these groups. Comparatively, the HFPL group in Exp. 2 only had 1% free lactose wt/wt due to differences in polylactose preparation and composition. This increased abundance of *Lactobacillus* species could provide the means for degradation by *Lactobacillus* species of longer chain polylactose fiber, such that *Bifidobacterium* species are able to utilize the shorter chain molecules. While this study does not provide adequate evidence to support cross-feeding or how it is occurring, the literature suggests this is a possibility. Further research is needed in order to better understand this effect, particularly when using polylactose as a substrate [121, 134, 135].

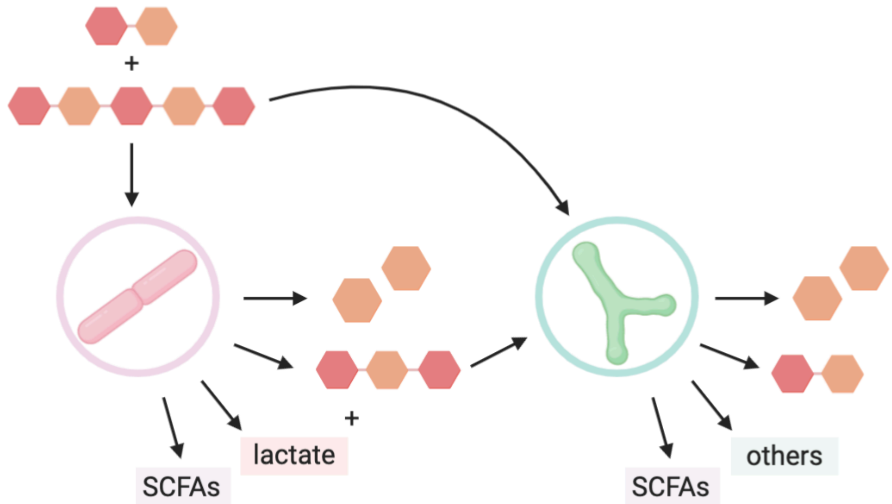

Figure 5.7 Hypothetical schematic of cross-feeding in Exp. 1 with the first polylactose preparation.

Glucose is in coral and galactose tangerine, indicating lactose (dp 2) and polylactose (dp 3-5). These substrates could be utilized by *Lactobacillus* species (pink) in the ceca to produce fermentation byproducts that could be utilized by *Bifidobacterium* (green), allowing both genera to increase in abundance upon feeding polylactose. Made with Biorender.

In addition to the difference in the amount of free lactose between the two polylactose preparations, the amount of "other materials" differed. In Exp. 1, the polylactose preparation had more other materials than in the preparation used for Exp. 2. Some of these unknown compounds could be responsible for the differences in the gut microbiomes of the two polylactose fed groups. Depending on the other materials present, they could also be responsible for cross-feeding, facilitating the use of polylactose by certain gut bacteria. Alternatively, they could act as prebiotics on their own. For example, gluconic acid, an oxidation product of glucose, has been demonstrated to increase fecal bifidobacterial in humans [136] and to stimulate butyrate production in the large intestine [137] and may therefore have prebiotic activity. Gluconic acid can also be linked to galactose, forming lactobionic acid. Lactobionic acid has been demonstrated to exhibit prebiotic activity [138] and is under investigation for food use [139]. While we know that in both polylactose preparations gluconic acid was below the detectable limit (data not shown), the presence of lactobionic acid has not been ruled out. Complete characterization of both polylactose preparations may aid in identifying the relationships between different polylactose preparations and the gut microbiome.

Overall, this analysis suggests that understanding the constituents in the preparations of polylactose dietary fiber may be quite important for understanding of the changes in the gut microbiome and host health effects. It also provides evidence that a certain threshold of beneficial gut bacteria needs to be met in order to see reductions in fatty liver and adiposity in rats fed high fat diets. Complete characterization of polylactose and cross-feeding experiments need to be conducted in order to provide adequate evidence for why each preparation behaves differently.

Chapter 6: Conclusions

This thesis investigated the prebiotic activity of polylactose, a novel dietary fiber, by determining fermentation in the cecum, changes in the gut microbiome, and the effect on adiposity, fatty liver, and prediabetes in rats fed high fat diets. Several commercial prebiotics, polydextrose, fructooligosaccharides (FOS), and galactooligosaccharides, were used for comparison of prebiotic activity with polylactose.

In the first study, we demonstrated the polylactose was vigorously fermented in the large intestine, as indicated by an increase in empty cecum weight and reduction in cecal pH. This corresponded to increased short chain fatty acid (SCFA) production in the ceca and beneficial changes in the gut microbiomes of polylactose fed animals. The increase in probiotic gut bacteria, *Bifidobacterium* and *Lactobacillus,* cecal SCFAs, and fermentation occurred to a greater extent than in polydextrose and FOS fed animals, indicating that polylactose is a more potent prebiotic. We also demonstrated that when fed in the context of a high fat diet, polylactose fed animals had reduced adiposity, as indicated by reduced epididymal fat pad weights and circulating leptin, compared to animals fed the high fat control diet. Additionally, the polylactose group had reduced hepatic lipid and cholesterol concentration, indicating a reduction in fatty liver compared to the high fat control group. These health benefits were also seen to a greater extent compared to the polydextrose and FOS groups. Together, this indicates that polylactose is a potent prebiotic dietary fiber.

In the second polylactose animal study, we failed to replicate the health benefit results of the first study. This is likely due to differences in the preparation of polylactose used, where it contains more fiber, less lactose, and less "other" materials when compared to the preparation used in the first study. While the polylactose mixture was still vigorously fermented and greater amounts of SCFAs were produced compared to all other high fat groups, the changes in the gut microbiome and health benefits were not consistent with findings from the first study. This suggests that the preparation and composition of polylactose determines its prebiotic activity.

When taken together, this research provides evidence that large increases in probiotic bacteria in the gut microbiome are needed in order to provide certain health benefits to the host. It also suggests that SCFAs are not the primary mediator of communication between the gut microbiome and the liver, as increased SCFA production does not correspond to reduced fatty liver. While polylactose is a promising solution for manipulating the gut microbiome to reduce fatty liver, further research is needed to better understand whether a mixture of fermentable carbohydrates is superior to a single fermentable carbohydrate, how fermentable carbohydrates are used by large intestinal bacteria, and how the gut microbiome communicates with the liver.

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Chapter 7: Appendices

Appendix 1. Unreported data for study 1

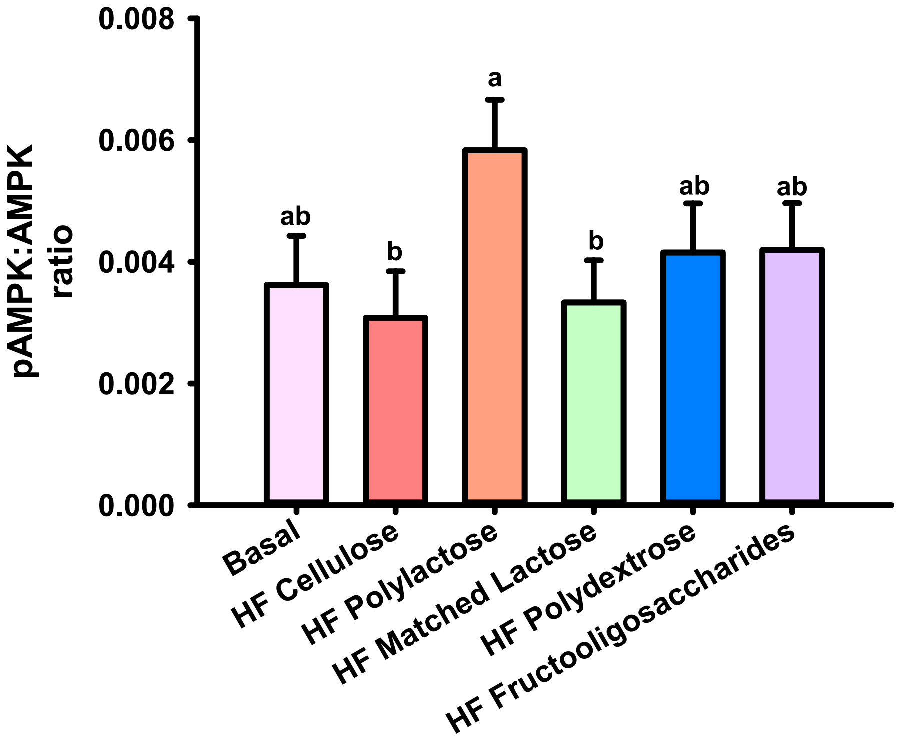

Figure 7.1 Ratio of pAMPK to AMPK in the liver of rats fed polylactose, polydextrose, and fructooligosaccharides.

Values are means ± SEMs, n=12. Bars that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

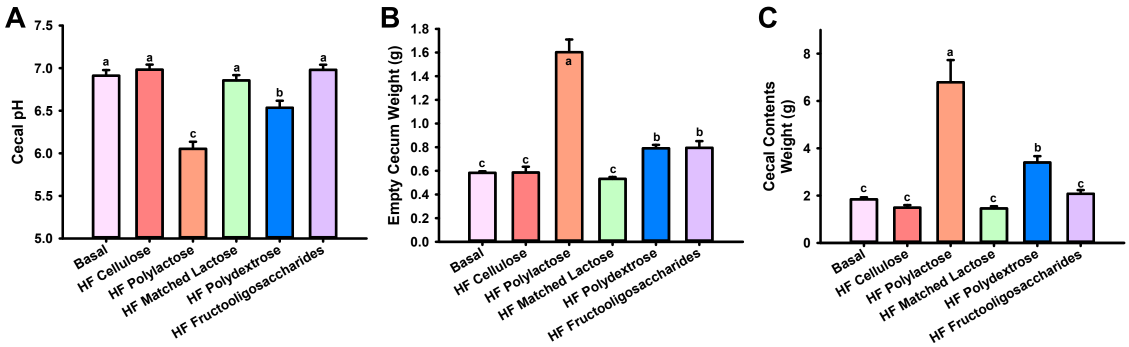

Figure 7.2 Measures of fermentation in the ceca of rats fed polylactose, polydextrose, and fructooligosaccharides.

(A) Cecal pH. (B) Weight of the empty cecum. (C) Weight of cecal contents. Values are means ± SEMs, n=12. Bars that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Table 7.1 Unreported data for polylactose animal study 1, Chapter 3

Variable	Basal	HFC	HFPL	HFML	HFPD	HFFOS
Initial body weight, *g*	197.4 ± 3.9	196.2 ± 3.9	192.4 ± 3.6	189.7 ± 3.9	188.8 ± 5.0	195.5 ± 5.6
Body weight week 1, *g*	258.3 ± 4.2 [ab]	263.3 ± 4.9[a]	242.1 ± 4.2[b]	253.8 ± 5.7 [ab]	248.9 ± 7.1[ab]	255.8 ± 6.1[ab]
Body weight week 2, *g*	319.3 ± 5.1[ab]	328.1 ± 7.1[a]	300.3 ± 6.5[b]	314.0 ± 8.8[ab]	306.7 ± 10.1[ab]	315.6 ± 8.2[ab]
Body weight week 3, *g*	358.8 ± 6.9[ab]	376.2 ± 9.0[a]	340.1 ± 8.0[b]	359.3 ± 11.2[ab]	348.8 ± 11.4[ab]	356.4 ± 10.4[ab]
Body weight week 4, *g*	396.4 ± 7.5[ab]	414.8 ± 11.1[a]	376.6 ± 8.2[b]	394.9 ± 12.8[ab]	389.8 ± 14.1[ab]	395.3 ± 11.5[ab]
Body weight week 5, *g*	420.9 ± 8.2	447.8 ± 11.9	408.8 ± 10.0	427.6 ± 14.5	422.2 ± 15.7	430.9 ± 12.3
Body weight week 6, *g*	439.5 ± 9.7	473.5 ± 13.3	434.0 ± 9.6	453.4 ± 14.7	453.3 ± 18.1	459.3 ± 13.3
Body weight week 7, *g*	455.9 ± 10.0	499.2 ± 15.7	462.0 ± 11.3	477.9 ± 16.9	473.9 ± 18.3	486.5 ± 14.4
Body weight week 8, *g*	480.9 ± 11.4	525.3 ± 15.0	486.5 ± 11.9	502.3 ± 17.7	504.6 ± 19.2	510.1 ± 14.6
Body weight week 9, *g*	484.7 ± 11.3	534.8 ± 14.8	490.7 ± 13.7	509.3 ± 18.2	512.3 ± 19.3	517.25 ± 14.6
Change in body weight, *g*	292.7 ± 12.6[b]	338.6 ± 12.6[a]	304.0 ± 10.3[ab]	328.1 ± 15.7[ab]	326.1 ± 15.1[ab]	327.5 ± 9.9[ab]
Oral glucose tolerance, baseline, *mg/dL*	115.0 ± 4.2	115.5 ± 5.0	115.0 ± 7.3	118.1 ± 4.0	125.2 ± 4.2	127.7 ± 3.8
Oral glucose tolerance, 15 min, *mg/dL*	167.3 ± 8.7	159.3 ± 8.9	155.8 ± 6.5	158.5 ± 6.2	163.0 ± 5.8	154.5 ± 6.6
Oral glucose tolerance, 30 min, *mg/dL*	154.6 ± 4.8[b]	161.4 ± 7.1[ab]	150.3 ± 5.7 [b]	165.3 ± 4.4[ab]	173.2 ± 7.5[a]	160.3 ± 4.6[ab]

Oral glucose tolerance, 60 min, *mg/dL*	139.8 ± 6.0	148.8 ± 5.9	135.0 ± 3.2	145.7 ± 5.1	149.2 ± 4.1	144.7 ± 5.5
Oral glucose tolerance, 90 min, *mg/dL*	127.3 ± 5.2	134.8 ± 4.2	121.3 ± 3.2	126.3 ± 4.5	135.1 ± 6.4	132.1 ± 6.1
Oral glucose tolerance, 120 min, *mg/dL*	123.8 ± 4.4[ab]	128.9 ± 3.8[a]	113.8 ± 5.1[b]	123.8 ± 5.8[ab]	129.5 ± 5.1[a]	132.1 ± 4.8[a]
Insulin tolerance, baseline, *mg/dL*	106.4 ± 4.5	110.5 ± 5.1	106.9 ± 7.1	113.8 ± 3.4	119.9 ± 3.2	121.2 ± 5.4
Insulin tolerance, 15 min, *mg/dL*	99.3 ± 4.4	104.8 ± 3.8	95.2 ± 4.9	103.1 ± 4.3	103.3 ± 5.3	102.8 ± 4.8
Insulin tolerance, 30 min, *mg/dL*	78.8 ± 3.5	72.8 ± 2.5	71.0 ± 5.1	78.3 ± 2.5	78.8 ± 1.8	80.2 ± 2.7
Insulin tolerance, 60 min, *mg/dL*	58.0 ± 3.1	55.3 ± 3.5	54.5 ± 4.5	57.3 ± 2.5	58.3 ± 3.6	58.8 ± 3.3
Insulin tolerance, 90 min, *mg/dL*	52.3 ± 3.9	48.1 ± 3.2	49.3 ± 6.6	49.1 ± 3.4	48.4 ± 2.9	52.7 ± 2.8
Insulin tolerance, 120 min, *mg/dL*	55.1 ± 6.2	52.8 ± 5.8	69.3 ± 9.1	55.5 ± 3.9	53.8 ± 4.1	59.01 ± 5.2
Insulin tolerance, AUC, 10^3 *min*mg/dL*	81.96 ± 3.86	79.32 ± 3.26	80.67 ± 7.79	81.87 ± 3.23	82.3 ± 2.97	84.84 ± 3.13
Pyruvate tolerance, baseline, *mg/dL*	107.2 ± 5.0[b]	115.8 ± 4.4[ab]	115.1 ± 4.2[ab]	112 ± 3.8[ab]	122.6 ± 4.5[a]	117.3 ± 3.8[ab]

Pyruvate tolerance, 15 min, *mg/dL*	140.4 ± 5.3	136.3 ± 3.7	146.3 ± 5.7	132.6 ± 3.0	140.4 ± 6.1	140.0 ± 5.7
Pyruvate tolerance, 30 min, *mg/dL*	147.0 ± 6.9	135.6 ± 3.1	145.3 ± 4.4	141.8 ± 5.3	147.9 ± 6.3	145.3 ± 4.9
Pyruvate tolerance, 60 min, *mg/dL*	134.5 ± 4.5	127.4 ± 2.9	140.9 ± 7.3	135.2 ± 3.4	139.5 ± 4.6	140.7 ± 6.8
Pyruvate tolerance, 90 min, *mg/dL*	124.6 ± 5.0	123.0 ± 6.8	131.7 ± 5.8	124.7 ± 7.2	128.3 ± 5.6	135.9 ± 5.5
Pyruvate tolerance, 120 min, *mg/dL*	121.7 ± 6.2	117.3 ± 4.0	122.1 ± 4.7	120.4 ± 3.9	127.1 ± 5.0	130.0 ± 4.8
Food intake, week 1, *g*	21.9 ± 1.0^{a}	18.5 ± 1.1^{b}	13.7 ± 0.8^{d}	16.5 ± 0.6^{bc}	15.2 ± 0.7^{cd}	15.2 ± 0.7^{cd}
Food intake, week 2, *g*	24.0 ± 1.0^{a}	19.3 ± 0.8^{b}	17.9 ± 2.7^{b}	17.2 ± 0.8^{b}	19.7 ± 1.7^{b}	16.7 ± 0.8^{b}
Food intake, week 3, *g*	18.7 ± 1.6	18.6 ± 0.8	18.3 ± 1.0	16.1 ± 0.9	17.8 ± 1.1	16.1 ± 1.5
Food intake, week 4, *g*	23.7 ± 1.5^{a}	21.0 ± 0.8^{ab}	19.8 ± 1.1^{b}	18.4 ± 0.8^{b}	20.2 ± 1.2^{b}	20.1 ± 0.8^{b}
Food intake, week 5, *g*	19.9 ± 1.6	20.0 ± 0.9	17.5 ± 1.1	17.6 ± 0.9	18.3 ± 1.2	20.9 ± 1.5
Food intake, week 6, *g*	23.6 ± 0.9	22.9 ± 1.8	20.9 ± 1.5	22.3 ± 1.5	21.7 ± 1.4	21.9 ± 1.4
Food intake, week 7, *g*	24.3 ± 1.6^{a}	22.0 ± 0.9^{ab}	18.3 ± 0.6^{c}	20.2 ± 1.3^{bc}	19.6 ± 1.1^{bc}	20.0 ± 0.9^{bc}
Food intake, week 8a, *g*	23.0 ± 0.9^{a}	21.3 ± 1.2^{ab}	21.8 ± 1.1^{ab}	20.0 ± 0.5^{b}	20.2 ± 0.7^{ab}	23.0 ± 0.8^{a}
Food intake, week 8b, *g*	21.1 ± 2.0^{a}	17.1 ± 1.4^{b}	16.3 ± 0.4^{b}	16.4 ± 0.7^{b}	16.2 ± 0.6^{b}	15.9 ± 0.5^{b}
Food intake, week 8c, *g*	19.6 ± 1.1^{ab}	19.2 ± 0.7^{ab}	17.3 ± 1.1^{b}	21.1 ± 0.9^{a}	18.0 ± 0.9^{b}	19.9 ± 0.7^{ab}

