Fiber Evaluation for Prebiotic Effects and Fermentation Kinetics – Sustainable Sources and Commercial Products

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Dedication

This thesis is dedicated to my grandfather, Daniel H. Carlson, who passed away on October 1, 2015 at the age of 74. Since the day I was born, my father and grandfather were the two most influential people in my life, teaching me the importance of working hard, studying hard and being there for your family. My whole life I had two role models, and they were always pushing me to be a better person every day. My grandfather taught me countless valuable life lessons that I will never forget.

My grandfather worked in masonry for most of his adult life, and was also a farmer in Chisago Lakes, Minnesota. His hobbies included playing cribbage, fishing for walleye, spending time with his horses and giving sleigh rides to everyone in the family, cutting trees and sawing them on his sawmill and building numerous log cabins, spending time with his family at our family's log cabin making homemade maple syrup, and above everything else, spending time with his family on the farm.

My grandfather never sat still, unless we were fishing for walleye, watching Tiger Woods golf on Sundays or cheering on the Minnesota Vikings. He always was working and getting things done, and taught me the importance of really working hard, no matter what you were working on. He taught me how to drive (way before turning 16), how to farm and everything that involves, how to build or fix just about anything and how to always be there for family and be willing to help with anything.

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Abstract

Dietary fibers are a broad subset of non-digestible carbohydrates that have many health benefits to consumers. When consumed in adequate amounts they can protect against cardiovascular disease, decrease the risk of diabetes, aid in weight loss and numerous other health benefits. One unique attribute that dietary fibers have is that when they reach the distal intestine or colon, some of these fibers can be fermented by the hundreds of species of bacteria present. When these fibers provide growth to the beneficial bacteria present in the gastrointestinal tract, they are referred to as prebiotics, or prebiotic dietary fibers. As the understanding of the gut micobiota and the gut microbiome expand, the importance of stimulating the beneficial microbiota present in the gut becomes increasingly more important. Because the human diet is so complex, understanding the fermentation process of individual compounds can be a difficult task.

The use of *in vitro* fermentation models is one way to understand the fermentation of dietary fibers, and whether or not they support the growth of beneficial taxa, and very closely mimics the function of the colon in that regard. Incubating fecal bacteria from human donors, exposing them to specific dietary fibers, and measuring their fermentation differences provides a snapshot of how these compounds may ferment *in vivo*. The primary objective of this research was to conduct a preliminary *in vitro* analysis of two emerging dietary fibers (wheat dextrin and partially hydrolyzed guar gum) to test their prebiotic capacity (Chapter 2), and to compare new dietary fibers (Oatwell, xylooligosaccharides, beta-glucans) to an established prebiotic dietary fiber (inulin) to

measure key fermentation differences *in vitro* (Chapter 5). The secondary objective of this research was to compare fermentation differences between donors exposed to these same compounds by measuring differences in the production of short chain fatty acids (Chapter 3), and the microbiota that were stimulated (Chapter 4). The final objective of this research was to determine if conventionally or organically grown plants in Minnesota provided a significant amount of dietary fiber, if it was fermentable, and whether or not the growing differences in these plants affected key nutrients and compounds, including: vitamin C, potassium, iron, sodium, calcium and nitrate-nitrogen.

Both wheat dextrin and partially hydrolyzed guar gum stimulated growth of two beneficial genera of bacteria, *Lactobacillus* and *Bifidobacterium*, indicating that both fibers are bifidogenic and lactogenic within 24 h of fermentation (Chapter 2). At 12 h wheat dextrin was significantly more bifidogenic (9.50 CFU $\log_{10}$/mL) than partially hydrolyzed guar gum (PHGG) (9.30 CFU $\log_{10}$/mL) ($p=0.052$), and also at 24 h wheat dextrin (9.41 CFU $\log_{10}$/mL) compared with PHGG (9.27 CFU $\log_{10}$/mL) ($p=0.043$). Wheat dextrin produced less total short chain fatty acids at both 12 h and 24 h than partially hydrolyzed guar gum, and produced significantly lower amounts of gas at 12 h and 24 h ($p<0.001$, $p<0.001$), a key marker for gastrointestinal tolerance.

Changes in short chain fatty acid concentration (SCFA) due to the fermentation of dietary fibers in the colon has been widely studied, but there are limited studies analyzing the differences in this production across multiple individuals (ages 23-68) exposed to the

same dietary fiber (Chapter 3). The objective of this project was to look at fermentation differences from six fecal donors all exposed to partially hydrolyzed guar gum in an *in vitro* model. With the six donors analyzed in this study, gas production varied from 59-80 mL/0.5g fiber at 12 h and 85-93 mL/0.5g fiber at 24 h between the six donors. At 12 h butyrate concentrations varied from 6.99 µmol/mL to 23.84 µmol/mL and from 8.78 µmol/mL to 22.84µmol/mL at 24 h. Total SCFA concentration at 24 h ranged from 42.85 µmol/mL to 91.17 µmol/mL. With over a 2-fold difference in SCFA production, significant differences were found between healthy individuals exposed to partially hydrolyzed guar gum *in vitro*.

Following this analysis, 16s rRNA sequencing was used to identify the fecal microbiota responsible for the fermentation of partially hydrolyzed guar gum (Chapter 4) comparing samples at baseline, 12 h and 24 h post-exposure. *Parabacteroides* increased from 3.48% of sequence reads to 10.62% of sequence reads after 24 h ($p = 0.0181$) and Bacteroidetes increased from 45.89% of sequence reads to 50.29% of sequence reads ($p = 0.0008$). Partially hydrolyzed guar gum stimulates growth of *Parabacteroides*, a genus of bacteria that have been inversely associated with irritable bowel syndrome (IBS) and ulcerative colitis. PHGG provides stimulation of beneficial Bacteroidetes (*Bacteroides* and *Parabacteroides*), which may be correlated with many positive health markers and outcomes *in vivo*.

Five other dietary fibers were analyzed to test their prebiotic capacity and fermentation differences, including: Oatwell, inulin, beta-glucan, xylooligosaccharide and a dried chicory root blend. Oatwell had the highest production of propionate at 12 h (4.76 μmol/mL) compared to inulin, dried chicory root blend and xylooligosaccharide samples (p<0.03). Its effect was similar to those of the beta-glucan samples. Oatwell and beta-glucan samples promoted the highest mean propionate production at 24 h. Xylooligosaccharide resulted in a significant increase in the genus *Bifidobacterium* after 24 h of fermentation (0 h: 0.67 OTUs; 24 h: 5.22 OTUs; p = 0.038). Inulin and the dried chicory root blend increased the beneficial genus *Collinsella*, consistent with findings in clinical studies. All prebiotic dietary fibers studied promoted the formation of beneficial markers due to the fermentation of each specific compound. All compounds provided different, significant fermentation patterns, and all provided beneficial effects that would promote host health *in vivo*.

Produce can provide a substantial amount of dietary fiber in the consumer diet, along with many other nutrients. Arugula (*Eruca sativa*), mizuna (*Brassica rapa* var. *nipponsinca*), red giant mustard (*Brassica juncea*) and spinach (*Spinacia oleracea* 'Tyee') are fresh produce crops high in nutritive value and provide shortfall and high interest nutrients addressed in the 2015 U.S. Dietary Guidelines. The primary objective of this project was to evaluate fertility treatments unique to these crops that optimize their nutritional capacity. Plants were grown using five different fertility treatments, including four organic treatments and one conventional control. The plant treatment combinations

were replicated three times and the entire experiment was duplicated. Fertility treatments had a high impact on vitamin C (with over a 3-fold difference in treatments in the first experiment), nitrate (over 10-fold difference among fertility treatments in some species) and potassium concentrations (over 5-fold difference among fertility treatments in some species) in analyzed plant tissue. No consistent differences were found for fiber, calcium, iron and sodium concentrations in tissue analyzed. This is the first study to analyze the impact that different production treatments can have on multiple deficient nutrients and compounds addressed by the U.S. Dietary Guidelines for high-impact, highly-consumed produce crops.

Based on *in vitro* assays, partially hydrolyzed guar gum, wheat dextrin, and xylooligosaccharides stimulate the growth of *Lactobacilli* and *Bifidobacteria,* the two most beneficial genera of bacteria in the gastrointestinal tract. All dietary fibers analyzed resulted in significant amounts of short chain fatty acids being produced from their fermentation, which was found in all three *in vitro* studies. Although they are all fermentable by fecal bacteria, within healthy donors there can be over a 2-fold total difference in acetate, propionate and butyrate production within 24 h of fermentation for these dietary fibers. For consumers who prefer to consume dietary fiber in whole foods instead of supplements or fortified products, red giant mustard and mizuna both offer between 2-4g fiber/serving, which is a "good source of dietary fiber" for the consumer. This includes both organic and conventionally grown red giant mustard and mizuna.

Like any other dietary component, moderation and variety is still the most important factor to consider. Different dietary fibers all support different functions and roles in the body, and may all be fermented differently depending on the consumer. Whole foods, fortified foods and supplements may all play a critical role in developing a healthy gut microbiome, and may all be needed for consumers to meet their recommended daily intake.