Fecal weight, 3d fecal collection, *g*	7.2 ± 0.2[a]	7.2 ± 0.3[a]	2.7 ± 0.3[d]	5.3 ± 0.1[b]	4.3 ± 0.2[c]	4.2 ± 0.2[c]
Colon area, *in*	9.5 ± 0.3	9.8 ± 0.2	9.8 ± 0.2	9.3 ± 0.5	9.5 ± 0.3	9.2 ± 0.4
Aberrant crypt foci (ACF) 1	9.6 ± 1.7[ab]	12.4 ± 1.9[a]	13.1 ± 1.7[a]	11.5 ± 2.3[a]	5.8 ± 1.1[b]	10.3 ± 1.7[ab]
ACF 2	11.4 ± 3.0	14.4 ± 3.2	12.7 ± 2.1	11.1 ± 2.4	10.0 ± 2.7	10.8 ± 1.9
ACF 3	7.7 ± 1.9	10.4 ± 2.0	8.7 ± 2.2	4.8 ± 0.9	5.4 ± 1.5	9.5 ± 1.8
ACF 4	3.5 ± 0.9[ab]	6.4 ± 1.6[a]	4.8 ± 1.6[ab]	2.5 ± 0.9[b]	3.3 ± 0.8[ab]	6.3 ± 1.3[a]
ACF 5	1.4 ± 0.5	3.9 ± 1.1	3.7 ± 1.4	2.4 ± 0.7	1.8 ± 0.7	2.9 ± 0.7
ACF 6	1.3 ± 0.6	1.7 ± 0.4	2.3 ± 0.9	1.0 ± 0.5	1.0 ± 0.3	1.2 ± 0.3
ACF 7	0.7 ± 0.2[ab]	1.5 ± 0.4[a]	1.3 ± 0.7[ab]	0.3 ± 0.1[b]	0.5 ± 0.3[ab]	0.6 ± 0.3[ab]
ACF 8+	0.8 ± 0.3[ab]	1.6 ± 0.4[a]	0.8 ± 0.5[ab]	0.4 ± 0.2[b]	0.4 ± 0.2[b]	0.6 ± 0.2[b]
Total ACF	36.3 ± 7.7[ab]	52.3 ± 8.6[a]	47.3 ± 8.6[ab]	34.01 ±5.5[ab]	28.3 ± 6.5[b]	42.2 ± 7.0[ab]
ACF per cm	3.7 ± 0.8[ab]	5.3 ± 0.8[a]	4.8 ± 0.9[ab]	3.6 ± 0.5[ab]	2.9 ± 0.6[b]	4.4 ± 0.7[ab]
Glycated hemoglobin, *%HbA1C*	7.6 ± 0.9	7.9 ± 0.5	6.6 ± 0.8	6.8 ± 0.7	5.7 ± 0.6	5.9 ± 0.7
Weight of cecal contents, *g*	1.8 ± 0.1[c]	1.5 ± 0.1[c]	6.8 ± 0.9[a]	1.5 ± 0.1[c]	3.4 ± 0.3[b]	2.1 ± 0.2[c]
Cecal acetate, *μmol/g*	25.6 ± 1.4[ab]	28.7 ± 3.1[ab]	24.5 ± 2.7[ab]	29.7 ± 1.5[a]	22.4 ± 1.7[b]	28.6 ± 1.8[ab]
Cecal propionate, *μmol/g*	11.4 ± 0.6[c]	11.1 ± 0.8[c]	17.7 ± 2.2[a]	13.3 ± 0.9[bc]	12.2 ± 0.9[c]	16.9 ± 1.7[ab]
Cecal isobutyrate, *μmol/g*	1.8 ± 0.1[ab]	1.8 ± 0.1[ab]	0.6 ± 0.1[c]	1.7 ± 0.1[ab]	1.4 ± 0.1[b]	2.0 ± 0.3[a]
Cecal isobutyrate, *μmol*	3.4 ± 0.2[abc]	2.9 ± 0.3[bc]	4.4 ± 0.7[ab]	2.5 ± 0.3[c]	4.7 ± 0.4[a]	4.4 ± 0.9[ab]

Cecal butyrate, *μmol/g*	10.3 ± 0.8^{ab}	9.6 ± 0.9^{ab}	2.2 ± 0.3^{d}	12.0 ± 1.3^{a}	5.8 ± 1.3^{c}	8.2 ± 0.6^{bc}
Cecal isovalerate, *μmol/g*	2.1 ± 0.1^{a}	2.1 ± 0.2^{a}	1.3 ± 0.1^{b}	2.0 ± 0.2^{ab}	2.0 ± 0.2^{ab}	2.7 ± 0.4^{a}
Cecal isovalerate, *μmol*	3.9 ± 0.2^{bcd}	3.3 ± 0.3^{cd}	8.9 ± 1.4^{a}	2.8 ± 0.3^{d}	6.6 ± 0.6^{ab}	6.0 ± 1.3^{bc}
Cecal valerate, *μmol/g*	2.1 ± 0.1^{a}	2.1 ± 0.2^{a}	0.03 ± 0.03^{c}	2.0 ± 0.1^{a}	1.6 ± 0.2^{ab}	1.3 ± 0.3^{b}
Cecal valerate, *μmol*	3.9 ± 0.2^{ab}	3.4 ± 0.4^{b}	0.3 ± 0.3^{c}	3.0 ± 0.3^{b}	5.1 ± 0.5^{a}	2.9 ± 0.8^{b}
Total short chain fatty acids, *μmol/g*	51.0 ± 2.9^{ab}	55.4 ± 4.6^{ab}	46.2 ± 5.0^{b}	60.7 ± 3.7^{a}	45.4 ± 2.8^{b}	59.7 ± 4.3^{a}
Firmicutes abundance, *%*	53.8 ± 2.6^{a}	50.1 ± 2.4^{ab}	22.7 ± 3.4^{d}	46.0 ± 3.3^{ab}	43.0 ± 2.8^{bc}	37.5 ± 1.6^{c}
Bacteroidetes abundance, *%*	21.6 ± 1.8^{b}	21.8 ± 1.4^{b}	29.0 ± 2.6^{a}	23.4 ± 2.4^{ab}	22.6 ± 1.7^{b}	20.1 ± 2.0^{b}
Prevotella abundance, *%*	0.08 ± 0.04	0.37 ± 0.2	0.0 ± 0.0	0.46 ± 0.3	0.0 ± 0.0	0.57 ± 0.5
Faecalibacterium abundance, *%*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0014 ± 0.0014	0.0 ± 0.0	0.0 ± 0.0
Bacteroides abundance, *%*	9.5 ± 1.3^{b}	10.5 ± 1.0^{ab}	15.0 ± 2.2^{a}	11.0 ± 1.8^{ab}	8.7 ± 0.9^{b}	9.9 ± 2.3^{ab}
AMPK total, *intensity*	8.4 ± 0.5	7.5 ± 0.8	7.5 ± 0.6	7.9 ± 1.0	7.0 ± 0.7	7.6 ± 0.7
Total AMPK corrected, *intensity*	58.3 ± 8.4	51.4 ± 8.5	58.5 ± 9.3	57.2 ± 12.1	48.1 ± 7.2	55.6 ± 9.4
pAMPK, *intensity*	0.028 ± 0.006^{ab}	0.018 ± 0.003^{b}	0.040 ± 0.004^{a}	0.022 ± 0.005^{b}	0.025 ± 0.004^{b}	0.027 ± 0.003^{b}
pAMPK corrected, *intensity*	0.193 ± 0.053^{b}	0.127 ± 0.020^{b}	0.346 ± 0.083^{a}	0.132 ± 0.025^{b}	0.173 ± 0.030^{b}	0.186 ± 0.028^{b}
B-actin, *intensity*	0.169 ± 0.019	0.166 ± 0.018	0.160 ± 0.022	0.179 ± 0.026	0.158 ± 0.012	0.160 ± 0.018

Ratio of pAMPK to AMPK	0.0036 ± 0.0008[ab]	0.0031 ± 0.0008[b]	0.0058 ± 0.0008[a]	0.0033 ± 0.0007[b]	0.0042 ± 0.0008[ab]	0.0042 ± 0.0008[ab]

Values are means ± SEMs, n=12. Means with a row that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test. Rows with no letters indicate no significant differences. Basal, normal fat cellulose control; HFC, high fat cellulose control; HFPL, high fat polylactose; HFML, high fat matched lactose; HFPD, high fat polydextrose; HFFOS, high fat fructooligosaccharides.

Appendix 2. Unreported data for study 2

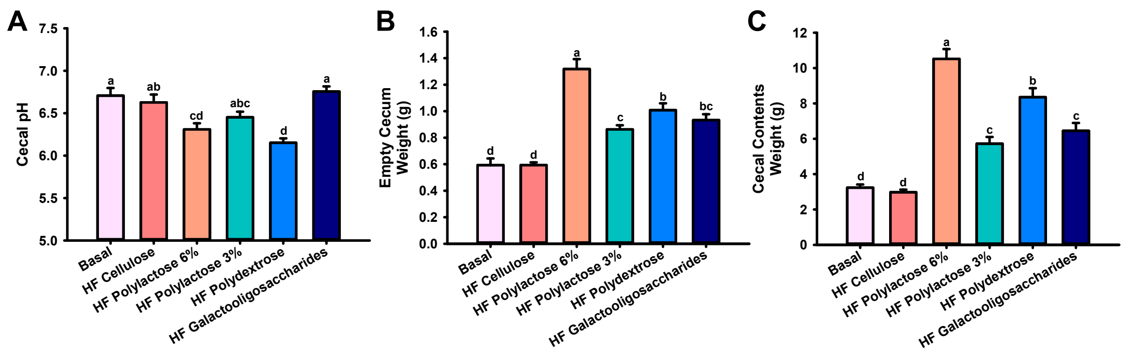

Figure 7.3 Measures of fermentation in the ceca of rats fed polylactose, polydextrose, and galactooligosaccharides.

(A) Cecal pH. (B) Weight of the empty cecum. (C) Weight of cecal contents. Values are means ± SEMs, n=12. Bars that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test.

Table 7.2 Unreported data for polylactose animal study 2, chapter 4

Variable	Basal	HFC	HFPL	HFPL3%	HFPD	HFGOS
Initial body weight, *g*	92.9 ± 2.4	98.0 ± 2.1	99.4 ± 1.9	92.4 ± 3.5	96.6 ± 2.9	96.9 ± 2.7
Body weight week 1, *g*	142.2 ± 4.8	147.6 ± 4.0	148.3 ± 4.7	140.9 ± 5.9	146.1 ± 4.4	143.8 ± 4.9
Body weight week 2, *g*	182.6 ± 4.8	194.7 ± 4.5	191.6 ± 5.3	184.2 ± 7.1	188.9 ± 4.6	184.3 ± 5.0
Body weight week 3, *g*	226.7 ± 4.7[b]	246.0 ± 5.1[a]	245.1 ± 6.0[ab]	235.3 ± 7.8[ab]	239.2 ± 5.0[ab]	235.4 ± 6.2[ab]
Body weight week 4, *g*	265.0 ± 4.5[b]	288.8 ± 6.3[a]	287.7 ± 6.6[a]	278.0 ± 8.0[ab]	281.7 ± 5.0[ab]	277.8 ± 6.4[ab]
Body weight week 5, *g*	299.1 ± 6.3[b]	330.8 ± 8.7[a]	330.3 ± 9.0[a]	316.8 ± 10.0[ab]	320.3 ± 6.4[ab]	315.8 ± 7.6[ab]
Body weight week 6, *g*	320.5 ± 6.5[b]	358.2 ± 9.5[a]	360.3 ± 10.0[a]	347.3 ± 10.0[a]	347.9 ± 6.9[a]	347.0 ± 8.3[a]
Body weight week 7, *g*	338.6 ± 7.5[b]	375.6 ± 10.6[a]	390.7 ± 12.6[a]	374.1 ± 10.9[a]	376.3 ± 8.6[a]	372.7 ± 9.6[a]
Body weight week 8, *g*	357.8 ± 8.0[b]	406.5 ± 11.4[a]	410.5 ± 12.5[a]	399.8 ± 10.3[a]	400.8 ± 8.2[a]	397.8 ± 9.4[a]
Body weight week 9, *g*	370.7 ± 7.6[b]	420.8 ± 11.7[a]	427.6 ± 13.4[a]	417.3 ± 11.8[a]	414.8 ± 8.7[a]	409.3 ± 9.2[a]
Body weight week 10, *g*	384.1 ± 8.5[b]	434.8 ± 12.6[a]	449.9 ± 14.9[a]	435.6 ± 12.3[a]	434.3 ± 9.1[a]	432.0 ± 10.7[a]
Food intake week 1, *g*	19.2 ± 1.1[a]	15.2 ± 1.0[b]	13.9 ± 0.9[b]	14.2 ± 0.9[b]	14.1 ± 0.5[b]	12.7 ± 1.4[b]
Food intake week 2, *g*	17.6 ± 1.3	16.9 ± 0.7	15.9 ± 0.6	16.9 ± 1.0	16.6 ± 0.8	16.4 ± 0.9
Food intake week 3, *g*	20.3 ± 0.7[a]	18.0 ± 0.6[b]	18.1 ± 0.8[b]	16.6 ± 0.7[b]	17.1 ± 0.4[b]	16.7 ± 0.6[b]
Food intake week 4, *g*	21.8 ± 1.2	20.3 ± 1.2	17.0 ± 3.8	19.7 ± 1.0	19.5 ± 1.0	19.2 ± 0.7

Food intake week 5, *g*	22.8 ± 1.9^{a}	19.9 ± 1.1^{ab}	17.7 ± 1.0^{bc}	16.5 ± 0.9^{c}	16.1 ± 0.8^{c}	16.6 ± 0.5^{c}
Food intake week 6, *g*	19.1 ± 1.0	17.9 ± 0.6	18.7 ± 0.9	17.7 ± 0.7	18.0 ± 0.9	18.8 ± 1.0
Food intake week 7, *g*	19.3 ± 1.9	18.8 ± 0.6	18.7 ± 1.0	18.8 ± 0.8	19.7 ± 1.3	18.2 ± 0.9
Fat mass by MRI, *g*	63.0 ± 3.4^{b}	80.3 ± 6.8^{ab}	80.9 ± 9.7^{ab}	85.4 ± 6.6^{a}	77.4 ± 5.4^{ab}	76.1 ± 6.9^{ab}
Lean mass by MRI, *g*	289.3 ± 6.0^{a}	320.8 ± 7.2^{b}	326.1 ± 7.1^{b}	312.4 ± 7.9^{b}	315.4 ± 6.5^{b}	319.0 ± 7.4^{b}
Free body water by MRI, *g*	1.2 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
Total body water by MRI, *g*	236.3 ± 4.7^{b}	262.0 ± 5.9^{a}	267.1 ± 5.8^{a}	255.7 ± 6.4^{a}	258.4 ± 5.3^{a}	261.2 ± 6.1^{a}
Body weight at MRI, *g*	370.7 ± 7.6^{b}	420.8 ± 11.7^{a}	427.6 ± 13.4^{a}	417.3 ± 11.8^{a}	414.8 ± 8.7^{a}	409.3 ± 9.2^{a}
Cecal contents weight, *g*	3.2 ± 0.2^{d}	3.0 ± 0.1^{d}	10.5 ± 0.6^{a}	5.7 ± 0.4^{c}	8.4 ± 0.5^{b}	6.5 ± 0.4^{c}
Acetate concentration, *µmol/mg*	41.4 ± 2.2^{a}	35.5 ± 2.4^{a}	20.8 ± 2.8^{b}	21.7 ± 1.7^{b}	16.0 ± 2.6^{b}	21.7 ± 2.3^{b}
Propionate concentration, *µmol/mg*	15.8 ± 1.1^{a}	12.4 ± 1.0^{ab}	12.5 ± 1.7^{ab}	11.4 ± 1.2^{ab}	9.5 ± 2.1^{b}	13.3 ± 1.6^{ab}
Isobutyrate concentration, *µmol/mg*	1.9 ± 0.2^{a}	1.6 ± 0.2^{ab}	1.1 ± 0.4^{abc}	1.0 ± 0.1^{bc}	0.8 ± 0.2^{c}	1.6 ± 0.2^{ab}
Cecal isobutyrate, *µmol*	5.9 ± 0.5	4.8 ± 0.5	11.5 ± 4.6	5.9 ± 0.6	6.4 ± 1.5	10.0 ± 1.0
Butyrate concentration, *µmol/mg*	16.1 ± 2.4^{a}	13.3 ± 1.1^{b}	2.1 ± 0.3^{c}	3.2 ± 0.2^{c}	4.0 ± 0.5^{c}	3.7 ± 0.3^{c}
Isovalerate concentration, *µmol/mg*	1.8 ± 0.3^{ab}	1.8 ± 0.2^{ab}	1.4 ± 0.2^{b}	1.5 ± 0.1^{b}	1.4 ± 0.2^{b}	2.1 ± 0.2^{a}

Cecal isovalerate, *μmol*	5.6 ± 0.7[b]	5.2 ± 0.6[b]	13.6 ± 1.5[a]	8.6 ± 0.9[b]	12.2 ± 1.6[a]	13.5 ± 1.2[a]
Valerate concentration, *μmol/mg*	2.2 ± 0.2[a]	2.2 ± 0.2[a]	0.0 ± 0.0[c]	0.2 ± 0.1[bc]	0.6 ± 0.2[b]	0.2 ± 0.2[bc]
Cecal valerate, *μmol*	6.9 ± 0.6[a]	6.4 ± 0.4[a]	0.1 ± 0.1[b]	0.8 ± 0.6[b]	5.5 ± 1.6[a]	1.2 ± 1.2[b]
Total short chain fatty acids, *μmol/mg*	79.1 ± 5.2[a]	66.8 ± 4.3[a]	38.0 ± 5.0[b]	39.0 ± 3.1[b]	32.3 ± 5.5[b]	42.7 ± 4.2[b]
Firmicutes abundance, *%*	50.7 ± 1.2[a]	46.6 ± 1.1[b]	9.7 ± 0.9[e]	15.7 ± 1.3[d]	19.3 ± 1.6[d]	24.3 ± 2.4[c]
Bacteroidetes abundance, *%*	31.0 ± 1.9[c]	31.8 ± 0.5[c]	44.4 ± 1.2[ab]	42.0 ± 1.9[b]	47.1 ± 1.3[a]	34.0 ± 2.4[c]
Cells gated by FACS, *n*	9180.6 ± 94.5	9366.8 ± 127.9	9104.6 ± 150.7		9245.1 ± 173.0	
Single cells gated by FACS, *n*	15890.6 ± 8788.4	7467.3 ± 125.1	7173.8 ± 147.3		7308.4 ± 171.6	
Lipid event containing cells by FACS, *n*	6814.6 ± 126.2[a]	7063.0 ± 172.5[a]	6266.6 ± 230.9[b]		7078.1 ± 200.1[a]	
Large adipocytes by FACS, *n*	880.9 ± 141.4	1037.9 ± 119.3	1051.3 ± 105.7		799.1 ± 73.7	
Medium adipocytes by FACS, *n*	2907.3 ± 185.0	3213.0 ± 146.5	2809.0 ± 157.1		3141.6 ± 126.0	
Small adipocytes by FACS, *n*	2509.1 ± 188.8[ab]	2559.1 ± 197.7[ab]	1998.5 ± 266.2[b]		2704.5 ± 210.5[a]	
Large lipid droplets by FACS, *n*	2125.4 ± 388.9[a]	1839.6 ± 330.6[a]	873.1 ± 197.0[b]		2071.4 ± 351.0[a]	
Medium lipid droplets by FACS, *n*	3399.3 ± 380.0	3664.5 ± 294.0	3233.8 ± 301.8		3661.3 ± 184.6	

Small lipid droplets by FACS, *n*	1051.8 ± 176.3^{b}	1538.9 ± 321.9^{ab}	1984.6 ± 280.1^{a}		1187.1 ± 206.1^{b}

Values are means ± SEMs, n=8-12. Means with a row that do not share a common letter are statistically different, $p < 0.05$, by ANOVA followed by Duncan's Multiple Range Test. Basal, normal fat cellulose control; HFC, high fat cellulose control; HFPL, high fat polylactose 6%; HFPL3%, high fat polylactose 3%; HFPD, high fat polydextrose; HFGOS, high fat galactooligosaccharides.

Appendix 3. Microbiome code for study 1

Microbiome PL1 code

```
abern041@cn1164 [~] % qsub -I -l walltime=8:00:00,nodes=4:ppn=128

qsub: waiting for job 19246393.mesabim1.msi.umn.edu to start

qsub: job 19246393.mesabim1.msi.umn.edu ready

abern041@cn1126 [~] % module load R/3.5.0

abern041@cn1126 [~] % R; version/3.5.0


R version 3.5.0 (2018-04-23) -- "Joy in Playing"

Copyright (C) 2018 The R Foundation for Statistical Computing

Platform: x86_64-pc-linux-gnu (64-bit)

Type 'q()' to quit R.

[Previously saved workspace restored]

> library(dada2); packageVersion("dada2")

Loading required package: Rcpp

Error: unexpected input in " packageVersion(?"

> path <- "~/microbiome/Gallaher2_Project_001"

> list.files(path)

 [1] "10_S6_R1_001.fastq"  "10_S6_R2_001.fastq"  "11_S57_R1_001.fastq"

 [4] "11_S57_R2_001.fastq" "12_S29_R1_001.fastq" "12_S29_R2_001.fastq"

 [7] "13_S21_R1_001.fastq" "13_S21_R2_001.fastq" "14_S70_R1_001.fastq"

[10] "14_S70_R2_001.fastq" "15_S34_R1_001.fastq" "15_S34_R2_001.fastq"

[13] "16_S16_R1_001.fastq" "16_S16_R2_001.fastq" "17_S66_R1_001.fastq"

[16] "17_S66_R2_001.fastq" "18_S39_R1_001.fastq" "18_S39_R2_001.fastq"
```

```
[19] "19_S31_R1_001.fastq" "19_S31_R2_001.fastq" "1_S1_R1_001.fastq"
[22] "1_S1_R2_001.fastq"   "20_S3_R1_001.fastq"  "20_S3_R2_001.fastq"
[25] "21_S44_R1_001.fastq" "21_S44_R2_001.fastq" "22_S26_R1_001.fastq"
[28] "22_S26_R2_001.fastq" "23_S75_R1_001.fastq" "23_S75_R2_001.fastq"
[31] "24_S49_R1_001.fastq" "24_S49_R2_001.fastq" "25_S41_R1_001.fastq"
[34] "25_S41_R2_001.fastq" "26_S13_R1_001.fastq" "26_S13_R2_001.fastq"
[37] "27_S54_R1_001.fastq" "27_S54_R2_001.fastq" "28_S36_R1_001.fastq"
[40] "28_S36_R2_001.fastq" "29_S8_R1_001.fastq"  "29_S8_R2_001.fastq"
[43] "2_S52_R1_001.fastq"  "2_S52_R2_001.fastq"  "30_S59_R1_001.fastq"
[46] "30_S59_R2_001.fastq" "31_S51_R1_001.fastq" "31_S51_R2_001.fastq"
[49] "32_S23_R1_001.fastq" "32_S23_R2_001.fastq" "33_S63_R1_001.fastq"
[52] "33_S63_R2_001.fastq" "34_S46_R1_001.fastq" "34_S46_R2_001.fastq"
[55] "35_S28_R1_001.fastq" "35_S28_R2_001.fastq" "36_S68_R1_001.fastq"
[58] "36_S68_R2_001.fastq" "37_S69_R1_001.fastq" "37_S69_R2_001.fastq"
[61] "38_S33_R1_001.fastq" "38_S33_R2_001.fastq" "39_S72_R1_001.fastq"
[64] "39_S72_R2_001.fastq" "3_S14_R1_001.fastq"  "3_S14_R2_001.fastq"
[67] "40_S56_R1_001.fastq" "40_S56_R2_001.fastq" "41_S38_R1_001.fastq"
[70] "41_S38_R2_001.fastq" "42_S77_R1_001.fastq" "42_S77_R2_001.fastq"
[73] "43_S2_R1_001.fastq"  "43_S2_R2_001.fastq"  "44_S43_R1_001.fastq"
[76] "44_S43_R2_001.fastq" "45_S5_R1_001.fastq"  "45_S5_R2_001.fastq"
[79] "46_S65_R1_001.fastq" "46_S65_R2_001.fastq" "47_S48_R1_001.fastq"
[82] "47_S48_R2_001.fastq" "48_S10_R1_001.fastq" "48_S10_R2_001.fastq"
[85] "49_S12_R1_001.fastq" "49_S12_R2_001.fastq" "4_S73_R1_001.fastq"
[88] "4_S73_R2_001.fastq"  "50_S53_R1_001.fastq" "50_S53_R2_001.fastq"
[91] "51_S15_R1_001.fastq" "51_S15_R2_001.fastq" "52_S74_R1_001.fastq"
```

```
 [94] "52_S74_R2_001.fastq" "53_S58_R1_001.fastq" "53_S58_R2_001.fastq"
 [97] "54_S20_R1_001.fastq" "54_S20_R2_001.fastq" "55_S22_R1_001.fastq"
[100] "55_S22_R2_001.fastq" "56_S62_R1_001.fastq" "56_S62_R2_001.fastq"
[103] "57_S25_R1_001.fastq" "57_S25_R2_001.fastq" "58_S7_R1_001.fastq"
[106] "58_S7_R2_001.fastq"  "59_S67_R1_001.fastq" "59_S67_R2_001.fastq"
[109] "5_S47_R1_001.fastq"  "5_S47_R2_001.fastq"  "60_S30_R1_001.fastq"
[112] "60_S30_R2_001.fastq" "61_S32_R1_001.fastq" "61_S32_R2_001.fastq"
[115] "62_S71_R1_001.fastq" "62_S71_R2_001.fastq" "63_S35_R1_001.fastq"
[118] "63_S35_R2_001.fastq" "64_S17_R1_001.fastq" "64_S17_R2_001.fastq"
[121] "65_S76_R1_001.fastq" "65_S76_R2_001.fastq" "66_S40_R1_001.fastq"
[124] "66_S40_R2_001.fastq" "67_S42_R1_001.fastq" "67_S42_R2_001.fastq"
[127] "68_S4_R1_001.fastq"  "68_S4_R2_001.fastq"  "69_S45_R1_001.fastq"
[130] "69_S45_R2_001.fastq" "6_S19_R1_001.fastq"  "6_S19_R2_001.fastq"
[133] "70_S27_R1_001.fastq" "70_S27_R2_001.fastq" "71_S9_R1_001.fastq"
[136] "71_S9_R2_001.fastq"  "72_S50_R1_001.fastq" "72_S50_R2_001.fastq"
[139] "7_S11_R1_001.fastq"  "7_S11_R2_001.fastq"  "8_S61_R1_001.fastq"
[142] "8_S61_R2_001.fastq"  "9_S24_R1_001.fastq"  "9_S24_R2_001.fastq"
[145] "Analysis"            "blanks"              "done"
[148] "filtered"            "Rplots.pdf"
```

```
> fnFs <- sort(list.files(path, pattern="_R1_001.fastq", full.names = TRUE))
> fnRs <- sort(list.files(path, pattern="_R2_001.fastq", full.names = TRUE))
> sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)
> plotQualityProfile(fnFs[1:2])
> plotQualityProfile(fnRs[1:2])
> filtFs <- file.path(path, "filtered1", paste0(sample.names, "_F_filt.fastq.gz"))
```

```
> filtRs <- file.path(path, "filtered1", paste0(sample.names, "_R_filt.fastq.gz"))
> names(filtFs) <- sample.names
> names(filtRs) <- sample.names
> out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(240,160),
+               maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE,
+               compress=TRUE, multithread=FALSE)
Creating output directory: /home/gallaher/abern041/microbiome/Gallaher2_Project_001/filtered1
> head(out)
                     reads.in reads.out
10_S6_R1_001.fastq    212595    171536
11_S57_R1_001.fastq   259367    216469
12_S29_R1_001.fastq   194327    153182
13_S21_R1_001.fastq   206672    169609
14_S70_R1_001.fastq   130741    108262
15_S34_R1_001.fastq    62980     39703
> errF <- learnErrors(filtFs, multithread=TRUE)
129884880 total bases in 541187 reads from 3 samples will be used for learning the error rates.
> errR <- learnErrors(filtRs, multithread=TRUE)
113727360 total bases in 710796 reads from 4 samples will be used for learning the error rates.
> plotErrors(errF, nominalQ=TRUE)
Warning messages:
1: Transformation introduced infinite values in continuous y-axis
2: Transformation introduced infinite values in continuous y-axis
> derepFs <- derepFastq(filtFs, verbose=TRUE)
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/10_F_filt.fastq.gz
Encountered 40816 unique sequences from 171536 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/11_F_filt.fastq.gz
Encountered 50820 unique sequences from 216469 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/12_F_filt.fastq.gz
Encountered 30105 unique sequences from 153182 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/13_F_filt.fastq.gz
Encountered 37429 unique sequences from 169609 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/14_F_filt.fastq.gz
Encountered 27468 unique sequences from 108262 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/15_F_filt.fastq.gz
Encountered 11808 unique sequences from 39703 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/16_F_filt.fastq.gz
Encountered 38193 unique sequences from 196780 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/17_F_filt.fastq.gz
Encountered 42090 unique sequences from 224754 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/18_F_filt.fastq.gz

Encountered 7367 unique sequences from 21249 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/19_F_filt.fastq.gz

Encountered 37246 unique sequences from 98594 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/1_F_filt.fastq.gz

Encountered 28022 unique sequences from 80632 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/20_F_filt.fastq.gz

Encountered 35783 unique sequences from 123971 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/21_F_filt.fastq.gz

Encountered 32964 unique sequences from 137590 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/22_F_filt.fastq.gz

Encountered 31855 unique sequences from 147032 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/23_F_filt.fastq.gz

Encountered 45619 unique sequences from 177292 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/24_F_filt.fastq.gz

Encountered 24791 unique sequences from 116980 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/25_F_filt.fastq.gz

Encountered 31065 unique sequences from 129464 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/26_F_filt.fastq.gz

Encountered 44834 unique sequences from 213417 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/27_F_filt.fastq.gz

Encountered 40960 unique sequences from 207568 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/28_F_filt.fastq.gz

Encountered 35294 unique sequences from 130344 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/29_F_filt.fastq.gz

Encountered 34735 unique sequences from 141636 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/2_F_filt.fastq.gz

Encountered 50343 unique sequences from 229284 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/30_F_filt.fastq.gz

Encountered 43062 unique sequences from 204390 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/31_F_filt.fastq.gz

Encountered 35891 unique sequences from 163778 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/32_F_filt.fastq.gz

Encountered 44630 unique sequences from 214461 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/33_F_filt.fastq.gz

Encountered 19354 unique sequences from 151570 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/34_F_filt.fastq.gz

Encountered 34578 unique sequences from 134379 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/35_F_filt.fastq.gz

Encountered 42162 unique sequences from 182342 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/36_F_filt.fastq.gz

Encountered 38961 unique sequences from 198484 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/37_F_filt.fastq.gz

Encountered 28234 unique sequences from 100100 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/38_F_filt.fastq.gz

Encountered 31961 unique sequences from 120999 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/39_F_filt.fastq.gz

Encountered 43743 unique sequences from 169548 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/3_F_filt.fastq.gz

Encountered 40631 unique sequences from 208026 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/40_F_filt.fastq.gz

Encountered 43262 unique sequences from 195343 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/41_F_filt.fastq.gz

Encountered 36266 unique sequences from 106267 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/42_F_filt.fastq.gz

Encountered 21574 unique sequences from 128230 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/43_F_filt.fastq.gz

Encountered 31906 unique sequences from 97731 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/44_F_filt.fastq.gz

Encountered 38137 unique sequences from 147459 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/45_F_filt.fastq.gz

Encountered 42911 unique sequences from 185040 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/46_F_filt.fastq.gz

Encountered 49062 unique sequences from 220855 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/47_F_filt.fastq.gz

Encountered 39038 unique sequences from 136559 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/48_F_filt.fastq.gz

Encountered 35905 unique sequences from 119227 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/49_F_filt.fastq.gz

Encountered 40111 unique sequences from 239515 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/4_F_filt.fastq.gz

Encountered 36834 unique sequences from 149244 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/50_F_filt.fastq.gz

Encountered 39351 unique sequences from 210473 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/51_F_filt.fastq.gz

Encountered 42991 unique sequences from 256579 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/52_F_filt.fastq.gz

Encountered 28188 unique sequences from 138857 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/53_F_filt.fastq.gz

Encountered 38829 unique sequences from 201049 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/54_F_filt.fastq.gz
Encountered 45133 unique sequences from 247657 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/55_F_filt.fastq.gz
Encountered 26001 unique sequences from 164289 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/56_F_filt.fastq.gz
Encountered 46268 unique sequences from 195729 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/57_F_filt.fastq.gz
Encountered 46975 unique sequences from 241272 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/58_F_filt.fastq.gz
Encountered 31601 unique sequences from 124346 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/59_F_filt.fastq.gz
Encountered 46124 unique sequences from 202743 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/5_F_filt.fastq.gz
Encountered 26569 unique sequences from 83888 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/60_F_filt.fastq.gz
Encountered 44034 unique sequences from 189592 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/61_F_filt.fastq.gz

Encountered 30479 unique sequences from 122101 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/62_F_filt.fastq.gz

Encountered 37156 unique sequences from 150043 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/63_F_filt.fastq.gz

Encountered 22570 unique sequences from 79340 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/64_F_filt.fastq.gz

Encountered 38440 unique sequences from 207088 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/65_F_filt.fastq.gz

Encountered 40632 unique sequences from 174710 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/66_F_filt.fastq.gz

Encountered 12654 unique sequences from 33173 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/67_F_filt.fastq.gz

Encountered 28773 unique sequences from 123163 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/68_F_filt.fastq.gz

Encountered 29618 unique sequences from 117929 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/69_F_filt.fastq.gz

Encountered 34670 unique sequences from 159835 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/6_F_filt.fastq.gz

Encountered 40778 unique sequences from 245879 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/70_F_filt.fastq.gz

Encountered 38967 unique sequences from 219560 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/71_F_filt.fastq.gz

Encountered 37003 unique sequences from 161148 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/72_F_filt.fastq.gz

Encountered 24937 unique sequences from 95381 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/7_F_filt.fastq.gz

Encountered 45970 unique sequences from 178116 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/8_F_filt.fastq.gz

Encountered 42601 unique sequences from 185204 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/9_F_filt.fastq.gz

Encountered 45347 unique sequences from 246671 total sequences read.

> derepRs <- derepFastq(filtRs, verbose=TRUE)
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/10_R_filt.fastq.gz

Encountered 50988 unique sequences from 171536 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/11_R_filt.fastq.gz

Encountered 62283 unique sequences from 216469 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/12_R_filt.fastq.gz

Encountered 46645 unique sequences from 153182 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/13_R_filt.fastq.gz

Encountered 41779 unique sequences from 169609 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/14_R_filt.fastq.gz

Encountered 33589 unique sequences from 108262 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/15_R_filt.fastq.gz

Encountered 8819 unique sequences from 39703 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/16_R_filt.fastq.gz

Encountered 49707 unique sequences from 196780 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/17_R_filt.fastq.gz

Encountered 51906 unique sequences from 224754 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/18_R_filt.fastq.gz

Encountered 6045 unique sequences from 21249 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/19_R_filt.fastq.gz

Encountered 34311 unique sequences from 98594 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/1_R_filt.fastq.gz

Encountered 32970 unique sequences from 80632 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/20_R_filt.fastq.gz

Encountered 42106 unique sequences from 123971 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/21_R_filt.fastq.gz

Encountered 36479 unique sequences from 137590 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/22_R_filt.fastq.gz

Encountered 31680 unique sequences from 147032 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/23_R_filt.fastq.gz

Encountered 55099 unique sequences from 177292 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/24_R_filt.fastq.gz

Encountered 24146 unique sequences from 116980 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/25_R_filt.fastq.gz

Encountered 29832 unique sequences from 129464 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/26_R_filt.fastq.gz

Encountered 54090 unique sequences from 213417 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/27_R_filt.fastq.gz

Encountered 52574 unique sequences from 207568 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/28_R_filt.fastq.gz

Encountered 31136 unique sequences from 130344 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/29_R_filt.fastq.gz

Encountered 39508 unique sequences from 141636 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/2_R_filt.fastq.gz

Encountered 63553 unique sequences from 229284 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/30_R_filt.fastq.gz

Encountered 55442 unique sequences from 204390 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/31_R_filt.fastq.gz

Encountered 46379 unique sequences from 163778 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/32_R_filt.fastq.gz
Encountered 53803 unique sequences from 214461 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/33_R_filt.fastq.gz
Encountered 27504 unique sequences from 151570 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/34_R_filt.fastq.gz
Encountered 31302 unique sequences from 134379 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/35_R_filt.fastq.gz
Encountered 49382 unique sequences from 182342 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/36_R_filt.fastq.gz
Encountered 48925 unique sequences from 198484 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/37_R_filt.fastq.gz
Encountered 35349 unique sequences from 100100 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/38_R_filt.fastq.gz
Encountered 28957 unique sequences from 120999 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/39_R_filt.fastq.gz
Encountered 54529 unique sequences from 169548 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/3_R_filt.fastq.gz

Encountered 50536 unique sequences from 208026 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/40_R_filt.fastq.gz

Encountered 56910 unique sequences from 195343 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/41_R_filt.fastq.gz

Encountered 31887 unique sequences from 106267 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/42_R_filt.fastq.gz

Encountered 30507 unique sequences from 128230 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/43_R_filt.fastq.gz

Encountered 37312 unique sequences from 97731 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/44_R_filt.fastq.gz

Encountered 35668 unique sequences from 147459 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/45_R_filt.fastq.gz

Encountered 49817 unique sequences from 185040 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/46_R_filt.fastq.gz

Encountered 59548 unique sequences from 220855 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/47_R_filt.fastq.gz
Encountered 36872 unique sequences from 136559 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/48_R_filt.fastq.gz
Encountered 36311 unique sequences from 119227 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/49_R_filt.fastq.gz
Encountered 52507 unique sequences from 239515 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/4_R_filt.fastq.gz
Encountered 44992 unique sequences from 149244 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/50_R_filt.fastq.gz
Encountered 51361 unique sequences from 210473 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/51_R_filt.fastq.gz
Encountered 54361 unique sequences from 256579 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/52_R_filt.fastq.gz
Encountered 35500 unique sequences from 138857 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/53_R_filt.fastq.gz
Encountered 50539 unique sequences from 201049 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/54_R_filt.fastq.gz

Encountered 56445 unique sequences from 247657 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/55_R_filt.fastq.gz

Encountered 32642 unique sequences from 164289 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/56_R_filt.fastq.gz

Encountered 55453 unique sequences from 195729 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/57_R_filt.fastq.gz

Encountered 56911 unique sequences from 241272 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/58_R_filt.fastq.gz

Encountered 37610 unique sequences from 124346 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/59_R_filt.fastq.gz

Encountered 52242 unique sequences from 202743 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/5_R_filt.fastq.gz

Encountered 20316 unique sequences from 83888 total sequences read.

Dereplicating sequence entries in Fastq file:

~/microbiome/Gallaher2_Project_001/filtered1/60_R_filt.fastq.gz

Encountered 54227 unique sequences from 189592 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/61_R_filt.fastq.gz
Encountered 25425 unique sequences from 122101 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/62_R_filt.fastq.gz
Encountered 45385 unique sequences from 150043 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/63_R_filt.fastq.gz
Encountered 17084 unique sequences from 79340 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/64_R_filt.fastq.gz
Encountered 47830 unique sequences from 207088 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/65_R_filt.fastq.gz
Encountered 48028 unique sequences from 174710 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/66_R_filt.fastq.gz
Encountered 10104 unique sequences from 33173 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/67_R_filt.fastq.gz
Encountered 24220 unique sequences from 123163 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/68_R_filt.fastq.gz
Encountered 36248 unique sequences from 117929 total sequences read.
```

```
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/69_R_filt.fastq.gz
Encountered 34364 unique sequences from 159835 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/6_R_filt.fastq.gz
Encountered 53641 unique sequences from 245879 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/70_R_filt.fastq.gz
Encountered 47491 unique sequences from 219560 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/71_R_filt.fastq.gz
Encountered 47419 unique sequences from 161148 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/72_R_filt.fastq.gz
Encountered 18213 unique sequences from 95381 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/7_R_filt.fastq.gz
Encountered 56259 unique sequences from 178116 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/8_R_filt.fastq.gz
Encountered 49191 unique sequences from 185204 total sequences read.
Dereplicating sequence entries in Fastq file:
~/microbiome/Gallaher2_Project_001/filtered1/9_R_filt.fastq.gz
Encountered 58152 unique sequences from 246671 total sequences read.
```

```
> names(derepFs) <- sample.names
```

```
> names(derepRs) <- sample.names

> dadaFs <- dada(derepFs, err=errF, multithread=TRUE)
```

```
Sample 1 - 171536 reads in 40816 unique sequences.

Sample 2 - 216469 reads in 50820 unique sequences.

Sample 3 - 153182 reads in 30105 unique sequences.

Sample 4 - 169609 reads in 37429 unique sequences.

Sample 5 - 108262 reads in 27468 unique sequences.

Sample 6 - 39703 reads in 11808 unique sequences.

Sample 7 - 196780 reads in 38193 unique sequences.

Sample 8 - 224754 reads in 42090 unique sequences.

Sample 9 - 21249 reads in 7367 unique sequences.

Sample 10 - 98594 reads in 37246 unique sequences.

Sample 11 - 80632 reads in 28022 unique sequences.

Sample 12 - 123971 reads in 35783 unique sequences.

Sample 13 - 137590 reads in 32964 unique sequences.

Sample 14 - 147032 reads in 31855 unique sequences.

Sample 15 - 177292 reads in 45619 unique sequences.

Sample 16 - 116980 reads in 24791 unique sequences.

Sample 17 - 129464 reads in 31065 unique sequences.

Sample 18 - 213417 reads in 44834 unique sequences.

Sample 19 - 207568 reads in 40960 unique sequences.

Sample 20 - 130344 reads in 35294 unique sequences.

Sample 21 - 141636 reads in 34735 unique sequences.

Sample 22 - 229284 reads in 50343 unique sequences.

Sample 23 - 204390 reads in 43062 unique sequences.
```

Sample 24 - 163778 reads in 35891 unique sequences.