Table of Contents

List of Tables

List of Figures

CHAPTER ONE

Literature Review – Health Benefits of Fiber, Prebiotics and Probiotics: A Review of Intestinal Health and Related Health Claims

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Executive Summary

Gastrointestinal health in regard to the gut microbiome is a rapidly emerging field and has many key components driving its emergence. Fiber, prebiotics and probiotics are all dietary components that can play a critical role in maintaining a healthy gut microflora. Fiber has long been appreciated for its influential role in cardiovascular disease, glycemic control and weight management through various physiological mechanisms. Prebiotics have been shown to play an influential role in irritable bowel symptoms/disease, colon cancer, cardiovascular disease and overall digestive health. Together, various types of fibers and prebiotics have been targeted and synthesized to influence the gut microbiome, specifically *Lactobacillus* and *Bifidobacterium* populations. *Lactobacillus* spp. and *Bifidobacterium* spp. are common markers for gut health because they have been shown to down-regulate inflammation in the gastrointestinal tract, alleviate IBS symptoms, stimulate immune functions, aid in mineral absorption and produce little, if any, gas or known carcinogenic substances. Probiotics have also been shown to display many of

these pro-health components, and include many species of bacteria outside of the commonly utilized *Lactobacillus* and *Bifidobacterium* populations. How these dietary components are defined in the scientific and political arenas will play a critical role in the success of the gut health field moving forward. Key changes in administrative definitions and requirements of these dietary components will influence consumption, awareness and understanding of these key influential components. The purpose of this review is to provide current accepted definitions for fiber, prebiotics and probiotics, as well as introduce key scientific studies describing the health benefits of these components, as well as current health claims.

Introduction

The gut health field is one of the fastest emerging scientific fields in the public health community. Rapid innovation in the biotechnology field has allowed for novel insights into the gut microbiome and an understanding of the challenges that are associated with analyzing this complex ecosystem.[1] The gut microbiome plays a critical role in maintaining a healthy lifestyle, driving the necessity to thoroughly understand the dietary components that play an influential role in the development of the host's microflora throughout all stages of life,[2] and all critical pathways influenced in the body.[3]

As science emerges quickly on the influence of dietary components on microflora development, there is an increasing demand to accurately define the regulated definitions for fiber, prebiotics and probiotics.[4–6] Clear definitions in the scientific community, as

well as for regulatory bodies, will play an influential role in a clear public understanding of these easily misunderstood definitions.[7,8] Accurate and expandable definitions will allow for clear categorization and development of key dietary components that influence not only the gut microflora, but how the public views and understands these critical components and concepts.

The health benefits of consuming fiber, prebiotics and probiotics are extensive, and in some cases overlapping.[6,9] Clear scientific studies have led to the development of authorized health claims for many of these dietary constituents under the jurisdiction of the largest regulatory bodies. The purpose of this review is to introduce and evaluate the definitions currently being used/proposed, the scientific studies and literature behind the health benefits of these dietary constituents, health claims that are allowed by regulatory bodies, and involve adoption of a definition similar to the one proposed by the Institute of Medicine (IOM, 2002) (i.e., total fiber [IOM, 2005]).

Fiber Definition

Fiber definitions in the US have been a highly debated topic over the past 30 years. Current changes for the 2015 U.S. Dietary Guidelines, according to the FDA's new proposal, is looking to adapt a similar definition as the 2002 Institute of Medicine (IOM) definition of *total fiber* (IOM, 2005). The Food and Nutrition Board's Dietary Reference Intake (DRI) Standing Committee put forth The Panel on the Definition of Dietary Fiber

(Dietary Fiber Panel) in the FDA's proposed revision to the Nutrition and Supplemental Facts Label in March 2013.[10] According to The Panel, the definition would include:

1. Non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units) and lignin that are intrinsic and intact in plants.
2. Isolated and synthetic non-digestible carbohydrates (with 3 or more monomeric units) that the FDA has granted to be included in the definition of dietary fiber, in response to a petition submitted to the FDA demonstrating that such carbohydrates have a physiological effect(s) that is beneficial to human health.
3. Isolated and synthetic non-digestible carbohydrates (with 3 or more monomeric units) that are the subject of an authorized health claim.

According to the FDA's current regulations and guidance, there are only two isolated non-digestible carbohydrates meeting this definition: beta-glucan and barley beta-fiber. Although no guidance has been given for analytical methods or documentation required to display 'physiological effect(s) beneficial to human health', there is speculation that the FDA will provide an inclusive list categorizing these components. Labeled fiber would then be quantified by the amount determined by the appropriate AOAC method, minus the amount of fiber not determined to have a 'physiological effect(s) beneficial to human health'. Currently, the FDA is proposing that manufacturers keep written records of all fiber that is contained in food products. The Daily Reference Value (DRV) that is used on the Nutrition Facts Panel is also proposed to increase from the current 25 g to 28

g. The proposed increase will be affected both by fiber content and definition, potentially affecting health claims already in place for various fibers currently in market. This proposed change and definition was also introduced in the Scientific Advisory Report of the 2015 Dietary Guidelines Committee.[11]

The U.S. has shifted from the past definition of dietary fiber that was classified with basic ethanol dissolution assays, to the current international trend of basing this definition and classification to include many new synthetic fibers, largely taking into consideration their beneficial impact to the health of the consumer. No new approved analytical methods have been unanimously agreed upon, but many definitions for fiber outside the U.S. have adapted to these new trends. Proposed protocols to determine whether or not fibers display 'physiological effect(s) beneficial to human health' will need to be addressed.