Sample 25 - 214461 reads in 44630 unique sequences.

Sample 26 - 151570 reads in 19354 unique sequences.

Sample 27 - 134379 reads in 34578 unique sequences.

Sample 28 - 182342 reads in 42162 unique sequences.

Sample 29 - 198484 reads in 38961 unique sequences.

Sample 30 - 100100 reads in 28234 unique sequences.

Sample 31 - 120999 reads in 31961 unique sequences.

Sample 32 - 169548 reads in 43743 unique sequences.

Sample 33 - 208026 reads in 40631 unique sequences.

Sample 34 - 195343 reads in 43262 unique sequences.

Sample 35 - 106267 reads in 36266 unique sequences.

Sample 36 - 128230 reads in 21574 unique sequences.

Sample 37 - 97731 reads in 31906 unique sequences.

Sample 38 - 147459 reads in 38137 unique sequences.

Sample 39 - 185040 reads in 42911 unique sequences.

Sample 40 - 220855 reads in 49062 unique sequences.

Sample 41 - 136559 reads in 39038 unique sequences.

Sample 42 - 119227 reads in 35905 unique sequences.

Sample 43 - 239515 reads in 40111 unique sequences.

Sample 44 - 149244 reads in 36834 unique sequences.

Sample 45 - 210473 reads in 39351 unique sequences.

Sample 46 - 256579 reads in 42991 unique sequences.

Sample 47 - 138857 reads in 28188 unique sequences.

Sample 48 - 201049 reads in 38829 unique sequences.

```
Sample 49 - 247657 reads in 45133 unique sequences.

Sample 50 - 164289 reads in 26001 unique sequences.

Sample 51 - 195729 reads in 46268 unique sequences.

Sample 52 - 241272 reads in 46975 unique sequences.

Sample 53 - 124346 reads in 31601 unique sequences.

Sample 54 - 202743 reads in 46124 unique sequences.

Sample 55 - 83888 reads in 26569 unique sequences.

Sample 56 - 189592 reads in 44034 unique sequences.

Sample 57 - 122101 reads in 30479 unique sequences.

Sample 58 - 150043 reads in 37156 unique sequences.

Sample 59 - 79340 reads in 22570 unique sequences.

Sample 60 - 207088 reads in 38440 unique sequences.

Sample 61 - 174710 reads in 40632 unique sequences.

Sample 62 - 33173 reads in 12654 unique sequences.

Sample 63 - 123163 reads in 28773 unique sequences.

Sample 64 - 117929 reads in 29618 unique sequences.

Sample 65 - 159835 reads in 34670 unique sequences.

Sample 66 - 245879 reads in 40778 unique sequences.

Sample 67 - 219560 reads in 38967 unique sequences.

Sample 68 - 161148 reads in 37003 unique sequences.

Sample 69 - 95381 reads in 24937 unique sequences.

Sample 70 - 178116 reads in 45970 unique sequences.

Sample 71 - 185204 reads in 42601 unique sequences.

Sample 72 - 246671 reads in 45347 unique sequences.
```

```
> dadaRs <- dada(derepRs, err=errR, multithread=TRUE)
```

Sample 1 - 171536 reads in 50988 unique sequences.

Sample 2 - 216469 reads in 62283 unique sequences.

Sample 3 - 153182 reads in 46645 unique sequences.

Sample 4 - 169609 reads in 41779 unique sequences.

Sample 5 - 108262 reads in 33589 unique sequences.

Sample 6 - 39703 reads in 8819 unique sequences.

Sample 7 - 196780 reads in 49707 unique sequences.

Sample 8 - 224754 reads in 51906 unique sequences.

Sample 9 - 21249 reads in 6045 unique sequences.

Sample 10 - 98594 reads in 34311 unique sequences.

Sample 11 - 80632 reads in 32970 unique sequences.

Sample 12 - 123971 reads in 42106 unique sequences.

Sample 13 - 137590 reads in 36479 unique sequences.

Sample 14 - 147032 reads in 31680 unique sequences.

Sample 15 - 177292 reads in 55099 unique sequences.

Sample 16 - 116980 reads in 24146 unique sequences.

Sample 17 - 129464 reads in 29832 unique sequences.

Sample 18 - 213417 reads in 54090 unique sequences.

Sample 19 - 207568 reads in 52574 unique sequences.

Sample 20 - 130344 reads in 31136 unique sequences.

Sample 21 - 141636 reads in 39508 unique sequences.

Sample 22 - 229284 reads in 63553 unique sequences.

Sample 23 - 204390 reads in 55442 unique sequences.

Sample 24 - 163778 reads in 46379 unique sequences.

Sample 25 - 214461 reads in 53803 unique sequences.

Sample 26 - 151570 reads in 27504 unique sequences.

Sample 27 - 134379 reads in 31302 unique sequences.

Sample 28 - 182342 reads in 49382 unique sequences.

Sample 29 - 198484 reads in 48925 unique sequences.

Sample 30 - 100100 reads in 35349 unique sequences.

Sample 31 - 120999 reads in 28957 unique sequences.

Sample 32 - 169548 reads in 54529 unique sequences.

Sample 33 - 208026 reads in 50536 unique sequences.

Sample 34 - 195343 reads in 56910 unique sequences.

Sample 35 - 106267 reads in 31887 unique sequences.

Sample 36 - 128230 reads in 30507 unique sequences.

Sample 37 - 97731 reads in 37312 unique sequences.

Sample 38 - 147459 reads in 35668 unique sequences.

Sample 39 - 185040 reads in 49817 unique sequences.

Sample 40 - 220855 reads in 59548 unique sequences.

Sample 41 - 136559 reads in 36872 unique sequences.

Sample 42 - 119227 reads in 36311 unique sequences.

Sample 43 - 239515 reads in 52507 unique sequences.

Sample 44 - 149244 reads in 44992 unique sequences.

Sample 45 - 210473 reads in 51361 unique sequences.

Sample 46 - 256579 reads in 54361 unique sequences.

Sample 47 - 138857 reads in 35500 unique sequences.

Sample 48 - 201049 reads in 50539 unique sequences.

Sample 49 - 247657 reads in 56445 unique sequences.

Sample 50 - 164289 reads in 32642 unique sequences.

```
Sample 51 - 195729 reads in 55453 unique sequences.

Sample 52 - 241272 reads in 56911 unique sequences.

Sample 53 - 124346 reads in 37610 unique sequences.

Sample 54 - 202743 reads in 52242 unique sequences.

Sample 55 - 83888 reads in 20316 unique sequences.

Sample 56 - 189592 reads in 54227 unique sequences.

Sample 57 - 122101 reads in 25425 unique sequences.

Sample 58 - 150043 reads in 45385 unique sequences.

Sample 59 - 79340 reads in 17084 unique sequences.

Sample 60 - 207088 reads in 47830 unique sequences.

Sample 61 - 174710 reads in 48028 unique sequences.

Sample 62 - 33173 reads in 10104 unique sequences.

Sample 63 - 123163 reads in 24220 unique sequences.

Sample 64 - 117929 reads in 36248 unique sequences.

Sample 65 - 159835 reads in 34364 unique sequences.

Sample 66 - 245879 reads in 53641 unique sequences.

Sample 67 - 219560 reads in 47491 unique sequences.

Sample 68 - 161148 reads in 47419 unique sequences.

Sample 69 - 95381 reads in 18213 unique sequences.

Sample 70 - 178116 reads in 56259 unique sequences.

Sample 71 - 185204 reads in 49191 unique sequences.

Sample 72 - 246671 reads in 58152 unique sequences.
```

```
> dadaFs[[1]]
```

```
dada-class: object describing DADA2 denoising results

831 sequence variants were inferred from 40816 input unique sequences.
```

```
Key parameters: OMEGA_A = 1e-40, OMEGA_C = 1e-40, BAND_SIZE = 16
> mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs, verbose=TRUE)
154094 paired-reads (in 3748 unique pairings) successfully merged out of 166476 (in 9958 pairings) input.
196092 paired-reads (in 5240 unique pairings) successfully merged out of 210675 (in 12871 pairings) input.
141406 paired-reads (in 2625 unique pairings) successfully merged out of 150701 (in 7046 pairings) input.
156693 paired-reads (in 4694 unique pairings) successfully merged out of 166555 (in 8487 pairings) input.
97087 paired-reads (in 3530 unique pairings) successfully merged out of 105470 (in 6817 pairings) input.
36455 paired-reads (in 1028 unique pairings) successfully merged out of 38486 (in 2267 pairings) input.
183970 paired-reads (in 4759 unique pairings) successfully merged out of 193615 (in 8677 pairings) input.
213056 paired-reads (in 5281 unique pairings) successfully merged out of 221310 (in 8733 pairings) input.
18983 paired-reads (in 656 unique pairings) successfully merged out of 20455 (in 1653 pairings) input.
81540 paired-reads (in 2778 unique pairings) successfully merged out of 93902 (in 9388 pairings) input.
63228 paired-reads (in 2631 unique pairings) successfully merged out of 76092 (in 8263 pairings) input.
105980 paired-reads (in 3945 unique pairings) successfully merged out of 118871 (in 9912 pairings) input.
125625 paired-reads (in 2635 unique pairings) successfully merged out of 134456 (in 6775 pairings) input.
136184 paired-reads (in 2491 unique pairings) successfully merged out of 143968 (in 5781 pairings) input.
157586 paired-reads (in 4458 unique pairings) successfully merged out of 172223 (in 11985 pairings) input.
108744 paired-reads (in 2058 unique pairings) successfully merged out of 114985 (in 4392 pairings) input.
120108 paired-reads (in 2799 unique pairings) successfully merged out of 126688 (in 5834 pairings) input.
197954 paired-reads (in 5145 unique pairings) successfully merged out of 209450 (in 10807 pairings) input.
193309 paired-reads (in 5254 unique pairings) successfully merged out of 204218 (in 9747 pairings) input.
118838 paired-reads (in 2988 unique pairings) successfully merged out of 127502 (in 7257 pairings) input.
128474 paired-reads (in 3961 unique pairings) successfully merged out of 137844 (in 8153 pairings) input.
207076 paired-reads (in 4727 unique pairings) successfully merged out of 223760 (in 11973 pairings) input.
188782 paired-reads (in 5688 unique pairings) successfully merged out of 199893 (in 10437 pairings) input.
150647 paired-reads (in 4567 unique pairings) successfully merged out of 159936 (in 8842 pairings) input.
196779 paired-reads (in 4565 unique pairings) successfully merged out of 209627 (in 10503 pairings) input.
148559 paired-reads (in 1222 unique pairings) successfully merged out of 150548 (in 1973 pairings) input.
124360 paired-reads (in 2826 unique pairings) successfully merged out of 131660 (in 6140 pairings) input.
166555 paired-reads (in 5391 unique pairings) successfully merged out of 177505 (in 10665 pairings) input.
```

185395 paired-reads (in 3873 unique pairings) successfully merged out of 194274 (in 7973 pairings) input.

85001 paired-reads (in 2768 unique pairings) successfully merged out of 96026 (in 8136 pairings) input.

108961 paired-reads (in 2166 unique pairings) successfully merged out of 117845 (in 6544 pairings) input.

148435 paired-reads (in 4787 unique pairings) successfully merged out of 163848 (in 12281 pairings) input.

192538 paired-reads (in 4101 unique pairings) successfully merged out of 203874 (in 9504 pairings) input.

177676 paired-reads (in 4330 unique pairings) successfully merged out of 190718 (in 10355 pairings) input.

90346 paired-reads (in 2633 unique pairings) successfully merged out of 102026 (in 8852 pairings) input.

120535 paired-reads (in 1889 unique pairings) successfully merged out of 126137 (in 3994 pairings) input.

79041 paired-reads (in 3020 unique pairings) successfully merged out of 92695 (in 9618 pairings) input.

134960 paired-reads (in 3014 unique pairings) successfully merged out of 144030 (in 7725 pairings) input.

167977 paired-reads (in 5231 unique pairings) successfully merged out of 179891 (in 11417 pairings) input.

202114 paired-reads (in 4399 unique pairings) successfully merged out of 216158 (in 11258 pairings) input.

120074 paired-reads (in 3329 unique pairings) successfully merged out of 132269 (in 8997 pairings) input.

102409 paired-reads (in 3383 unique pairings) successfully merged out of 114191 (in 9252 pairings) input.

229580 paired-reads (in 4872 unique pairings) successfully merged out of 236459 (in 7903 pairings) input.

131883 paired-reads (in 3427 unique pairings) successfully merged out of 144486 (in 9708 pairings) input.

197970 paired-reads (in 5111 unique pairings) successfully merged out of 207209 (in 8917 pairings) input.

244154 paired-reads (in 5472 unique pairings) successfully merged out of 253544 (in 8994 pairings) input.

131641 paired-reads (in 3389 unique pairings) successfully merged out of 136610 (in 5877 pairings) input.

189084 paired-reads (in 5366 unique pairings) successfully merged out of 197882 (in 9079 pairings) input.

233666 paired-reads (in 5219 unique pairings) successfully merged out of 243782 (in 9446 pairings) input.

158555 paired-reads (in 2290 unique pairings) successfully merged out of 162182 (in 4070 pairings) input.

172050 paired-reads (in 4323 unique pairings) successfully merged out of 190413 (in 12107 pairings) input.

224750 paired-reads (in 5277 unique pairings) successfully merged out of 236430 (in 11093 pairings) input.

110533 paired-reads (in 3162 unique pairings) successfully merged out of 120090 (in 8193 pairings) input.

185359 paired-reads (in 4846 unique pairings) successfully merged out of 197485 (in 10664 pairings) input.

74475 paired-reads (in 2081 unique pairings) successfully merged out of 81237 (in 5689 pairings) input.

172196 paired-reads (in 5615 unique pairings) successfully merged out of 184600 (in 11872 pairings) input.

113767 paired-reads (in 2176 unique pairings) successfully merged out of 119892 (in 5441 pairings) input.

134711 paired-reads (in 3714 unique pairings) successfully merged out of 145738 (in 9143 pairings) input.

```
73247 paired-reads (in 1994 unique pairings) successfully merged out of 77840 (in 4525 pairings) input.
195319 paired-reads (in 3661 unique pairings) successfully merged out of 203857 (in 7803 pairings) input.
158945 paired-reads (in 4179 unique pairings) successfully merged out of 170340 (in 9271 pairings) input.
28318 paired-reads (in 946 unique pairings) successfully merged out of 31664 (in 2737 pairings) input.
116665 paired-reads (in 2647 unique pairings) successfully merged out of 120952 (in 4721 pairings) input.
104677 paired-reads (in 3972 unique pairings) successfully merged out of 114361 (in 7650 pairings) input.
151237 paired-reads (in 2769 unique pairings) successfully merged out of 157148 (in 5595 pairings) input.
231702 paired-reads (in 4225 unique pairings) successfully merged out of 242199 (in 8590 pairings) input.
208356 paired-reads (in 4709 unique pairings) successfully merged out of 216078 (in 7977 pairings) input.
146347 paired-reads (in 4855 unique pairings) successfully merged out of 157036 (in 9144 pairings) input.
90209 paired-reads (in 2233 unique pairings) successfully merged out of 93782 (in 4024 pairings) input.
155592 paired-reads (in 5048 unique pairings) successfully merged out of 171625 (in 13061 pairings) input.
166542 paired-reads (in 3553 unique pairings) successfully merged out of 180557 (in 9764 pairings) input.
230357 paired-reads (in 4733 unique pairings) successfully merged out of 242155 (in 10633 pairings) input.
> head(mergers[[1]])
sequence
1
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT
AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG
TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT
GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT
GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGAAACC
CCAGTAGTCC
2
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT
AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG
TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT
```

GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT

GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGAAACC

CCTGTAGTCC

3

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT

AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG

TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT

GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT

GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGATACC

CCAGTAGTCC

4

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT

AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG

TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT

GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT

GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGAAACC

CCGGTAGTCC

5

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT

AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG

TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT

GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT

GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGAAACC

CTAGTAGTCC

```
6
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGT
AAAGAGCATGTAGGCGGCTTAATAAGTCGAGCGTGAAAATGCGGGGCTCAACCCCG
TATGGCGCTGGAAACTGTTAGGCTTGAGTGCAGGAGAGGAAAGGGGAATTCCCAGT
GTAGCGGTGAAATGCGTAGATATTGGGAGGAACACCAGTGGCGAAGGCGCCTTTCT
GGACTGTGTCTGACGCTGAGATGCGAAAGCCAGGGTAGCGAACGGGATTAGAAACC
CTTGTAGTCC
  abundance forward reverse nmatch nmismatch nindel prefer accept
1       900       1       1    109         0      0      1   TRUE
2       815       1       2    109         0      0      1   TRUE
3       801       1       3    109         0      0      1   TRUE
4       779       1       4    109         0      0      1   TRUE
5       772       1       6    109         0      0      1   TRUE
6       765       1       7    109         0      0      1   TRUE
```

```
> seqtab <- makeSequenceTable(mergers)
> dim(seqtab)
```

```
[1]    72 66060
```

```
> table(nchar(getSequences(seqtab)))
```

```
  240   242   247   248   250   254   257   259   260   261   265   266   267
  111     2     1     4     1     1     2     2     1     2     2    19     1
  268   270   271   272   277   279   280   282   283   288   289   290   291
    2     1     1     1     1    14    16     3     1     1     5    23 13344
  292   293   294   295   297   298   303   304   306   307   308   312   313
51507   869    39     1     1     1     1     1     1     1     1     2     1
  316   317   318   323   325   327   329   337   338   343   345   346   347
```

```
  2   3   1   1   2   1   1   1   7   3  12   1   3
349 352 353 357 358 368 369 377 378 379 380
  1   2   1   1   1   1   2   1   4  19   1
```

```
> seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE,
verbose=TRUE)
```

```
Identified 55226 bimeras out of 66060 input sequences.
```

```
> dim(seqtab.nochim)
```

```
[1]    72 10834
```

```
> sum(seqtab.nochim)/sum(seqtab)
```

```
[1] 0.8774831
```

```
> getN <- function(x) sum(getUniques(x))
> track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN), sapply(mergers, getN),
rowSums(seqtab.no> im))
> colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR", "merged", "nonchim")
> rownames(track) <- sample.names
> head(track)
```

```
   input filtered denoisedF denoisedR merged nonchim
10 212595   171536    169116    168526 154094  137191
11 259367   216469    213592    213106 196092  173780
12 194327   153182    151914    151787 141406  129229
13 206672   169609    167928    168017 156693  129030
14 130741   108262    106573    107000  97087   80709
15  62980    39703     39131     38975  36455   32902
```

```
> taxa <- assignTaxonomy(seqtab.nochim, "~/mbpl2/Gallaher_Project_004/GTDB_bac-
arc_ssu_r86.fa.gz", multithread=TRUE)
```

```
> taxa.print <- taxa

> rownames(taxa.print) <- NULL

> head(taxa.print)
```

```
     Kingdom    Phylum         Class            Order
[1,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
[2,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
[3,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
[4,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
[5,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
[6,] "Bacteria" "Firmicutes_C" "Negativicutes" "Acidaminococcales"
     Family               Genus              Species
[1,] "Acidaminococcaceae" "Acidaminococcus" NA
[2,] "Acidaminococcaceae" "Acidaminococcus" NA
[3,] "Acidaminococcaceae" "Acidaminococcus" NA
[4,] "Acidaminococcaceae" "Acidaminococcus" NA
[5,] "Acidaminococcaceae" "Acidaminococcus" NA
[6,] "Acidaminococcaceae" "Acidaminococcus" NA
```

```
> write.table(taxa, "~/microbiome/Gallaher2_Project_001/taxa_mbpl1.txt")

> write.table(seqtab.nochim, "~/microbiome/Gallaher2_Project_001/mbpl1_seqtabnochim.txt")

> write.table(track, "~/microbiome/Gallaher2_Project_001/tracking.txt")
```

IN R

```
> mbpl1_seqtabnochim <- read.csv("~/Desktop/mbpl1/mbpl1_seqtabnochim.txt", sep="")

>   View(mbpl1_seqtabnochim)

> taxa_mbpl1 <- read.csv("~/Desktop/mbpl1/taxa_mbpl1.txt", sep="")
```

```
>   View(taxa_mbpl1)

> library(phyloseq); packageVersion("phyloseq")

[1] ‘1.30.0’

> library(Biostrings); packageVersion("Biostrings")

[1] ‘2.54.0’

> library(ggplot2); packageVersion("ggplot2")

[1] ‘3.2.1’

> mbpl1_otu <- as.matrix(mbpl1_seqtabnochim)

> mbpl1_taxa <- as.matrix(taxa_mbpl1)

> library(readxl)

> mbpl1metadata <- read_excel("Desktop/mbpl1/metadata.xlsx")

> View(mbpl1metadata)

> row.names(mbpl1metadata) <- mbpl1metadata$Sample

> View(mbpl1metadata)

> View(mbpl1metadata)

> mbpl1ps <- phyloseq(otu_table(mbpl1_otu, taxa_are_rows=FALSE),

sample_data(mbpl1metadata), tax_table(mbpl1_taxa))

> mbpl1ps

phyloseq-class experiment-level object

otu_table()   OTU Table:         [ 10834 taxa and 72 samples ]

sample_data() Sample Data:       [ 72 samples by 3 sample variables ]

tax_table()   Taxonomy Table:    [ 10834 taxa by 7 taxonomic ranks ]

> dna <- Biostrings::DNAStringSet(taxa_names(mbpl1ps))

> names(dna) <- taxa_names(mbpl1ps)

> mbpl1ps <- merge_phyloseq(mbpl1ps, dna)
```

```
> taxa_names(mbpl1ps) <- paste0("ASV", seq(ntaxa(mbpl1ps)))
> mbpl1ps
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 10834 taxa and 72 samples ]
sample_data() Sample Data:       [ 72 samples by 3 sample variables ]
tax_table()   Taxonomy Table:    [ 10834 taxa by 7 taxonomic ranks ]
refseq()      DNAStringSet:      [ 10834 reference sequences ]
> mbpl1ps.prop <- transform_sample_counts(mbpl1ps, function(mbpl1_otu)
mbpl1_otu/sum(mbpl1_otu))
> mbpl1_norm = transform_sample_counts(mbpl1ps, function(OTU) OTU/sum(OTU))
> ord.pcoambpl1.bray <- ordinate(mbpl1ps.prop, method="PCoA", distance="bray")
> plot_ordination(mbpl1ps.prop, ord.pcoambpl1.bray, color="Diet", title="Bray PCoA")
> mbpl1_Bray_PCoA <- plot_ordination(mbpl1ps.prop, ord.pcoambpl1.bray, color="Diet",
title="Bray PCoA")
> View(mbpl1_Bray_PCoA)
> View(mbpl1_Bray_PCoA)
> plot_ordination(mbpl1ps.prop, ord.pcoambpl1.bray, color="Diet", title="Bray PCoA")


> mbpl1Dist = phyloseq::distance(mbpl1ps.prop, "bray")
> mbpl1bray = ordinate(mbpl1ps.prop, "PCoA", mbpl1Dist)
> adonis(mbpl1Dist ~ Diet, as(sample_data(mbpl1ps.prop), "data.frame"), method = "bray")
Call:
adonis(formula = mbpl1Dist ~ Diet, data = as(sample_data(mbpl1ps.prop),      "data.frame"),
method = "bray")
```

```
Permutation: free
Number of permutations: 999
Terms added sequentially (first to last)
          Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
Diet       5    4.8441 0.96883  6.6233 0.33412  0.001 ***
Residuals 66    9.6542 0.14628         0.66588
Total     71   14.4983                 1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> library(ggplot2)
> p <- plot_ordination(mbpl1ps.prop, ord.pcoambpl1.bray, color="Diet", title="Bray PCoA")
> p
> p + geom_point(size=3)
> library(RColorBrewer)
> mbpl1Colors <- c("#00807f", "#40007f", "#024fa0", "#dfc0ff", "#c6e0fc", "#febffe")
> p + geom_point(size=3) + scale_color_manual(values = mbpl1Colors) + theme_bw()
```

#NEW COLORS

```
> mbpl1Colors <- c("#FE807F", "#DAC1FB", "#C3FFC3", "#0080FF", "#FE9E7F", "#FFDFFF")
> p + geom_point(size=7) + scale_color_manual(values = mbpl1Colors) + theme_bw()
```

MANUSCRIPT

```
> p2 <- plot_ordination(mbpl1ps.prop, ord.pcoambpl1.bray, shape="Diet", title="Bray Curtis PCoA")
```

> p2 + geom_point(size=5, aes(shape=Diet)) + theme_bw() + scale_shape_manual(values=c(0, 3, 5, 8, 2, 1))

p2 + geom_point(size=5, colour="black", fill="black", aes(shape=Diet)) + theme_bw() + scale_shape_manual(values=c(0, 3, 5, 8, 2, 1))

EXTRACTING TAXA IN R

> mbpl1_lacto = subset_taxa(mbpl1_norm, Genus=="Lactobacillus")

> mbpl1_L_df <- as.data.frame(otu_table(mbpl1_lacto))

> write.csv(mbpl1_L_df, "~/Desktop/mbpl1_lacto.csv")

> mbpl1_bifido = subset_taxa(mbpl1_norm, Genus=="Bifidobacterium")

> mbpl1_Bifido_df <- as.data.frame(otu_table(mbpl1_bifido))

> write.csv(mbpl1_Bifido_df, "~/Desktop/mbpl1_bifido.csv")

> mbpl1_firmicutes = subset_taxa(mbpl1_norm, Phylum=="Firmicutes_A")

> mbpl1_firmicutes_df <- as.data.frame(otu_table(mbpl1_firmicutes))

> write.csv(mbpl1_firmicutes_df, "~/Desktop/mbpl1_firmicutes.csv")

> mbpl1_firmicutesB = subset_taxa(mbpl1_norm, Phylum=="Firmicutes_B")

> mbpl1_firmicutesB_df <- as.data.frame(otu_table(mbpl1_firmicutesB))

> write.csv(mbpl1_firmicutesB_df, "~/Desktop/mbpl1_firmicutesB.csv")

> mbpl1_firmicutesC = subset_taxa(mbpl1_norm, Phylum=="Firmicutes_C")

> mbpl1_firmicutesC_df <- as.data.frame(otu_table(mbpl1_firmicutesC))

> write.csv(mbpl1_firmicutesC_df, "~/Desktop/mbpl1_firmicutesC.csv")

```
> mbpl1_Bacteroid = subset_taxa(mbpl1_norm, Phylum=="Bacteroidota")
> mbpl1_Bacter_df <- as.data.frame(otu_table(mbpl1_Bacteroid))
> write.csv(mbpl1_Bacter_df, "~/Desktop/mbpl1_Bacter.csv")

> mbpl1_prevotella = subset_taxa(mbpl1_norm, Genus=="Prevotella")
> mbpl1_prevotella_df <- as.data.frame(otu_table(mbpl1_prevotella))
> write.csv(mbpl1_prevotella_df, "~/Desktop/mbpl1_prevotella.csv")

> mbpl1_faecalibacterium = subset_taxa(mbpl1_norm, Genus=="Faecalibacterium")
> mbpl1_faecalibacterium_df <- as.data.frame(otu_table(mbpl1_faecalibacterium))
> write.csv(mbpl1_faecalibacterium_df, "~/Desktop/mbpl1_faecalibacterium.csv")

> mbpl1_bacteroides = subset_taxa(mbpl1_norm, Genus=="Bacteroides")
> mbpl1_bacteroides_df <- as.data.frame(otu_table(mbpl1_bacteroides))
> write.csv(mbpl1_bacteroides_df, "~/Desktop/mbpl1_bacteroides.csv")

> mbpl1_bacteroides_b = subset_taxa(mbpl1_norm, Genus=="Bacteroides_B")
> mbpl1_bacteroides_b_df <- as.data.frame(otu_table(mbpl1_bacteroides_b))
> write.csv(mbpl1_bacteroides_b_df, "~/Desktop/mbpl1_bacteroides_b.csv")

> mbpl1_gammaproteobacteria = subset_taxa(mbpl1_norm, Class=="Gammaproteobacteria")
> mbpl1_gammaproteobacteria_df <- as.data.frame(otu_table(mbpl1_gammaproteobacteria))
> write.csv(mbpl1_gammaproteobacteria_df, "~/Desktop/mbpl1_gammaproteobacteria.csv")
```

```
> mbpl1_akker= subset_taxa(mbpl1_norm, Genus=="Akkermansia")

> mbpl1_akker_df <- as.data.frame(otu_table(mbpl1_akker))

> write.csv(mbpl1_akker_df, "~/Desktop/mbpl1_Akkermansia.csv")
```

Appendix 4. SAS code for study 1

```
SAS CODE FOR POLYLACTOSE ANIMAL STUDY 1
*Polylactose animal study, 2017;
Data All_animals (Label='All Dietary Groups');
Title 'Polylactose animal study';
Input Animal Diet Block BW_0 BW_1 BW_2 BW_3 BW_4 BW_5 BW_6 BW_7 BW_8
BW_9 BW_final Cecal_pH Cecum_weight Colon_RNA_weight Fat_pad_weight
Liver_weight Liver_RNA_weight OGTT_0 OGTT_15 OGTT_30 OGTT_60 OGTT_90
OGTT_120 OGTT_AUC ITT_0 ITT_15 ITT_30 ITT_60 ITT_90 ITT_120 ITT_AUC
PTT_0 PTT_15 PTT_30 PTT_60 PTT_90 PTT_120 FI_1 FI_2 FI_3 FI_4 FI_5 FI_6
FI_7 FI_8a FI_8b FI_8c Fecal_wt Colon_length Colon_width Colon_area
ACF_1 ACF_2 ACF_3
ACF_4 ACF_5 ACF_6 ACF_7 ACF_8 ACF_total ACF_cm HbA1C Leptin
Liver_lipids
Adiponectin Liver_cholesterol Plasma_insulin Plasma_TAG BW_change
Cecal_contents_wt
Acetate_conc Acetate_amt Propionate_conc Propionate_amt
Isobutyrate_conc
Isobutyrate_amt Butyrate_conc Butyrate_amt Isovalerate_conc
Isovalerate_amt Valerate_conc
Valerate_amt SCFA_total_conc SCFA_total_amt Firmicutes_abundance
Bacteroidetes_abundance
FB_ratio Bifido_abundance Lactobacillus_abundance Akkermansia_abundance
Prevotella_abundance Faecalibacterium_abundance
Bacteroides_abundance Bacteroides_B_abundance
Total_Bacteroides_abundance Gammaproteobacteria_abudance AMPK_p AMPK
AMPK_total
Total_AMPK_corrected pAMPK pAMPK_corrected b_actin pAMPK_AMPK_ratio;

*Diet Groups:
1=Normal fat cellulose
2=High fat cellulose
3=High fat polylactose
4=High fat matched lactose
5=High fat polydextrose
6=High fat fructooligosaccharides;


Label Diet='Diet Group'
Block='Block'
BW_0='Initial Body Weight at start of experimental diets in grams'
BW_1='Body Weight Week 1 in grams'
BW_2='Body Weight Week 2 in grams'
BW_3='Body Weight Week 3 in grams'
BW_4='Body Weight Week 4 in grams'
BW_5='Body Weight Week 5 in grams'
BW_6='Body Weight Week 6 in grams'
BW_7='Body Weight Week 7 in grams'
BW_8='Body Weight Week 8 in grams'
BW_9='Body Weight Week 9 in grams'
BW_final='Final Body Weight in grams'
Cecal_pH='Cecal pH'
Cecum_weight='Weight of empty cecum in grams'
```

```
Colon_RNA_weight='Weight of colon segment for RNA-seq in grams'
Fat_pad_weight='Epididymal fat pad weight in grams'
Liver_weight='Liver weight in grams'
Liver_RNA_weight='Weight of liver segment for RNA-seq in grams'
OGTT_0='Fasting blood glucose for oral glucose tolerance test in mg/dl'
OGTT_15='Blood glucose 15 min after glucose administration in mg/dl'
OGTT_30='Blood glucose 30 min after glucose administration in mg/dl'
OGTT_60='Blood glucose 60 min after glucose administration in mg/dl'
OGTT_90='Blood glucose 90 min after glucose administration in mg/dl'
OGTT_120='Blood glucose 120 min after glucose administration in mg/dl'
OGTT_AUC='Area under the curve for oral glucose tolerance test'
ITT_0='Fasting blood glucose for insulin tolerance test in mg/dl'
ITT_15='Blood glucose 15 min after insulin administration in mg/dl'
ITT_30='Blood glucose 30 min after insulin administration in mg/dl'
ITT_60='Blood glucose 60 min after insulin administration in mg/dl'
ITT_90='Blood glucose 90 min after insulin administration in mg/dl'
ITT_120='Blood glucose 120 min after insulin administration in mg/dl'
ITT_AUC='Area under the curve for insulin tolerance test'
PTT_0='Fasting blood glucose for pyruvate tolerance test in mg/dl'
PTT_15='Blood glucose 15 min after pyruvate administration in mg/dl'
PTT_30='Blood glucose 30 min after pyruvate administration in mg/dl'
PTT_60='Blood glucose 60 min after pyruvate administration in mg/dl'
PTT_90='Blood glucose 90 min after pyruvate administration in mg/dl'
PTT_120='Blood glucose 120 min after pyruvate administration in mg/dl'
FI_1='Food intake week 1 in grams'
FI_2='Food intake week 2 in grams'
FI_3='Food intake week 3 in grams'
FI_4='Food intake week 4 in grams'
FI_5='Food intake week 5 in grams'
FI_6='Food intake week 6 in grams'
FI_7='Food intake week 7 in grams'
FI_8a='Food intake week 8 in grams, day one of fecal collection'
FI_8b='Food intake week 8 in grams, day two of fecal collection'
FI_8c='Food intake week 8 in grams, day three of fecal collection'
Fecal_wt='3 day fecal weight in g'
Colon_length='Length of colon segment for ACF counting'
Colon_width='Width of colon segment for ACF counting'
Colon_area='Area of colon segment for ACF counting'
ACF_1='ACF 1'
ACF_2='ACF 2'
ACF_3='ACF 3'
ACF_4='ACF 4'
ACF_5='ACF 5'
ACF_6='ACF 6'
ACF_7='ACF 7'
ACF_8='ACF 8 or more'
ACF_total='Total ACF'
ACF_cm='Total ACF per cm squared'
HbA1C='Percent HbA1C, glycated hemoglobin'
Leptin='Plasma Leptin in ng/mL'
Liver_lipids='g lipids/1g liver'
Adiponectin='Plasma Adiponectin in ng/mL'
Liver_cholesterol='Liver cholesterol in umol/g liver'
Plasma_insulin='Fasting insulin in ng/mL'
```

```
Plasma_TAG='Plasma triacyglycerols in mmol/L'
BW_change='Change in body weight in g'
Cecal_contents_wt='weight of cecal contents in g'
Acetate_conc='umol acetate/g cecal contents'
Acetate_amt='umol acetate in cecal contents'
Propionate_conc='umol propionate/g cecal contents'
Propionate_amt='umol propionate in cecal contents'
Isobutyrate_conc='umol isobutyrate/g cecal contents'
Isobutyrate_amt='umol isobutyrate in cecal contents'
Butyrate_conc='umol butyrate/g cecal contents'
Butyrate_amt='umol butyrate in cecal contents'
Isovalerate_conc='umol isovalerate/g cecal contents'
Isovalerate_amt='umol isovalerate in cecal contents'
Valerate_conc='umol valerate/g cecal contents'
Valerate_amt='umol valerate in cecal contents'
SCFA_total_conc='Concentration of total SCFAs in cecal contents umol/g'
SCFA_total_amt='Amount of total SCFAs in cecal contents umol'
Firmicutes_abundance='% Abundance of Firmicutes, Phylum'
Bacteroidetes_abundance='% Abundance of Bacteroidetes, Phylum'
FB_ratio='Ratio of Firmicutes:Bacteroidetes, Phylum'
Bifido_abundance='% Abundance of Bifidobacterium, Genus'
Lactobacillus_abundance='Abundance of Lactobacillus, Genus'
Akkermansia_abundance='Abundance of Akkermansia muciniphila, Species'
Prevotella_abundance='Abudance of Prevotella, Genus'
Faecalibacterium_abundance='Abundance of Faecaliabacterium, Genus'
Bacteroides_abundance='Abundance of Bacteroides, general, Genus'
Bacteroides_B_abundance='Abundance of Bacteroides B, Genus'
Total_Bacteroides_abundance='Abundance of total Bacteroides, Genus'
Gammaproteobacteria_abudance='Abundance of Gammaproteobacteria, Class'
AMPK_p='AMPK intensity 70kDa'
AMPK='AMPK intensity 62kDa'
AMPK_total='Total AMPK intensity'
Total_AMPK_corrected='AMPK intensity corrected'
pAMPK='pAMPK intensity'
pAMPK_corrected='pAMPK intensity corrected'
b_actin='b-actin intensity (loading control)'
pAMPK_AMPK_ratio='Ratio of pAMPK to AMPK'
;
Output All_animals;

Cards;
1      1      1      184    242    300    321    370    407    432    448    479
       494    492    6.63   0.577  0.1350        5.01   15.409        0.25   137
       158    169    129    98     109    15645  124    117    83     64     54
       45     8767.5        97     133    119    124    130    125    21.4   20.1
       13.4   23.4   21.6   20     23.6   21.9   18.9   22.5   7      5      2
       10     9      7      6      0      2      0      1      1      26     2.6
       13.13  7.29   0.138111111 20739.85042 19.01559796 8.777936963
       1.462051013 308    1.6172        21.87  35.368164     12.635
       20.433322     2.072  3.3508384     8.493  13.7348796    2.676  4.3276272
       2.725  4.40687       50.471        81.6217012    54.3519027    20.1068323
       2.703155917 0      0      4.686227341 0      0      2.026783854
       6.766048529 8.792832383 1.443373174 1.01   5      6.01   33.57541899
       0.00309       0.01726257    0.179  0.000514143
```

2 1 1 178 236 290 336 375 412 430 435 472
463 472 6.62 0.566 0.1444 4.83 12.842 0.258 129
231 158 109 116 112 16417.5 134 113 71 56
53 70 8617.5 141 137 141 123 122 121 18.8
21.8 20.2 20.7 24.7 23.2 28.7 21 19.8 14.2 6.4 5
2 10 18 23 19 5 1 5 1 1 73
7.3 5.02 10.60 0.056111111 37731.28293 20.79808435 6.82234957
3.006628027 294 2.1699 35.237 76.4607663 12.212
26.4988188 1.645 3.5694855 10.453 22.6819647 1.837
3.9861063 1.781 3.8645919 63.165 137.0617335 53.5955914
26.2249018 2.043690833 0 0 2.974137931 0 0
2.447621126 11.42186818 13.86948931 3.798559581 1.79 7.62 9.41
61.1038961 0.0285 0.185064935 0.154 0.003028693
3 1 1 192 252 316 332 375 402 420 450 480
469 468 6.75 0.617 0.1669 2.99 11.278 0.2094
108 184 158 132 111 109 16050 112 91 73 43
33 41 6742.5 86 112 119 121 97 96 18.8
20.5 9.7 25.8 19.7 20.3 22.9 19.7 21.9 21 7.2 5
2 10 20 31 15 8 5 2 0 3 84
8.4 6.50 2.20 0.0867 32929.38969 24.76928495 5.203438395
1.746810184 276 1.8185 21.058 38.293973 13.032
23.698692 2.219 4.0352515 9.966 18.123171 2.776 5.048156
2.826 5.139081 51.877 94.3383245 52.09983 22.6174731
2.303521254 0.0131687 0.8994773 8.30313128 0 0
3.455916453 10.04883858 13.50475503 1.943809867 0.952 6.74 7.692
71.88785047 0.076 0.710280374 0.107 0.009880395
4 1 1 184 243 309 362 410 451 471 500 530
541 560 6.79 0.682 0.2131 5.47 19.926 0.2428
113 165 167 162 113 123 17175 96 100 84 47
56 62 8130 114 172 152 147 152 141 22.8 24.9
19.6 22.5 23.4 22.3 25.7 26.2 25 23.2 8.2 5 2
10 14 13 7 5 0 2 2 0 43 4.3
10.99 7.03 0.160444444 9131.864463 30.9779853 3.12640801
1.536936427 376 1.9254 22.839 43.9742106 10.282
19.7969628 1.74 3.350196 11.718 22.5618372 1.849
3.5600646 1.927 3.7102458 50.355 96.953517 53.1180344
22.8841604 2.321170341 0 0.3643565 5.063269435 0.400363499
0 4.513734097 3.956482974 8.470217071 1.166798121 2.43 5.48
7.91 30.30651341 0.0432 0.165517241 0.261 0.005461441
5 1 1 187 258 328 362 425 451 480 469 515
529 537 6.96 0.580 0.1528 7.09 16.328 0.258 138
164 156 141 136 126 17205 117 108 75 52 45
38 7665 137 129 133 134 129 141 23.7 23.3 16.3
29.3 24.8 23.9 31.4 25.4 23.7 20.8 7.6 5 2 10
8 19 18 9 1 6 1 1 63 6.3 7.49
20.12 0.1142 13743.89447 23.63413459 4.421777222 1.666115827
350 1.8325 30.271 55.4716075 12.088 22.15126
1.839 3.3699675 16.534 30.298555 1.996 3.65767 2.205
4.0406625 64.933 118.9897225 70.7329889 13.5125668
5.234607898 0 0.1226027 0.979361882 0 0 1.81714686
2.672446507 4.489593368 0.925358321 0.513 9.52 10.033
94.6509434 0.00752 0.070943396 0.106 0.000749527
6 1 1 216 277 331 376 421 445 461 478 485
483 495 7.03 0.511 0.1927 5.56 17.446 0.2583