In 2009, The World Health Organization (WHO), the United Nations Food and Agriculture Organization (FAO) and the Codex Alimentarius Commission (CODEX)[12] have recently stated: "Dietary fiber denotes carbohydrate polymers with 10 or more monomeric units that are not hydrolyzed by the endogenous enzymes found in the small intestine of humans, belonging to the categories below."

1. Edible carbohydrate polymers naturally occurring in the consumed food.
2. Carbohydrate polymers that have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have physiological benefit

to health, as demonstrated by generally accepted scientific evidence to competent authorities.

3. Synthetic carbohydrate polymers that have been shown to have a physiological benefit to health, as demonstrated by generally accepted scientific evidence to competent authorities.

Noting, this also includes lignin and others compounds quantified by AOAC 991.43 and that the decision to include carbohydrates with 3-9 monomeric units should be left to the discretion of national authorities.

Currently, the European Food Safety Authority (EFSA) has accepted the most recent CODEX method along with Health Canada and Food Standards Australia and New Zealand (FSANZ). All three regulatory bodies also include the footnote to include substances with 3-9 monomeric units, and accept AOAC 2009.01 as the method to correctly measure total dietary fiber with the CODEX definition. AOAC 2011.09 can also be used to measure CODEX total dietary fiber.

As the concept of dietary fiber merges with the notion of 'physiological effect(s) beneficial to human health', we must take into consideration how this will influence future definitions and distinctions of prebiotics and what they mean to consumer health and the food industry, both scientifically and politically. Definitions also need to be defined as flexible enough to take into consideration the improvements in the

biotechnology and microbiology fields, so technological advances do not outpace current regulatory changes.

Prebiotic Definition

The first active and published definition of the word "prebiotic" was defined in 1995 as, 'nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health',[13] and eight years later changed to include, 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits'.[14] In 2010 the International Scientific Association for Prebiotics and Probiotics (ISAPP) widened that definition to include focus on the functionality of prebiotics, 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'.[15]

Important to note is that all prebiotics can be classified as fiber, although not all fibers are considered prebiotic.[16] Characteristics of functional prebiotics include: the ability to resist the low pH of the stomach, resist hydrolysis by mammalian enzymes, resist absorption in the upper gastrointestinal tract, the ability to be fermented by intestinal microbiota and selectively stimulate the growth and/or activity of intestinal bacteria associated with host health and overall well-being.[13,14] It is generally accepted that beneficial bacteria includes bifidobacteria and also lactobacilli. Increased growth of lactobacilli due to stimulation of

prebiotics is often less prevalent because of lower overall concentrations compared to bifidobacteria in the gastrointestinal tract.

The need to develop universal methods for specific, quantifiable results in clinical studies will result if fiber regulations need to show 'physiological effect beneficial to human health' in the U.S. The general notion of beneficial stimulation of Bifidobacteria and Lactobacillus, as typically used to determine beneficial prebiotic capacity of molecules, will need quantifiable target ranges for stimulated populations of bacteria that are representative for universal populations in the U.S.

Probiotic Definition

The FAO/WHO defines probiotics as, "live mircroorganims that, when administered in adequate amounts, confer a health benefit to the host" which is a generally accepted definition.[5,17] In the U.S., the FDA may define a probiotic as a drug, biological product, dietary supplement, food or food ingredient.[18] Both the FDA and EFSA have a similar stance, and have generally accepted the FAO/WHO definition of probiotics. Unlike Canada, the FDA and EFSA have yet to allow any health claim associated with probiotic products.

Health Benefits of Fiber

Cardiovascular disease

Dietary fiber, especially soluble fiber, has been consistently shown to protect against CVD, but the exact functional components and mechanisms remain slightly unclear.[19] Soluble fiber has been shown to decrease serum cholesterol, primarily by decreasing low-density liproprotein cholesterol.[20] Bazzano et al[21] conducted an epidemiologic follow-up assessment study with an average time of 19 years of follow up, including 1843 cases of CHD and 3762 cases of CVD and found that a higher intake of soluble fiber (5.9 g/d compared to 0.9 g/d) reduces risk of CHD. Bazzano et al[21] conducted a pooled analysis of ten prospective cohort studies in the U.S. and Europe and found that the consumption of dietary fiber from cereals and fruits is inversely associated with risk of CHD. Viscosity influences the protective effects against CVD and CHD, but other mechanisms include decreases in C-reactive protein[22–25] and decreases in blood pressure[26–28] due to higher consumption of dietary fiber.