107 125 120 127 120 123 14632.5 90 67 69
46 36 31 6157.5 102 142 127 122 111 97
29.9 32.3 9 23.3 25 28.6 34.1 25.6 40.5 22.4 7.5
5 2 10 8 9 4 2 0 0 0 1
24 2.4 6.76 11.10 0.133636364 23961.44017 46.38145075
4.790988736 1.685943805 279 1.2914 . . . .
. . . .
51.8430261 15.5974122 3.323822275 0.00970652 0.0354288
18.79474101 0 0 1.402592612 0.566860959 1.969453571
4.782889341 2.19 5.58 7.77 28.15217391 0.0467 0.169202899
0.276 0.006010296
7 2 1 186 258 326 375 422 462 497 527 553
563 566 6.88 0.527 0.1310 7.48 17.193 0.2556
144 169 197 160 134 129 18802.5 111 133 64
54 35 44 7597.5 115 141 130 131 135 124
20.2 18.5 18 17.8 23.2 18.1 21.3 20.4 18.5 17.2 7.2
5 2 10 22 19 17 4 6 1 1 1
71 7.1 6.80 18.35 0.1876 43195.10794 50.95357869
6.936962751 1.229496855 380 1.6694 24.272 40.5196768
13.831 23.0894714 2.251 3.7578194 9.297 15.5204118 2.881
4.8095414 2.791 4.6592954 55.323 92.3562162 45.4502533
20.9928355 2.165036414 0 0 9.830180761 1.955244462 0
1.827842561 4.843520489 6.67136305 2.757127012 1.42 4.93 6.35
24.14448669 0.00209 0.007946768 0.263 0.000329134
8 2 1 206 276 343 396 445 484 510 540 565
567 570 6.73 0.578 0.1740 5.92 25.541 0.234 138
179 158 193 144 134 19395 135 120 91 67 49
48 9060 117 140 143 136 123 108 16 19.8 19.9
21.4 22.5 16.7 21 25.4 27 18.2 7.4 5 2 10
13 37 19 9 3 4 2 4 91 9.1 9.68
19.37 0.125 35758.12992 20.69054701 6.285100287 0.812589526 364
1.8403 33.74 62.091722 12.732 23.4306996 2.365
4.3523095 15.934 29.3233402 2.791 5.1362773 3.027
5.5705881 70.589 129.9049367 62.6747078 11.7121537
5.351253869 0 . 5.170193192 0.10971413 0 1.869910389
3.329585335 5.199495724 0.589457903 1.81 8.06 9.87 63.26923077
0.0191 0.122435897 0.156 0.001935157
9 2 1 203 273 340 388 435 470 503 530 545
555 550 6.88 0.488 0.1634 7.77 17.853 0.2714
97 154 167 158 142 133 17790 85 94 67 45
39 41 6690 102 126 120 137 132 127 17.9 16.9
19.4 20.6 24.1 18.2 20 19.5 15.4 18.6 7 5 2
10 8 17 24 20 13 4 4 2 92 9.2
8.67 15.51 0.146222222 39117.47325 40.92744907 6.356733524
1.44647485 347 1.5833 29.669 46.9749277 10.76
17.036308 1.631 2.5823623 10.731 16.9903923 1.783
2.8230239 2.16 3.419928 56.734 89.8269422 48.8634572
23.2841954 2.098567563 0 0 13.5787839 0 0
1.423963684 11.7156851 13.13964879 3.377374268 3.5 8.48 11.98
113.0188679 0.0161 0.151886792 0.106 0.001343907
10 2 1 177 237 280 312 340 365 390 411 435
444 460 7.01 0.510 0.1772 6.38 14.094 0.2066
124 233 202 141 133 126 19080 115 105 73 64
59 83 9015 108 160 136 140 130 124 15.7 15.3

```
        13.6  16.5  15.1  13.7  23.5  15.2  13.8  14.2  5.5   5     2
        10    22    32    13    11    4     1     2     1     86    8.6
        8.37  27.84 0.2023      23835.99668 33.32528159 10.5669587
        1.908273911 283   0.7911      .     .     .     .     .     .
        .     .     .     .     .     .     .     .     51.8124367
        18.8438017  2.749574503 0     0.212842    5.937707284 0.290106494
        0     2.084684855 7.554431413 9.639116269 1.169172905 2.35  4.72
        7.07  35.70707071 0.0182      0.091919192 0.198 0.002574257
11      2     1     193   267   344   393   438   480   516   546   570
        588   583   7.13  0.475 0.1971      6.64  19.026      0.2494
        104   135   150   137   124   109   15645 110   103   78    47
        38    39    7260  129   136   145   135   129   117   21.5  19.7
        20.8  19.4  22.4  23.4  27.3  23.2  18.1  21.3  7.9   5     2
        10    13    15    6     10    6     1     1     2     54    5.4
        9.72  9.60  0.215555556 10626.55437 40.01156206 4.82853567
        0.814725523 390   1.6418      18.763      30.8050934  9.659
        15.8581462  1.487 2.4413566   10.615      17.427707   1.806
        2.9650908   2.129 3.4953922   44.459      72.9927862  36.5536886
        27.3483715  1.336594707 0     0     7.41857521  0.560478766 0
        1.912763264 11.80745771 13.72022097 2.428933134 1.4   2.09  3.49
        28.60655738 0.0158      0.129508197 0.122 0.004527221
12      2     1     195   269   345   398   440   476   495   525   543
        551   536   6.78  0.594 0.2152      5.53  19.205      0.288 109
        122   123   107   97    113   13230 113   84    73    32    37
        28    6240  94    128   124   111   102   88    28.6  26.3  23.5
        24.3  19.2  20    28.4  26.3  26.3  20.5  8.6   5     2     10
        16    11    12    6     2     1     1     1     50    5.0   7.97
        6.37  0.277375    5902.463425 68.75975937 3.833541927 0.782455214
        341   2.0062      28.147      56.4685114  13.033      26.1468046
        1.769 3.5489678   8.81  17.674622   1.74  3.490788    1.989
        3.9903318   55.488      111.3200256 52.6336968  22.7503115
        2.313537412 0.0077382   0     6.43586192  0     0     3.708146004
        12.4507657  16.1589117  2.36247282  2.31  5.81  8.12  29
        0.0238      0.085 0.28  0.002931034
13      3     1     183   228   276   314   358   389   420   441   473
        482   485   5.91  1.749 0.0850      5.30  16.073      0.328 151
        188   159   139   111   124   16890 106   123   98    89    92
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17.7 21.7 21.3 32.8 22.3 20.7 15.8 18.8 5.6 5 2
10 7 10 4 2 2 2 1 1 29 2.9
9.33 22.88 0.2313 8725.280696 47.83912323 6.949937422
1.417562724 339 1.417 29.243 41.437331 12.31 17.44327
1.906 2.700802 10.072 14.272024 2.36 3.34412 2.098
2.972866 57.989 82.170413 40.7177172 14.5562507
2.797266826 0.1243274 1.1726559 14.95608757 0 0
1.247252365 3.805412717 5.052665082 5.299330621 1.2 3.33 4.53
13.6036036 0.0217 0.065165165 0.333 0.004790287
59 4 2 171 218 250 275 302 322 346 364 390
390 400 6.85 0.547 0.1952 2.48 10.74 0.297 123 157
155 128 102 114 15375 110 77 71 47 34 39
6592.5 138 124 145 131 84 134 12.1 12.5 11
13.2 14.1 21.1 16.8 17.1 12.4 15.1 4.8 2 2.2 4.4
1 2 2 0 1 0 0 0 6 1.4 7.03
6.74 0.088181818 6379.281255 18.2835693 5.779724656 1.064156138
229 1.3308 36.119 48.0671652 17.798 23.6855784
2.322 3.0901176 17.799 23.6869092 2.708 3.6038064 2.823
3.7568484 79.569 105.8904252 26.4803873 35.9244329
0.737113579 0.0288377 0.0147256 13.05673667 0 0
1.433909474 20.65087341 22.08478289 2.804621398 0.46 1.97 2.43
28.2887078 0.0106 0.123399302 0.0859 0.00436214
60 4 2 214 282 362 416 462 496 517 538 560
566 570 6.63 0.495 0.1881 7.85 18.571 0.257 110
180 185 134 102 112 16447.5 110 99 74 55
60 60 8325 112 130 156 131 108 120 15.6 16.6
21.4 22 14.5 30.1 14.9 20.5 14.5 18 5.9 5 2
10 4 1 1 0 0 0 0 0 6 0.6
7.50 24.35 0.248 32107.59143 55.13078059 7.312891114 0.906045635
356 1.2287 24.587 30.2100469 11.31 13.896597 0.992
1.2188704 8.552 10.5078424 1.273 1.5641351 1.672 2.0543864

48.386 59.4518782 36.9155448 25.156501 1.467435587
0.0221594 0 8.266162539 0 0 4.011550607 6.895047366
10.90659797 3.187496538 2.83 3.83 6.66 28.22033898 0.0274
0.116101695 0.236 0.004114114
61 5 2 179 227 287 330 374 408 442 464 496
493 503 6.97 0.743 0.2004 6.22 18.009 0.271 122
187 182 146 158 148 19155 110 102 79 61 46
56 8182.5 122 160 173 146 132 114 12.8 31.4
18.2 14 15 21.3 18 20.1 16 18.3 5.3 4 2
8 1 0 0 1 1 1 0 0 4 0.5
5.80 20.74 0.214111111 . 51.96173617 5.597421203 1.067617051
324 2.3944 24.221 57.9947624 12.081 28.9267464
1.496 3.5820224 4.239 10.1498616 1.968 4.7121792 1.402
3.3569488 45.407 108.7225208 43.8307201 26.20407
1.67266841 0.024782 0 15.79564409 0 0 7.498451127
4.019444312 11.51789544 4.976409474 1.75 4.19 5.94 28.55769231
0.00786 0.037788462 0.208 0.001323232
62 5 2 200 269 327 369 406 448 483 500 527
536 541 6.76 0.895 0.2012 5.69 15.983 0.268 135
166 178 166 140 137 18742.5 119 100 85 53
41 47 7830 136 150 148 138 120 125 16.7 17
20.1 18.5 19 22 20.5 22.1 17.4 16.8 4.4 5 2
10 6 16 6 3 7 2 0 0 40 4.0
3.49 9.39 0.114111111 28656.96447 18.72866818 8.73495702
0.776567998 341 4.171 10.826 45.155246 6.294 26.252274
0.993 4.141803 3.776 15.749696 1.631 6.802901 1.117
4.659007 24.637 102.760927 32.4550194 24.8458052
1.306257501 2.3291079 0.9498739 12.33387855 0 0
5.044128672 3.651264227 8.695392899 6.08174879 2.18 8.41 10.59
79.02985075 0.0166 0.123880597 0.134 0.001567517
63 5 2 226 295 378 434 500 546 600 624 660
660 672 6.58 0.925 0.2222 11.33 23.771 0.285 117
153 175 155 148 143 18345 109 99 89 79 59
69 9480 136 124 130 149 139 114 15.2 17 19.2
27.1 20.9 26.3 22.5 22.8 18.5 22.6 4.6 4.5 2 9
2 4 2 0 1 1 3 0 13 1.4 6.31
31.27 0.211909091 23132.22533 28.724548 15.64040115 1.707513972
446 3.3369 22.197 74.0691693 11.864 39.5889816
0.98 3.270162 3.358 11.2053102 1.422 4.7450718 1.168
3.8974992 40.989 136.7761941 49.7971694 20.0516842
2.483440738 0 0.619009 12.18335887 0 0 2.186063283
6.027825355 8.213888638 7.34697557 2.89 8.1 10.99 99.90909091
0.0428 0.389090909 0.11 0.003894449
64 5 2 196 261 328 360 392 420 460 483 510
516 522 6.78 0.889 0.1943 4.23 14.091 0.306 104
165 150 141 120 125 16335 103 84 72 43 44
36 6802.5 132 150 156 141 133 125 16.7 19.5
22.6 20.6 18.9 21.9 16.8 19.3 11.2 22.2 2.8 5 2
10 2 0 0 0 0 0 0 0 2 0.2
2.75 8.51 0.203230769 13171.97415 53.38725111 3.533166458
1.141297868 326 5.1502 14.858 76.5216716 8.911
45.8934322 1.453 7.4832406 5.721 29.4642942 2.008 10.3416016
1.279 6.5871058 34.23 176.291346 54.4876361 15.924946
3.421527213 0 0.0531416 8.037945343 0 0 5.09141481

0.102891128 5.194305937 3.310606832 2.82 3.42 6.24 27.73333333
0.0354 0.157333333 0.225 0.005673077
65 5 2 181 238 300 347 391 429 456 489 516
526 518 6.93 0.645 0.2170 6.64 15.121 0.301 143
209 238 179 177 144 22402.5 135 94 77 53
53 52 8115 130 157 156 164 111 150 13.8 18.1
21.6 22.7 27.6 31.5 29.9 21.4 18.5 18.4 5.9 5 2
10 3 4 3 5 0 2 1 1 19 1.9
6.00 22.54 0.184888889 13319.9177 38.03536283 11.78723404
0.949089195 337 3.0353 22.385 67.9451905 10.845
32.9178285 1.232 3.7394896 4.451 13.5101203 1.486 4.5104558
1.344 4.0794432 41.743 126.7025279 41.0832893 23.4751306
1.750077135 0.2158466 0.0630156 13.31585314 0 0
4.327072816 1.618125321 5.945198137 7.808142777 1.03 2.25 3.28
29.02654867 0.025 0.221238938 0.113 0.007621951
66 5 2 186 253 323 362 418 450 475 449 519
536 547 6.64 0.714 0.1809 8.11 17.66 0.303 122 165
192 150 145 144 18720 130 103 71 47 34 51
7312.5 122 153 138 132 139 119 13.6 33.1 23
17.6 21.6 27.8 19.4 18.7 15.5 21.5 4.3 5 2 10
7 7 3 1 0 0 0 0 18 1.8 7.42
13.12 0.177888889 13171.97415 46.04803087 5.204005006 0.744951914
361 2.8055 22.391 62.8179505 12.287 34.4711785
1.108 3.108494 4.237 11.8869035 1.426 4.000643 1.396
3.916478 42.845 120.2016475 57.0867203 9.6544285
5.913008761 0.0963131 0 11.26863659 0 0 0.820587895
1.186577802 2.007165697 11.01051739 3.85 4.41 8.26 43.02083333
0.0242 0.126041667 0.192 0.002929782
67 6 2 212 280 351 409 460 499 527 559 581
591 597 7.07 0.823 0.1805 9.37 21.122 0.279 139
179 176 191 188 175 21682.5 140 132 99 89
68 70 11017.5 140 192 192 202 178 167 13.1
21.3 19.2 20.5 25.8 27.4 24.6 24.7 16.3 23.2 4.7 4.5
2 9 15 7 6 1 1 0 0 1 31
3.4 3.42 29.47 0.153777778 28853.17973 24.93265651 8.871060172
1.573392732 385 2.3044 34.15 78.69526 27.582
63.5599608 4.081 9.4042564 7.385 17.017994 5.952 13.7157888
0 0 79.15 182.39326 39.9200737 18.920895 2.109840666
0.3255143 0.4236694 19.3395565 5.82820857 0 2.653192043
2.208489413 4.861681457 7.785300775 1.88 6.05 7.93 37.94258373
0.0165 0.078947368 0.209 0.002080706
68 6 2 155 218 267 295 322 348 371 390 417
421 442 7.08 0.852 0.2256 4.77 13.535 0.282 117
174 165 146 141 138 17880 81 90 70 58 51
54 7612.5 95 138 134 128 127 114 11 12.8
13.8 14.6 13.3 21 18.8 16.4 14.5 19.8 3.6 3 2
6 0 0 0 2 0 0 0 1 3 0.5
7.11 15.73 0.086111111 31092.05206 21.49391424 5.518624642
0.755516456 287 2.5173 20.226 50.9149098 11.006
27.7054038 0.678 1.7067294 8.197 20.6343081 1.395 3.5116335
0 0 41.502 104.4729846 33.8029846 19.1024618
1.769561691 0.3418172 0 7.816538237 0 0 1.619761085
3.956504651 5.576265736 8.956326001 1.81 8.8 10.61 102.0192308
0.0128 0.123076923 0.104 0.001206409

```
69   6    2    209  271  330  375  404  435  473  490  507
     520  526  7.05 0.749 0.1978     5.09 19.91 0.294 133 136
     163  138  125  112  16275 100   100  77   51   51   65
     8017.5    115  122  129  124  112  117  13.8 18.1 16.7
     22.1 29.1 27.2 24   22.6 12.6 22.1 4.8  5    1.5  7.5
     11   6    9    4    3    1    0    0    34   4.5  4.39
     7.82 0.299777778 35280.86657 39.62163843 5.153942428 0.881357897
     317  1.6897    21.093    35.6408421  11.122    18.7928434
     1.422 2.4027534  6.43 10.864771  1.888 3.1901536  1.562
     2.6393114  43.517    73.5306749  37.3178697  13.1048085
     2.847647083 3.3128247  .    9.252470684 0.607398832 0
     2.502483189 4.873482396 7.375965585 10.96747915 1.28  7.03  8.31
     69.83193277 0.0233    0.195798319 0.119 0.002803851
70   6    2    220  282  359  410  456  492  526  568  596
     600  606  6.94 1.355 0.1948     6.28 21.694    0.322 140
     139  132  142  123  138  16125 111  111  86   66   65
     73   9457.5    125  143  136  141  135  126  15.3 19
     24.2 19.1 23.4 24.6 22.6 24.8 13.9 24   4.3  5    2
     10   11   8    5    6    0    1    0    0    31   3.1
     10.06 10.63 0.187888889 8294.990584 35.43782586 4.972465582
     0.576404452 386  3.1754    38.326    121.7003804 25.179
     79.9533966 3.274 10.3962596  11.029    35.0214866  4.523
     14.3623342 2.521 8.0051834  84.852    269.4390408 43.2095394
     16.8380919  2.566177905 0.5161875  0.5451377  7.955296521
     0.36925161  0    1.655077483 4.567576868 6.222654351 11.67512413
     1.7  2.97 4.67 19.62184874 0.0247    0.103781513 0.238
     0.005289079
71   6    2    195  264  339  393  432  470  507  531  558
     564  558  7.11 0.703 0.1972     8.00 19.448    0.305 120
     146  160  151  143  138  17580 126  119  76   58   57
     69   8925 125  154  153  133  141  139  17.8 18.1 20.4
     21.3 22.9 27   20.4 23.3 15.9 22.2 5.2  5    2    10
     3    6    8    5    3    3    2    1    31   3.1  7.75
     24.34 0.272444444 13590.79627 40.84432724 6.28660826  0.997246841
     363  2.8138    22.083    62.1371454  13.526    38.0594588
     1.981 5.5741378  7.858 22.1108404  2.697 7.5888186  2.005
     5.641669   50.15 141.11207  32.7035004  24.5956373  1.329646392
     0.0981023  0.044517   7.419498442 0    0    2.398971163
     4.666040131 7.065011294 11.18777926 1.45  2.65  4.1  32.28346457
     0.0328     0.258267717 0.127 0.008
72   6    2    187  248  316  365  392  426  448  472  487
     493  502  7.24 0.838 0.1973     4.17 19.81 0.253 136 166
     148  155  109  130  16710 132  101  86   46   34   42
     7470 123  125  132  123  125  121  12.4 16   15.9 23
     27.8 28.5 13.3 26   16   18.2 3.7  4    2    8    2
     2    1    0    0    0    0    0    5    0.6  6.84 16.05
     0.157909091 12653.80452 39.87858637 2.863579474 1.188172732 315
     1.7288     29.002    50.1386576  11.394    19.6979472  0.478
     0.8263664  5.318 9.1937584  0.653 1.1289064  0    0
     46.845     80.985636  24.1341734  37.619149  0.641539589
     0.3444518  0.2667936  15.17466839 0    0    2.95226524
     27.67889575 30.63116099 5.762992096 0.953 5.76  6.713 38.80346821
     0.0227     0.131213873 0.173 0.003381499
```

```
proc sort data = All_animals; by Diet;

proc format;
      value dietfmt
1='Normal fat cellulose'
2='High fat cellulose'
3='High fat polylactose'
4='High fat matched lactose'
5='High fat polydextrose'
6='High fat fructooligosaccharides';


proc print data= All_animals; by Diet; format Diet dietfmt.;

proc means data=All_animals n mean stderr min max; by Diet; format Diet
dietfmt.;

proc glm data= All_animals;
title 'One Way Analysis of Variance in all rats';
format Diet dietfmt.;
class Diet;
model BW_0--pAMPK_AMPK_ratio=Diet;
Means Diet / Duncan;
run;
proc corr data= All_animals;
title 'Correlation analysis in all rats';
var Cecum_weight Cecal_pH SCFA_total_conc SCFA_total_amt FB_ratio;
with Acetate_conc Acetate_amt Propionate_conc Propionate_amt
Isobutyrate_conc
Isobutyrate_amt Butyrate_conc Butyrate_amt Isovalerate_conc
Isovalerate_amt Valerate_conc Valerate_amt SCFA_total_amt;
run;
quit;
```

Appendix 5. SAS code for study 2

```
/*Second polylactose animal study, 2019. Breann Abernathy
Modified 7/27/2020 by DDG
Includes re-analysis of bifidobacteria and lactobacillus abundance
using zero-inflated gamma model
using PROC FMM. */
Data All_animals (Label='All Dietary Groups');
Title 'Polylactose animal study 2';
Input Animal Diet Block BW_0 BW_1 BW_2 BW_3 BW_4 BW_5 BW_6 BW_7 BW_8
BW_9 BW_10 BW_final change_BW FI_1 FI_2 FI_3 FI_4 FI_5 FI_6 FI_7
MRI_fat_g MRI_Lean_g MRI_freeWater_g MRI_totalWater_g MRI_BW_g
MRI_bodyfat_percent     MRI_lean_percent
Cecal_pH Cecum_weight Fat_pad_weight Liver_weight Fasted_glucose
Liver_lipids Leptin Serum_insulin
Liver_cholesterol Cecal_contents_wt Acetate_conc Acetate_amt
Propionate_conc Propionate_amt Isobutyrate_conc
Isobutyrate_amt Butyrate_conc Butyrate_amt Isovalerate_conc
Isovalerate_amt Valerate_conc
Valerate_amt SCFA_total_conc SCFA_total_amt Firmicutes_abundance
Bacteroidetes_abundance
FB_ratio Bifido_abundance Lactobacillus_abundance Akkermansia_abundance
FACS_Cells FACS_Singlets Lipid_events Large_cells Large_cell_percent
Medium_cells Medium_cell_percent
Small_cells Small_cell_percent Large_droplets Large_droplet_percent
Medium_droplets Medium_droplet_percent
Small_droplets Small_droplet_percent Energy_intake;

*Diet Groups:
1=Normal fat cellulose
2=High fat cellulose
3=High fat polylactose
4=High fat polyactose2
5=High fat polydextrose
6=High fat galactooligosaccharides;


Label Diet='Diet Group'
Block='Block'
BW_0='Initial Body Weight at start of experimental diets in grams'
BW_1='Body Weight Week 1 in grams'
BW_2='Body Weight Week 2 in grams'
BW_3='Body Weight Week 3 in grams'
BW_4='Body Weight Week 4 in grams'
BW_5='Body Weight Week 5 in grams'
BW_6='Body Weight Week 6 in grams'
BW_7='Body Weight Week 7 in grams'
BW_8='Body Weight Week 8 in grams'
BW_9='Body Weight Week 9 in grams'
BW_10='Body Weight Week 10 in grams'
BW_final='Final Body Weight in grams'
change_BW='change in body weight from week 1-final'
FI_1='Food intake week 1 in grams'
FI_2='Food intake week 2 in grams'
```

```
FI_3='Food intake week 3 in grams'
FI_4='Food intake week 4 in grams'
FI_5='Food intake week 5 in grams'
FI_6='Food intake week 6 in grams'
FI_7='Food intake week 7 in grams'
MRI_fat_g='fat mass by MRI in grams'
MRI_Lean_g='lean mass by MRI in grams'
MRI_freeWater_g='free water by MRI in grams'
MRI_totalWater_g='total water by MRI in grams'
MRI_BW_g='body weight in grams on day of MRI'
MRI_bodyfat_percent='percent body fat by MRI'
MRI_lean_percent='percent lean mass by MRI'
Cecal_pH='Cecal pH'
Cecum_weight='Weight of empty cecum in grams'
Fat_pad_weight='Epididymal fat pad weight in grams'
Liver_weight='Liver weight in grams'
Fasted_glucose='fasted serum glucose in mg/mL'
Liver_lipids='g lipids/1g liver'
Leptin='Plasma Leptin in pg/mL'
Serum_insulin='Fasted serum insulin in uIU/mL'
Liver_cholesterol='Liver cholesterol in umol/g liver'
Cecal_contents_wt='weight of cecal contents in g'
Acetate_conc='umol acetate/g cecal contents'
Acetate_amt='umol acetate in cecal contents'
Propionate_conc='umol propionate/g cecal contents'
Propionate_amt='umol propionate in cecal contents'
Isobutyrate_conc='umol isobutyrate/g cecal contents'
Isobutyrate_amt='umol isobutyrate in cecal contents'
Butyrate_conc='umol butyrate/g cecal contents'
Butyrate_amt='umol butyrate in cecal contents'
Isovalerate_conc='umol isovalerate/g cecal contents'
Isovalerate_amt='umol isovalerate in cecal contents'
Valerate_conc='umol valerate/g cecal contents'
Valerate_amt='umol valerate in cecal contents'
SCFA_total_conc='Concentration of total SCFAs in cecal contents umol/g'
SCFA_total_amt='Amount of total SCFAs in cecal contents umol'
Firmicutes_abundance='% Abundance of Firmicutes, Phylum'
Bacteroidetes_abundance='% Abundance of Bacteroidetes, Phylum'
FB_ratio='Ratio of Firmicutes:Bacteroidetes, Phylum'
Bifido_abundance='% Abundance of Bifidobacterium, Genus'
Lactobacillus_abundance='Abundance of Lactobacillus, Genus'
Akkermansia_abundance='Abundance of Akkermansia muciniphila, Species'
FACS_Cells="Number of cells counted by FACS"
FACS_Singlets="Number of singlets by FACS"
Lipid_events="Number of lipid events or adipocytes by FACS"
Large_cells='Number of large adipocytes by FACS'
Large_cell_percent='Percentage of large adipocytes'
Medium_cells='Number of medium adipocytes by FACS'
Medium_cell_percent='Percentage of medium adipocytes'
Small_cells='Number of small adipocytes by FACS'
Small_cell_percent='Percentage of small adipocytes'
Large_droplets='Number of cells with large lipid droplets'
Large_droplet_percent='Percentage of adipocytes with large lipid
droplets'
```

```
Medium_droplets='Number of cells with medium lipid droplets'
Medium_droplet_percent='Percentage of adipocytes with medium lipid
droplets'
Small_droplets='Number of cells with small lipid droplets'
Small_droplet_percent='Percentage of adipocytes with small lipid
droplets'
Energy_intake='Average energy intake, kJoules'
;

Output All_animals;
/*Outlier in lactobaccillus abundance set to zero. Value was for animal
#33 (obs #63). Value set to missing was 2.21107628. */
Cards;
0      1      1      .      .      196.9 244.7 285    317    333    335    365
       378    388    386    .      .      19.9   21.8   19.5   22.3   16.4   12.6
       76.67 274.7 0.65   226.29      378    20.2831      72.6720      6.01
       1.1226      3.619 10.8209      93.175      0.08001843  6413.076923
       7.402298851 0.044077261 2.6652      38.978      103.8841656
       18.301      48.7758252  2.819 7.5131988   13.581      36.1960812
       3.223 8.5899396   3.052 8.1341904   79.954      213.0934008
       51.17571506 29.95109421 1.708642586 0      0      0      9175   7253
       6913   877    12.68624331 3389   49.02357876 2712   39.23043541 1540
       22.27686967 4623   66.8740055   953    13.78562129 339.0492446
1      1      1      83.6   119.9 156.2 202.7 242    268    291    310    312
       343    344    342    258.4 14.5   17     16.7   21.1   19.5   17.9   24
       65.41 259.57      1.56   212.84      343    19.0700      75.6764
       6.63   0.4272      5.756 14.1132      80.52 0.147068995 3008.214286
       5.642256    0.043324508 2.6711      .      .      .      .      .
       .      .      .      .      .      .      .      .      .      51.28373589
       31.42170508 1.632111808 0      0      0.491018171 .      .      .
       .      .      .      .      .      .      .      .      .      .      .
       .      321.916437
2      1      1      91.8   136.6 179.4 232    267    295    318    336    357
       363    373    369    277.2 19.2   18.2   19.3   15.4   16.5   17.7   23.3
       52.38 294.3 0.74   238.34      363    14.4298      81.0744      6.82
       0.545 4.913 11.2367      83.89 0.095034911 3302.857143 7.327754
       0.049453502 3.0803      .      .      .      .      .      .      .
       .      .      .      .      .      .      .      53.26030007 27.33031674
       1.948762635 0.022227514 0      0.214336747 9683   6813   6559   457
       6.967525537 2842   43.32977588 3137   47.82741272 2120   32.3220003
       3646   55.58774203 880    13.41667937 308.6610543
3      1      1      80     124    160.2 212.4 258    285    311    323    342
       356    377    378    298    17.5   22.8   22.3   20.8   23.6   19.5   23.1
       48.9   291.1 1.84   237.43      356    13.7360      81.7697      7.16
       0.5045      3.581 10.9609      89.02 0.107594937 1952.857143
       6.37112     0.034190305 4.0991      .      .      .      .      .
       .      .      .      .      .      .      .      .      .      42.55713403
       49.93251435 0.852293032 0.013725894 0.025164139 0.812115389 .
       .      .      .      .      .      .      .      .      .      .      .
       .      .      .      364.8386286
4      1      1      81.5   125.1 166    213.1 255    288    310    323    351
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12      2       1       94.6    131.9 174.6 218.4 253     284     306     324     341
        352     361     357     262.4 12.9    15.4    14.6    15.9    17.5    16      16.9
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13      3       1       101.2 139.9 186.5 241.1 290     323     348     372     392
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18    3     1     95.8  137.2 185.8 244.3 296   340   371   398   410
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	415	435	433	350	11	12.2	14.5	19.1	16.4	21.1	26.1
	59.35	333.39	1.29	272.11	415	14.3012	80.3349
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	67.86	323.07	1	264.39	412	16.4709	78.4150
	6.89	0.8	4.8	13.7238	86.42	0.114086366	5086.153846
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	58.47	306.87	0.87	250.47	385	15.1870	79.7065
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      69.69  298.79        0.74   242.36        388    17.9613       77.0077
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40    1      2      102.6  157    196    237    273    317    340    363    388
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	425	441	439	336.8	13.5	16.8	18.3	21.3	17.8	16.1	15.4
	95.62	310.69	1.47	254.24	425	22.4988	73.1035
	6.15	0.7337	7.89	17.1046	112.81	0.211168164
	7127.857143	5.642256	0.139560642	5.7825	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
	12.73252719	41.00387862	0.310520068	0	0	1.268537531	9301
	7456	7367	770	10.45201575	2914	39.55477128	2695	36.58205511
	1352	18.35211076	3535	47.98425411	1848	25.08483779	340.55898
63	5	2	109	166	212	266	304	347	372	413	435
	449	473	469	360	15.76	18.6	18.9	16.7	16.4	18.7	19.2
	88.1	337.95	1.44	277.03	449	19.6214	75.2673
	6.14	0.8535	6.44	15.696	109.205	0.201663396
	5074.615385	1.425287356	0.1265808	6.9228	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
	17.9245283	55.25548627	0.32439364	0	0	0.408939995	.
	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	355.0155944
64	5	2	92.4	144	179	229	267	314	340	370	392
	409	434	440	347.6	14.6	15.6	16.3	23.8	15	18.4	18.5
	103.58	283.85	1.12	233.4	409	.	.	5.84	0.73
	9.2	14.612	108.365	0.164051748	10999.61538	6.712643678
	0.050487767	7.8973	27.556	217.6179988	24.962
	197.1324026	1.511	11.9328203	7.134	56.3393382	2.678	21.1489694
	1.457	11.5063661	65.298	515.6778954	13.41992603	52.11288115
	0.257516486	0	0.049509276	1.662929202	9154	6611	6342	988
```

15.5786818 3196 50.39419741 2127 33.53831599 1587 25.02365184
3781 59.6184169 1106 17.4392936 349.1300953
65 5 2 104.9 157 200 246 286 332 363 392 416
432 457 455 350.1 16.4 15.8 15.5 20.4 19.4 21.7 20.6
53.13 356.26 2.15 292.52 432 . . 6.46
1.0614 5.03 14.9701 92.025 0.244230769 1670.769231
7.057471264 0.065804239 8.2444 30.21 249.063324 12.98
107.012312 1.528 12.5974432 6.077 50.1012188 2.232 18.4015008
1.455 11.995602 54.482 449.1714008 20.77756919 50.01513959
0.415425596 0.059347181 0.184097378 0.004844668 8785 7779 7585
773 . 3809 . 2561 . 4007 . 3178 . 338
. 370.8435873
66 5 2 105.6 160 202 248 285 328 358 382 402
411 425 423 317.4 14.2 18 19 18.7 15.2 18.2 16
72.86 316.04 3.03 259.73 411 17.7275 76.8954
6.19 0.8639 6.84 14.2427 97.295 0.207195572
3524.615385 7.402298851 0.078163162 7.9373 13.989
111.0348897 7.041 55.8865293 0 0 3.472 27.5583056 1.101
8.7389673 0 0 25.603 203.2186919 15.2737446
42.13117076 0.362528368 0 0 1.094270132 . . .
. . . .
. 340.8446838
67 6 2 97.6 148 184 237 276 310 348 370 385
399 425 425 327.4 13.2 12.2 16.5 17.6 17.4 18.3 15.8
66.63 313.34 1.09 256.61 399 16.6992 78.5313
6.46 1.0177 7.32 14.1166 91.31 0.145075813 4124.285714
4.77673 0.084256828 8.3296 15.185 126.484976 7.072
58.9069312 0.754 6.2805184 2.558 21.3071168 0.982 8.1796672
0 0 26.551 221.1592096 11.7356351 41.33536431
0.283912705 0.534822713 0.011847339 18.63924854 . . .
. . . .
. 317.1312649
68 6 2 105.6 160 205 253 289 334 368 409 429
451 467 468 362.4 14.4 19 16.3 19.8 17.8 19.4 16.4
60.71 370.3 1.28 303.17 451 13.4612 82.1064 6.76
1.0746 5.79 17.0314 88.67 0.197876251 2527.857143
4.958946 0.138215136 6.8963 27.026 186.3794038
14.653 101.0514839 1.696 11.6961248 3.757 25.9093991 2.414
16.6476682 1.812 12.4960956 51.358 354.1801754 15.24436899
37.06422457 0.411296045 0.975563101 0.12672756 24.02204581 .
. . . .
. . . 351.7014299
69 6 2 113.2 172 214 272 315 356 393 418 442
453 470 469 355.8 19.3 20 20.6 19.4 19.4 27.5 19.3
126.33 305.71 1.51 250.15 453 . . 6.47
0.8831 8.88 17.8026 93.39 0.207339628 10440 2.689655172
0.105763881 5.6783 24.186 137.3353638 16.964
96.3266812 1.067 6.0587461 2.622 14.8885026 1.587 9.0114621
0 0 46.426 263.6207558 22.86387339 27.01948587
0.846199424 1.703869728 0.054889763 26.07035038 . . .
. . . .
. 415.6990905
70 6 2 102.4 162 203 264 306 356 389 420 441
389 485 484 381.6 17.6 18.1 18.7 24.5 18.5 21.7 16.5

```
      89.39 352.38      0.48  288.87      389   22.9794     90.5861
      6.55  1.0256      7.17  18.055      117.41      0.300517034
      7159.230769 3.379310345 0.043114465 9.1825      .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      22.84981589 27.78143083 0.822485207 1.665789935 0.144660705
      9.696650885 .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     387.4144101
71    6     2     103.3 153   192   245   285   333   364   396   421
      442   456   455   351.7 14.6  20.3  17.7  18.4  16.5  20    15
      106.31      316.27      1.3   260.09      442   24.0520
      71.5543     6.59  0.8563      10.8  15.8368     114.83
      0.257757624 7751.538462 9.471264368 0.082814206 4.7625
      25.395      120.9436875 14.839      70.6707375  1.684 8.02005
      4.088 19.4691     2.301 10.9585125  0     0     48.307
      230.0620875 20.52153762 29.41543476 0.697645226 0.353973286
      0.052204693 21.77572354 .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     349.9872068
72    6     2     102.3 152   184   223   256   290   310   333   348
      360   373   369   266.7 0     13.9  15.9  17.9  15.6  15.9  14.2
      72.65 268.52      0.97  220.23      360   20.1806     74.5889
      6.99  0.7082      5.56  12.7342     93.965      0.130317475
      4388.076923 4.528735632 0.07566592  3.7944      .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      33.66470987 36.35800466 0.925922921 0     0.06194873  5.920528629
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     266.8473887

proc sort data = All_animals; by Diet;

proc format;
      value dietfmt
1='Normal fat cellulose'
2='High fat cellulose'
3='High fat polylactose'
4='High fat polylactose 3%'
5='High fat polydextrose'
6='High fat galactooligosaccharides';


proc print data= All_animals; by Diet; format Diet dietfmt.;

proc means data=All_animals n mean stderr min max; by Diet; format Diet
dietfmt.;

proc glm data= All_animals;
title 'One Way Analysis of Variance in all rats';
format Diet dietfmt.;
class Diet;
model BW_0--Energy_intake =Diet;
Means Diet / Duncan;
run;
```