In the U.S. the FDA states that there is sufficient evidence for beta-glucans in oats and barley, and also psyllium husk, to authorize health claims to reduce the risk of heart disease for foods containing 0.75-1.7 g of soluble fiber.[29,30] EFSA has approved function health claims for beta-glucans from oat, oat bran, barley and barley bran for food products that contain at least 1 g of beta-glucans from these sources (pure or mixed), while informing the consumer that they must consume 3 g daily to contribute to the maintenance of normal blood cholesterol levels.[31]

Glycemic Response and Diabetes Control

The relationship between fiber consumption and the development and control of type II diabetes includes many working theories and mechanisms. Mechanisms include increases in satiety cues that may lead to decreased caloric intake, thus, lesser weight gain, the ability of soluble, viscous fibers to attenuate glucose absorption rates, increase absorption and availability of nutrients and stimulate beneficial bacteria in the colon that may play an unknown role in the onset of type II diabetes.

Hopping et al[32] conducted a large-scale prospective multiethnic cohort study with 75,512 participants over a span of 14 years. Total dietary fiber intake was inversely associated with overall diabetes risk for all populations analyzed (Men HR: 0.75; 95% CI: 0.67, 0.84; P-trend <0.001 and Women HR: 0.95; 95% CI: 0.85, 1.06; P-trend = 0.05), and people who consumed greater than 15 g of fiber a day had significantly lower diabetes risk ($p<0.05$). Meyer et al[33] conducted a prospective cohort of 35,988 women for 6 years. Intakes of 17 g/d of insoluble fiber and 8 g/d of cereal fiber led to a decreased risk of the onset of type II diabetes ($p=0.0012$ and $p=0.0001$, respectively), while there was no positive association between soluble fiber intake. Schulze et al[34] conducted a similar cohort analysis of 91,249 young women for 8 years and found that low cereal fiber intake (<4.4 g/d) resulted in higher risk of developing type II diabetes.

Short-term intervention studies have displayed mixed results depending on dosage, fiber type and population analyzed. A recent meta-analysis of 34 oat and barley fiber studies

concluded that 3 g of beta-glucans from intact foods and 4 g of beta-glucans from processed foods is enough to significantly lower post-prandial glucose concentrations with 4 g of beta-glucans resulting in a decreased glycemic response of 27 ± 3 mmol · min/L.[35] Analysis of many short-term studies found no correlations between fiber intake and post-prandial glucose concentrations.[36] Mechanisms for fiber intake and type II diabetes prevention remain largely unclear with mixed results short-term, while long-term studies have found many positive associations between fiber intake and decreased risk of onset of type II diabetes.

Weight Maintenance

Fiber intake and obesity prevention has been extensively reviewed.[37] It is generally recognized that dietary fiber intake supports weight loss and maintenance, although exact mechanisms remain unclear. Supported hypotheses include promotions in satiety cues,[38] alterations in absorption of select macronutrients,[39] altering patterns and concentrations of gut hormones[40–46] and overall decreases in caloric intake. Heaton et al[47] first introduced three primary hypothesized mechanisms of decreasing absorption efficiency in the small intestine, fiber displacing higher caloric nutrients in foods and promotion of saliva and gastric juice secretion due to increased mastication. Many mechanisms remain unclear and inconsistent at best. Cross-sectional observational studies have shown consistently an inverse relationship between fiber intake and BMI and body fat across many ages and populations.[48–51] Several observational studies have shown that obese adults have significantly lower dietary fiber intake.[52,53]

US Fiber Health Claims

Currently, the FDA has allowed approved health claims for soluble fibers in foods from barley, psyllium and oats.[19] There are only twelve allowed health claims allowed on labels in the U.S., with three of them (only two discussed) dedicated to dietary fiber (Table 1-4). Additional labeling describing quantities of fiber (nutrient content claims) on packages are allowed.[54] To claim a product is "High" in dietary fiber, the product must contain 20% or more of the daily value (DV) per RACC, while a "Good Source" claim requires 10-20%.

European Fiber Health Claims

European health claims are covered by the Nutrition and Health Claims Regulation (NHCR) which includes: social media, print media, websites, presentations and product labels. The European Commission (EC) has overall authorization. Allowable health claims are categorized as: General Function (Article 13(1)), Emerging Science (Article 13(5)), Disease Risk Reduction (Article 14(1)a), Children's Health (Article 14(1)b). Health claims are authorized for wheat, barley, oat, rye and sugar beet fiber (Table 1-5).

Health Benefits of Prebiotics

Inflammatory Bowel Disease

A recent randomized, double-blind, crossover study with twenty subjects with an ileal pouch-anal anastomosis receiving 24 g of inulin or a placebo for three weeks was

conducted. After three weeks, subjects exhibited increased butyrate concentrations (11.7 mmol/g in placebo and 18.9 mmol/g in inulin group), decreased *Bacteroides fragilis* (7.68 CFU/g in placebo and 6.77 CFU/g in inulin group) and lowered concentrations of bile acids in the feces (1.66 μmol/g in placebo and 1.42 μmol/g in treatment group), while reducing inflammation of the mucosa of the ileal reservoir under histological and endoscopical analysis.[55] A randomized, double-blind, placebo-controlled trial with 103 patients with active Crohn's disease fed a 15 g/d supplement of FOS or placebo for four weeks was conducted, and found no significant differences in *Bifidobacteria* or *Faecalibacterium prausnitzii,* concluding no significant clinical benefit to supplementation with 15 g/d of FOS.[56] Limited studies have shown consistent findings on alleviation of IBD symptoms with prebiotics. Many successful studies have utilized similar paired prebiotic and probiotic treatments with consistent success in lessening symptoms of IBD and related health issues.[57]

Colon Cancer

Inulin, oligofructose, lactulose and resistant starch are all prebiotics that have been analyzed for their role in colorectal cancer risk.[58] Roncucci et al[59] researched the effect of supplementing 209 adults who previously had polyps removed consuming 20g/d of lactulose for three years. Significant reduction in adenoma recurrence was found at the end of the study, of the 61 subjects analyzed only 9 had recurrence while 61 saw no recurrence post-treatment, although no placebo or comparison group was included in this study. Langlands et al[60] conducted a 2-week study with 29 healthy adults, divided into an

experimental and control group. Oligofructose (7.5g) and 7.5 g of inulin were consumed daily in this study, with the control group not consuming a supplement. Bifidobacteria increased from 6.6 to 7.3 $\log_{10}$ CFUs ($p<0.001$) and lactobacilli increased from 3.0 to 3.7 $\log_{10}$ CFUs ($p=0.02$). MCM2 and Ki67 markers were measured for changes in cell proliferation, although no significant differences were found. Limberg et al[61] conducted a randomized, phase II chemoprevention trial with 85 subjects over 40 with previously resected colon cancer or multiple/advanced adenomas. No changes were seen for any measurements (aberrant crypt foci, Ki67 and caspase -3) for subjects consuming 12 g/d of oligofructose-enriched inulin. Many studies have researched the potential influence of resistant starches on colorectal cancer. Worthley et al[62] conducted a randomized, double-blind, placebo-controlled 4-week crossover trial. No significant differences in epithelial proliferation or crypt height were noted for the subjects consuming 25 g/d of RS2. Wacker et al[63] found no effect on cell proliferation determined by bromodeoxyuridine labeling for the 12 volunteers consuming 50-60 g/d of RS in starchy foods during their two 4-week periods of a supplemented, controlled diet. Many studies with varying amounts of RS supplements have all found similar results.[64–67] Prebiotics have displayed many potential protective effects against colorectal cancer in various animal models, but there has been limited evidence in human studies.[68] The current hypotheses include effects of SCFAs, stimulated immunity of the host and many anticarcinogenic pathwways.

Cardiovascular Disease

Causey et al[69] conducted a randomized, double-blind crossover study with twelve men with hypercholesterolemia divided into two controlled diets. One included a pint of ice cream with 20 g of inulin, and one a pint of ice cream with sucrose. Daily intake of 20 g of inulin significantly reduced serum triglycerides by 40 mg/dL ($p=0.05$), increased butyrate concentrations (0.91mmol/L in control phase and 1.96 mmol/L in inulin phase) and did not significantly alter any change in transit time. Brighenti et al[70] researched the effects of inulin in ready-to-eat cereals in twelve healthy male volunteers. Volunteers consumed 50 g of rice-based cereal with 18% inulin daily for three periods throughout four weeks. They found no changes in fecal SCFAs or pH, but found plasma total cholesterol decreased significantly ($p<0.05$) and triacylglycerol decreased ($p<0.05$) at the end of each test period while bifidobacteria concentration increased ($p<0.05$). Gluconic acid, germinated barley, oligodextrans, lactose, glutamine, hemicellulose rich substrates and many types of resistant starches are all prebiotic compounds that have been targeted for their cholesterol-lowering effects. Although identified, no specific daily dosage has been established for these effects, resulting in conflicting study results.[71]

Prebiotic Regulatory Status

Authoritative governing branches have been strained to keep up with the rapidly growing field of prebiotics. In the U.S., commonly consumed prebiotics include inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) primarily due to their tenure

in the U.S. are being regarded as safe and effective,[72] thus increasing their consumption. All foods and ingredients are regulated under the Food Drug and Cosmetic Act, which clearly states that the safety of introduced ingredients and foods is the responsibility of the company manufacturing the product. For items that are not Generally Recognized As Safe (GRAS), they are required to obtain FDA approval before products can be sold commercially. Although approval may be obtained for ingredients that act as what is generally defined as "prebiotic", neither the FDA in the U.S. nor EFSA in the European Union have established a legal definition for a prebiotic.[73,74] Cited above, EFSA currently uses the FAO definition of a prebiotic, indicating the need to display a health benefit.[73,75] Challenges exist for regulatory bodies in that the scientific community does not have a universally agreed upon definition for the term prebiotic, a key first step in addressing these regulatory issues.[4] Challenges will also be faced as fibers are required to display "physiological effects beneficial to human health", as this may blend into the current opinions and definitions at stake.