```
/*Bifidobacteria and Lactobaccilus abundances had many values of zero,
particularly in NFC and HFC groups. Thus ANOVA is not appropriate to
use.
Proc FMM seems to be an appropriate approach, based on my readings.*/
proc FMM data=All_animals;
title 'zero-inflated gamma model of Bifido abundance';
class diet;
format Diet dietfmt.;
model Bifido_abundance=diet/dist=gamma;
model + / dist=constant;
probmodel diet;
run;
proc FMM data=All_animals;
title 'zero-inflated gamma model of Lactobaccillus abundance';
class diet;
format Diet dietfmt.;
model Lactobacillus_abundance=diet/dist=gamma;
model + / dist=constant;
probmodel diet;
run;
proc corr data= All_animals;
title 'Correlation analysis in all rats';
var Cecum_weight Cecal_pH SCFA_total_conc SCFA_total_amt;
with Acetate_conc Acetate_amt Propionate_conc Propionate_amt
Isobutyrate_conc
Isobutyrate_amt Butyrate_conc Butyrate_amt Isovalerate_conc
Isovalerate_amt Valerate_conc Valerate_amt SCFA_total_amt FB_ratio;
run;
quit;
```

Appendix 6. Microbiome sample processing method

1. Harvest cecal contents fresh and flash freeze on dry ice, store at -80C until ready for use.
2. Contact UMGC to set up project, receive quote.
3. Follow Qiagen DNA stool kit instructions for sample extractions.
4. Roughly quantify concentration of DNA in eluates on NanoSpec in 211.
5. Fill out UMGC sample submission form, containing sample information, concentrations, etc.
6. Plate samples in PCR skirted 96-well plate, leaving empty wells as a "barcode." Sample wells are denoted on the form.
7. Submit to UMGC (BioSci building, St. Paul campus). Plate should be adequately labeled and hard copy of submission form attached (email form to UMGC staff prior to dropping off samples).

Appendix 7. Sample processing method for RNA-seq

1. Harvest tissue fresh, place in RNA later, let sit for 4 hours (or instructed on RNA-later spec sheet), store at -80C until ready for use.
2. Contact UMGC to set up project, receive quote.
3. Follow Qiagen RNeasy midi kit instructions for sample extractions (I used the regular mRNA method in tandem with the small RNA method just in case we decided to do miRNA sequencing as well).
4. Roughly quantify concentration of RNA in eluates on NanoSpec in 211.
5. Fill out UMGC sample submission form, containing sample information, concentrations, etc.
6. Plate samples in PCR skirted 96-well plate, leaving empty wells as a "barcode." Sample wells are denoted on the form.
7. Submit to UMGC (CCRB, East Bank). Plate should be adequately labeled and hard copy of submission form attached (email form to UMGC staff prior to dropping off samples).

Appendix 8. Adipocyte isolation and flow cytometry method

The following adipocyte isolation and staining methods were adapted from Majka et al.

1. Epididymal fat pads were excised from the rat immediately, rinsed with deionized water, weighed, flash frozen on dry ice, and stored at -80*C until further analysis.
2. Frozen adipose tissue was minced with a razor on a glass plate, until fragments were approximately 1 mm.
3. Minced tissue was then weighed (approximately 0.75 g) in a 50 mL screw cap conical polypropylene centrifuge tube.
4. Digestion buffer (Krebs-Ringers HEPES with 2.5 mM glucose, 2% fetal bovine serum (FBS, Gibco, 35-010-CV), 200 uM adenosine, and 1 mg/mL collagenase) was added at 1 mL/0.25 g tissue.
    a. Krebs-Ringers HEPES contains 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 10 mM HEPES, 1.2 mM KH2PO4, and 1.2 mM MgSO4
5. Adipose-digestion buffer mixture was incubated on an orbital shaker at 100 rpm for 1 hour at 37*C.
6. After collagenase digestion, the slurry was passed through a 150-um mesh Celltrics filter (Celltrics, 04-004-2329) into a fresh 15 mL screw cap conical polypropylene centrifuge tube. The 50 mL digestion tube was then rinsed with wash buffer at an equal volume, which was then applied to the mesh filter.
    a. Wash buffer contains Hanks balanced salt solution (HBSS, LifeTechnologies, 1417509), 2% FBS, 200 uM adenosine, at pH 7.4.

7. The filtrate was centrifuged at 150 x g for 8 minutes at ambient temperature. As adipocytes contain a substantial amount of lipid, they are LESS dense than water, therefore, will float to the top.
8. Using a transfer pipette, adipocytes (top layer) were transferred to a clean 15 mL conical tube, and the same volume of wash buffer was added
    a. This should represent approximately 3x the volume of the transferred adipocytes
9. The adipocytes and wash buffer were then centrifuged again at 150 x g for 8 minutes, and the adipocytes were transferred to a 12 x 75 mm polypropylene tube

In order to assess lipid containing events and lipid droplet size, the isolated adipocytes were stained with LipidTox deep red. LipidTox was used instead of other lipid-staining compounds as it is functional on live, dead, or fixed cells, and in the absence of solvents. Furthermore, LipidTox is available in green (505 nm), red (609 nm), and far-red (655) emissions, allowing for versatility and avoidance of overlap with other fluorochromes commonly used in flow cytometry applications. The staining procedure immediately followed the isolation procedure.

1. LipidTox Deep Red (LifeTechnologies, H34477) was added to the suspension at a concentration of approximately 1 uL LipidTox/1 mL suspended adipocytes. Samples were gently inverted to ensure complete mixing.
2. Cells were incubated at 37*C for 25 minutes with mixing by flicking the tubes approximately halfway through.

Cells were also stained with DyeCycle Violet in order to identify intact cells containing nuclei, as well as appropriately select for singlets. DyeCycle Violet stains DNA in the cell, therefore, events containing both a nucleus and lipid droplet were considered to be nuclei. DyeCycleViolet staining directly followed LipidTox staining.

1. DyeCycle Violet (LifeTechnologies, V35003) was added to the LipidTox stained cell suspension at a concentration of approximately 2 uL DyeCycle Violet/1 mL suspension. Samples were gently inverted to ensure complete mixing.
2. Cells were incubated at 37*C for 30 minutes, with gentle inversion approximately halfway through.
3. Following incubation, cells were transferred to FACS tubes and taken to flow cytometry at room temperature.

FACS method/gating scheme

1. On the BD LSRII H4760 (BD Biosciences), set voltage parameters on the lasers (FSC, SSC, 633 C (measures LipidTox), and DyeCycle Violet) to FSC 494 (area, height, width), SSC 250 (area, height, width), 633 C 333 (Red laser C 633nm; log, area, height), DyeCycle Violet 300 (Violet laser F, 407nm; log, area, height)
2. Forward Scatter Area (FSC-A, x-axis) x Side Scatter Area (SSC-A, y-axis) to determine cells. Adipocytes are large (FSC = high) and round (SSC = medium)
3. Gate adipocytes into cell events by DyeCycleViolet (nuclear stain)-A by DCV-H.
4. Use SSC-W by A to determine singlets

5. Check spread on FSC-W by FSC-H
6. Gate lipid events using LipidTox A (or 633C-A, which denotes a common dye used with the same laser)
7. Gate in three vertical quadrants (3) for LipidTox (droplet size, y axis is 633C-A) and horizontal quadrants (3) for cell size (FSC-A, x axis)
8. Analyze using the percent of lipid events

Appendix 9. AMPK and p-AMPK Western blotting method

Reagents

Extraction buffer RIPA lysis buffer with phosphatase inhibitors

- 2 mL 5X RIPA + 8 mL ddH2O + 100 uL protease & phosphatase inhibitor
- From protein isolation method, can use leftovers

BME (2-mercaptoethanol)/Laemmli buffer

- 4X Laemmli Buffer from Bio-Rad (#161-0747)
- Add 2-mercaptoethanol (BME) according to spreadsheet at 10% of Laemmli Buffer for 2.5% BME/tube

1X Tris/glycine/SDS buffer

- Purchased in concentrate from Bio-Rad (#1610772)

Transfer Buffer

- From Bio-Rad "Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose" (#170-4270), 5X
- 600 mL ddH2O + 200 mL 5X transfer buffer + 200 mL 200 proof ethanol

PBS, pH 7.4

- 10X premade from Gibco, dilute to 1X
- Will need PBS-T, too (add 1% Tween)

Other Materials

- boil-safe microcentrifuge tubes
- gel-loading tips, 200 µL

- Bio-Rad Mini-Protean Precast TGX Gels, 4-15% (#4561086)
- Bio-Rad "Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose" 170-4270
    - 1 L 5X transfer buffer
    - 80 transfer stacks
    - 40 nitrocellulose membranes
- LI-COR Chameleon Duo Pre-Stained Protein Ladder 928-60000
- Blocking solution (5% non-fat dry milk in PBS)
- Primary antibodies (dilute in PBS-T + 5% non-fat dry milk)
    - AMPK Mouse: 1:1000 dilution (CellSignaling, 27933)
    - pAMPK Rabbit: 1:500 dilution (CellSignaling, 2535S)
    - B-actin Rabbit: 1:5,000 dilution (for 3h RT) (CellSignaling, 4967S)
- Secondary antibodies (near-IR from LI-COR, dilute in PBS-T)
    - Goat-anti-Mouse, LI-COR 925-68070
    - Goat-anti-Rabbit, LI-COR 926-32211

Method

1. Thaw whole-protein isolates on ice.
2. Make BME/Laemmli Buffer, add to each tube
3. Add lysis buffer and protein sample to the tubes as designated in the spreadsheet. Centrifuge (quick spin)
4. While samples are boiling, prepare the cassette and box for electrophoresis.
    a. Rinse all components with DI water.
    b. Remove tape from the bottom of the cassette

    c. Assemble the chamber with clamps, dam, and cassette (no tape). 1 goes on red side. Words face inside. Red matches to red.
    d. Fill the chamber with 1X Tris/glycine/SDS buffer.
    e. Make sure the red and black electrodes/connections line up.
    f. Remove comb from the gel cassette and fix the lanes if needed.
5. Load the samples and molecular weight markers with loading tips onto the gel in the appropriate lanes. Avoid bubbles
6. Run gels on a fixed voltage of 200V for 30 minutes (30-35).
7. Remove the chamber and place the gel cassette in the buffer in the box. Rinse the other components.
8. Prepare the nitrocellulose membrane with transfer stacks.
    a. Place the nitrocellulose membrane in a plastic tray and the transfer stacks in another, using forceps
    b. Add 30 mL of transfer buffer to the nitrocellulose membrane and 50 mL to the transfer stacks.
    c. Place on rocker for 5 minutes.
9. Prepare the transfer assembly box.
    a. Rinse the transfer assembly box with DI water and dry the electrodes with a paper towel.
    b. Pipette transfer buffer from trays onto the bottom of the transfer box (2-3 mL).
    c. Place first transfer stack in the center.

d. Place membrane on top of transfer stack.

e. Pipette 1 mL of transfer buffer on top of the membrane; avoid bubbles.

f. Open the gel cassette and gently place on top of the membrane.

    i. Do not move once in place.

g. Pipette 1-2 mL of transfer buffer to ‘rinse.’

h. Place 2nd transfer stack on top and roll to remove any bubbles.

i. Place the top of the transfer box on top and lock into place.

10. Turn on the BIO-RAD Trans Turbo Blot system and select “Turbo” on the home screen and select 1 TGX gel.
    a. Settings should read: 1 MINI TGX or MIXED MW, 2.5 A, 25 V, 3 minutes
11. Place in slot A and hit RUN for slot A.
12. Rinse the membrane with DI water several times.
13. Add Ponceau Stain and quickly pour back into container.
14. Wrap in plastic wrap and scan for confirmation of a successful transfer.
15. Cut the membrane to fit in black box
16. Rinse membrane with PBS
    a. 5-7 minute rinses, 3 times
    b. During these PBS rinses, prepare the 5% milk blocking buffer
        i. 1 g of dry milk in 20 mL PBS
17. Cover with blocking buffer. Incubate for 1 h, room temp, on rocker.
18. Dilute primary antibody in 0.375 g of dry nonfat milk in 7.5 mL of PBS-T

a. 7.5 µL of 1° AMPK and 15 µL of 1° p-AMPK in 7.5 mL of primary dilution buffer for 1:1000 AMPK 1° & 1:500 p-AMPK 1°

19. Incubate membranes with 10 mL of the 1° antibody solution on a rocker overnight at 4° C.
20. Discard the primary solution, wash the membrane with PBS-T at room temp on rocker
    a. 2 brief rinses
    b. 4x 5 min
21. Dilute the 2° antibodies in PBS-T (no milk). All 2° antibodies are light sensitive-cover during incubation.
    a. 1:10,000 dilution (1 µL of 2° in 10 mL of PBS-T).
22. Discard final wash and add the required amount of 2°. Incubate the membrane for 1 h at room temperature, in dark, on rocker.
23. Wash the membrane with PBS-T at room temp on rocker
    a. 2 brief rinses
    b. 2x 5 min PBS-T
    c. 2x 5 minutes in PBS (to remove Tween)
24. Use LI-COR to scan membrane, scan 700 and 800 channels for 10 minutes each

25. For loading control, rewash membrane
26. Incubate with loading control 1° antibody (b-actin, 1:1000 in 5% NFDM + PBS-T) for overnight at 4°C on rocker.

27. Wash, 4x, 5 min each, PBS-T
28. Incubate with 2° for 1h at RT on rocker (in dark)
29. Wash the membrane with PBS-T at room temp on rocker
    a. 2 brief rinses
    b. 2x 5 min PBS-T
    c. 2x 5 minutes in PBS (to remove Tween)
30. Use LI-COR to scan membrane, scan 800 channel for 10 minutes

Appendix 10. Index of reagents, file paths, stored samples

Reagents used + inventory

Item Description	Company	Number	Use	Location/stock + notes
Filters	Celltrics	04-004-2329	Adipocyte sizing	Filter cabinet
LipidTox deep red	LifeTechnologies	H34477	Adipocyte sizing	Chemical freezer
DyeCycle Violet	LifeTechnologies	V35003	Adipocyte sizing	Chemical freezer
HBSS	LifeTechnologies	1417509	Adipocyte sizing	Chemical freezer
FBS		35-010-CV	Adipocyte sizing	Chemical freezer
Rats	Envigo lab		Animals	75-99g, Wistar, Male
Diet ingredients	Dyets	Various	Animals	Cold room
Polydextrose	Danisco	8104705	Animals	Cold room
Fructooligosaccharides	Ingredion	11101300	Animals	Contact: Maria Stewart, gifted
Galactooligosaccharides	Vivinal GOS		Animals	Dry storage - pilot plant
Dimethylhydrazine	Sigma-aldrich (?)		Animals	Carcinogen dessicator
Glucometer + strips	AlphaTrak		Animals	Animal cabinet
Glucose			Animals	Gallaher shelf
Pyruvate (sodium)	Sigma-aldrich		Animals	Chemical fridge
Insulin			Animals	Used
Formaldehyde			Animals	South Solvents
EDTA disodium			Animals	Gallaher shelf

Total dietary fiber kit	Megazyme	K-INTDF	Animals	Chemical fridge/Schoenfuss lab
Ambersep 200 H+ resin	Megazyme	G-AMBH	Animals	Cold room/Schoenfuss lab
Amberlite FPA OH- resin	Megazyme	G-AMBOH	Animals	Cold room/Schoenfuss lab
Activated carbon	Norit	GAC 1240 PLUS	Animals	Schoenfuss lab
LDL inhibtor, poloxamer 407	Spectrum	P1166-500GM	Animals	Gallaher shelf
Methylene blue			Colon work	Gallaher shelf
Ethanol			General	Solvents cabinet
Citric acid			General	Gallaher shelf
Benzoic acid			General	Gallaher shelf
Glucose oxidase			Glucose assay	Chemical freezer
Glucose peroxidase			Glucose assay	Chemical freezer
Reduced sodium p-diphenylamine sulfonate			Glucose assay	Gallaher shelf
Harris hematoxylin	Fisher Scientific	ES4470	Immunohistochemistry	Gallaher shelf
Pierce chromogenic endotoxin quant kit	ThermoScientific	A39553	Kit assays	LPS kit - DO NOT USE (doesn't work)
Insulin ELISA kit	Millipore	EZRMI-13K	Kit assays	Used
LPS kit	ThermoScientific	A39553	Kit assays	Cold room - see lab notebook PL1-2

Glycated hemoglobin kit	Diazyme	DZ168A	Kit assays	Chemical fridge
Leptin ELISA	Millipore	EZRL-83K	Kit assays	Used
Adiponectin ELISA 1	Sigma-aldrich	RAB1136	Kit assays	DO NOT USE
Adiponectin ELISA 2	abcam	ab108784	Kit assays	Used
Leptin ELISA - PL2	Invitrogen	KRC2281	Kit assays	Used
Insulin ELISA - PL2	Invitrogen	ERINS	Kit assays	Used
Triglyceride reagent kit	Pointe Scientific	T7532-120	Lipids	Chemical fridge
Chlorofrom			Lipids	Solvents cabinet
Methanol			Lipids	Solvents cabinet
Sodium phosphate dibasic			Lipids	Gallaher shelf
Phenol crystals			Lipids	Chemical freezer?
4-aminoantipyrine	ThermoScientific	10184309	Lipids	Gallaher shelf
Sodium cholate			Lipids	Gallaher shelf
Potassium cholate			Lipids	Gallaher shelf
Triton-X			Lipids	Gallaher shelf
Cholesterol oxidase	Sigma aldrich	C8649-100UN	Lipids	Chemical freezer
Cholesterol esterase	Alfa Aesar	J64282	Lipids	Chemical freezer
Triglycerides standards	Pointe Scientific	T7531-STD	Lipids	Used
Horseradish peroxidase	SigmaAldrich	P6782	Lipids	Chemical freezer

QIAmp DNA stool mini kit	Qiagen	51504	Microbiome	Gallaher shelf
RIPA lysis buffer	Alfa Aesar	J62524	Protein Isolations	Chemical fridge
Protease & Phophatase Inhibitor	abcam	201120	Protein Isolations	Chemical freezer
BCA protein assay kit	ThermoScientific	23225	Protein Isolations	Breann's old bench
RNA later	Invitrogen	AM7020	RNA	Gallaher shelf
RNAse away	Molecular Bioproducts	7003	RNA	Gallaher shelf
Rneasy Plus Mini Kit	Qiagen	73404	RNA	Gallaher shelf
Adipose RNA extraction kits	Qiagen	1023539	RNA	Gallaher shelf
Allprotect tissue reagent	Qiagen	76405	RNA	Gallaher shelf
Filter tips	Millex	SLGNX13NL	Short Chain Fatty Acids	Filter cabinet
SCFA standards	Restek	35272	Short Chain Fatty Acids	
Diethyl Ether	SigmaAldrich	309966	Short Chain Fatty Acids	South Solvents
2-Ethylbutyric acid	SigmaAldrich	109959	Short Chain Fatty Acids	Chemical fridge
Laemmli buffer	Bio-Rad	161-0747	Western blots	Breann's old bench
BME (2-mercaotiethanol)	Sigma-aldrich	Serial #	Western blots	South Solvents
Tris/glycine/SDS running buffer	Bio-Rad	1610772	Western blots	Ryu lab
Trans-blot turbo RTA	Bio-Rad	170-4270	Western blots	Breann's old bench

transfer kit, nitrocellulose				
PBS, 7.4	Gibco		Western blots	Ryu lab
LI-COR Chameleon Duo Pre-stained protein ladder	LI-COR	928-60000	Western blots	Ryu lab
Mini-Protean Precast TGX gels, 4-15%	Bio-Rad	4561086	Western blots	Chemical fridge
AMPK mouse antibody	CellSignaling	27933	Western blots	Chemical freezer
pAMPK rabbit antibody	CellSignaling	2535S	Western blots	Chemical freezer
B-actin rabbit antibody	CellSignaling	4967S	Western blots	Chemical freezer
Goat anti-mouse IR 700 antibody	LI-COR	925-68070	Western blots	Chemical fridge
Goat anti-rabbit IR 800 antibody	LI-COR	926-32211	Western blots	Chemical fridge
Non-fat dry milk			Western blots	Ryu lab

Samples stored

Study	Category	Sample type	Number	Location
Polylactose 1	Tissue	Liver	72+	Biological freezer (old)
Polylactose 1	Tissue	Adipose	72+	Biological freezer (old)
Polylactose 1	Section (slides - unstained)	Colon		Microscope shelf
Polylactose 1	Section (slides - stained)	Colon		Microscope shelf (grey box)
Polylactose 1	RNA eluates	Liver	32	Biological freezer (old)
Polylactose 1	RNA eluates	Colon	32	Biological freezer (old)
Polylactose 1	DNA eluates	Cecal contents	72+	Biological freezer (old)
Polylactose 1	Colons	Destained, whole	72+	Fridge
Polylactose 1	Cecal contents		72+	Biological freezer (old)
Polylactose 1	RNAlater preserved tissue	Liver	72+	Biological freezer (old)
Polylactose 1	RNAlater preserved tissue	Colon	72+	Biological freezer (old)
Polylactose 1	Tissue	Feces	72	Chemical freezer
Polylactose 2	Tissue	Liver	72+	Biological freezer (old)
Polylactose 2	Tissue	Adipose	72+	Biological freezer (old)
Polylactose 2	Section (slides - unstained)	Liver		Microscope shelf
Polylactose 2	Section (slides - unstained)	Adipose		Microscope shelf
Polylactose 2	Section (slides - stained)	Liver	72	Microscope shelf (white box)
Polylactose 2	Section (slides - stained)	Adipose	72	Microscope shelf (white box)

Polylactose 2	Protein isolates	Liver	72+	Biological freezer (old)
Polylactose 2	Cell isolates	Adipocytes		Biological freezer (old)
Polylactose 2	DNA eluates	Cecal contents	72+	Biological freezer (old)
Polylactose 2	RNAlater preserved tissue	Liver	72+	Biological freezer (old)
Polylactose 2	RNAlater preserved tissue	Adipose	72+	Biological freezer (old)
Polylactose 2	Tissue	Feces	72	Chemical freezer

"+" denotes that there are also stored practice samples. All practice samples are in their own bag in the freezer
All samples in the biological freezer are in the righthand side

File paths

Study	Category	Filepath	Notes	Number of raw data files
Polylactose 1	Microbiome	/panfs/roc/data_release/2/umgc/gallaher/miseq/180402_M04141_0203_000000000-BNVG7/Gallaher2_Project_001		144
Polylactose 1	RNA-seq	/panfs/roc/data_release/2/umgc/gallaher/novaseq/180622_A00223_0018_BH2FFLDRXX/Gallaher_Project_003		128
Polylactose 2	Microbiome	/panfs/roc/data_release/2/umgc/gallaher/miseq/190823_M00784_0303_000000000-CLMF9/Gallaher_Project_004		144

File #s = forward and reverse (samples = 1/2 of number below)