Health Benefits of Probiotics

Currently, there are no health claims associated with probiotic products in the U.S., or recommendations for probiotic consumption. In Europe, EFSA has taken a similar approach. Canada has accepted and regulated claims and health benefits for identified probiotics (Table 1-1), and have identified probiotic species known to promote health benefits in the gastrointestinal tract and body.[76–78] Health impacts of probiotics have been well-documented (Table 1-2 and 1-3) and include the ability to reduce severity of

symptoms of IBS,[79–81] preventing and reducing various types of diarrhea,[82–84] improving overall lipid profiles[85] and many more.[86] Commonly used microorganisms as probiotics usually belong to the *Lactobacillus* and *Bifidobacterium* genera, but many others are commonly used, including: *Streptococcus thermophilis, Streptococcus diacetylactis Streptococcus intermedius, Lactococcus lactis, Leuconstoc mesenteroides, Saccharomyces bourlardii, Saccharomyces cerevisae* and *Escherichia coli* strain *Nissle.*

Fiber, Prebiotic and Probiotic Consumption

Fiber consumption in the U.S and other countries is typically half of what is recommended by governing regulating bodies. In the U.S., current intake of fiber is typically around 17 g/d, while recommendations are 25 g/d and 38 g/d for women and men, respectively. Similar consumption patterns are seen in Europe and other industrialized countries.[87] Fortified foods have evolved to help bridge the gap between consumption and recommendations, but further progress needs to be made. Changes in definitions and analytical methods have also been suggested as a means to address the 'fiber gap' in many countries.[88]

Inulin is a prebiotic that occurs naturally in leeks, asparagus, onions, wheat, garlic, chicory, oats, soybeans and Jerusalem artichokes. Estimated consumption in U.S and European diets is several grams a day for naturally occurring prebiotics (inulin and FOS).[89,90] At this dosage, it may be improbable that naturally-occurring prebiotics may have any beneficial effect. Without universal definitions of prebiotics and inclusive lists

of ingredients included, epidemiological tracking of prebiotic consumption patterns will be difficult to obtain.

Probiotic consumption is hard to quantitate due to its diverse origin across many food categories. Probiotic milk products, a common subset of probiotic products, are very widely consumed.[91] In Finland, sour milk is reportedly consumed by over 90% of respondents.[92] Japan and Belgium have much lower consumption rates, with less than 5% of respondents consuming fermented dairy products.[93,94]

The Evolution of Intestinal Health

Microbiome Composition

Many ecosystems co-exist throughout the human gastrointestinal tract. Outside of the distal intestine, diverse ecosystems exist on the skin, parts of the oral cavity and also the urogenital tract. The oral cavity microbiota contains over 500 diverse bacterial species.[95] Depending on exposure and quality of hygiene, evidence suggests that these bacteria are responsible for a wide array of systemic diseases.[96] The stomach also contains between 10^2 to 10^3 CFU/mL of stomach contents, primarily due to the low pH of the stomach.[97] The distal intestine is the primary home of the human gut microbiome, a diverse and complex ecosystem containing between 10^{10} to 10^{12} CFU/g of cells, belonging to hundreds of species of bacteria.[98–101] It is estimated that up to 30 g of bacteria are produced for every 100 g of carbohydrate fermented in the distal intestine.[6] By volume, bacteria compose 30% of the human colon.[102] There are many abundant genera of

bacteria in the distal intestine, including: bacteroides, eubacteria, fusobacteria, bifidobacteria, peptostreptococci, clostridia, lactobacilli and streptococci.[103] The gut microflora serves as the primary, interchangeable interface between the diet and the host's health, and has recently been associated with many diseases and conditions.[104] Advances in clinical studies and biotechnology are leading the way to successful modulation of the gut microflora, improving knowledge of many disease states and pioneering diets and functional foods that will continue to aid in health promotion.

Influences on the Gut Microbiome and Intestinal Health

Establishment of a healthy gut microbiota starts immediately at birth, although this progression is highly debated, partly due to the complexity of defining what a "healthy microbiome" entails. It is well-established that fiber and other non-digested food contents play a large role in the influence and development of the host's microflora.[105] Other influential factors include: age, stress, infection, geography and many other environmental componenets.[106–110] Independent of the geography of populations, specific species of *Bifidobacteria* have been shown to decrease with increasing age.[110] Stress has also been shown to influence certain bacteria over extended periods of time.[111] Differences in delivery between either caesarean section or the vaginal canal play a critical role in the early development of the microbiota,[112–114] and leads to both changes in bacterial composition and also the timing of advanced colonization.[114–117] At ages 1-3, most individuals have colonization most similar to adult populations.[113,118] Studies have shown that breast-fed infants have a microflora dominated by *bifidobacteria* populations,

[114,119,120] while other studies have shown *bifidobacteria* to be much less prevalent.[121,122] Extreme biodiversity exists among individuals, making identifying a healthy microbiome difficult. As dietary constituents continue to aim at improving gut health, continued research is needed to examine the full effects of exogenous factors on host development.

Influential Roles of Lactobacilli and Bifidobacteria

Because of the many health-promoting properties of these genera of bacteria they are commonly used markers of microflora health, and common targets for dietary stimulation. Lactobacilli have been shown to down-regulate mucosal inflammation in the gastrointestinal tract.[123] Lactobacilli play a role in helping digest lactose for lactose-intolerant individuals, alleviate constipation, improve IBS symptoms and potentially help prevent traveler's diarrhea.[124] Bifidobacteria and lactobacilli also inhibit the growth of harmful bacteria, stimulate immune functions throughout the body, aid in mineral absorption and help in the synthesis of vitamins.[125] Bifidobacteria reside naturally in the gastrointestinal tract of healthy human adults and have a strong affinity to ferment select oligosaccharides, making them a common marker for prebiotic capacity. Bifidobacteria are a unique genus of bacteria in that no gas is formed as an end product of metabolism.[126] Similar to the *Lactobacillus* genera, these bacteria are saccharolytic, an often used marker for beneficial bacteria.[102] Bifidobacteria also don't produce any known carcinogenic substances *in vivo*. Bifidobacteria concentrations have been negatively associated with obesity and weight gain.[127–130] Specific species might play a critical role in this association, as not all species of bifidobacteria may have identical influence.[131]

Decreased levels of bifidobacteria and lactobacilli have been positively associated with the development of allergy diseases in the first five years of life.[132,133] Decreases in bifidobacteria, along with decreases in bacterial diversity, have been associated with higher inflammation and IBS.[134,135] The mechanisms behind disease states and lactobacilli and bifidobacteria are unclear, but sufficient studies show these bacteria are highly associated with improved health.

Conclusions

Fiber, prebiotics and probiotics have all been shown to play an influential part in developing and maintaining a healthy microbiota throughout all stages of life. As gut microbiome research continues to advance our understanding of the significance and importance of this diverse ecosystem, critical importance should be placed on public awareness of this topic. Categorization and definitions of these critical dietary components will be influential in advancing the understanding of the gut microbiome. Health claims should continue to place importance on the roles that these dietary components can play in maintaining a healthy lifestyle and their importance in various diseases and conditions. As science advances our understanding of the critical components that influence the gut microbiome through the diet, regulation of these dietary components will play a critical role in the perception and consumption of fiber, prebiotics and probiotics.

Table 1-1. *Lactobacillus* and *Bifidobacterium* microorganisms most commonly used as probiotic supplements worldwide.

Bifidobacterium	*Lactobacillus*
B. adolescentis	L. acidophilus
B. animalis	L. casei
B. animalis subsp. animalis	L. crispatus
B. animalis supsp. lactis	L. fermentum
B. bifidum	L. gasseri
B. breve	L. johnsonii
B. infantis	L. plantarum
B. lactis	L. reuteri
B. longum	L. rhamnosus
B. thermophilum	L. salivarius

Table 1-2. Bacterial species with accepted non-strain-specific probiotic claims in foods in Canada.[77,78,136–138]

Bacterial Species	Eligible Claims	Conditions	Substantiation Requirements
Bifidobacterium adolescentis *Bifidobacterium animalis* subsp. *animalis* *Bifidobacterium animalis* subsp. *lactis* *Bifidobacterium bifidum* *Bifidobacterium breve* *Bifidobacterium infantis* *Bifidobacterium longum*	"Probiotic that naturally forms part of the gut flora" "Provides live microorganisms that naturally form part of the gut flora/contribute to healthy gut flora" "Probiotic that contributes to healthy gut flora"	At least 1.0 x 10^9 colony forming units of one or more eligible microorganisms per serving Must declare genus, species and strain in labeling Recommended to included ATCC assigned number	None
Lactobacillus acidophilus *Lactobacillus casei* *Lactobacillus fermentum* *Lactobacillus gasseri* *Lactobacillus johnsonii* *Lactobacillus paracasei* *Lactobacillus plantarum* *Lactobacillus rhamnosus* *Lactobacillus salivarius*			None
Bacteria not listed above	Claims need to be validated and wording of claim specific	Need strain-specific human efficacy evidence Require genus, species and strain in labeling	Yes. Strain-specific human efficacy evidence required.

Table 1-3. Probiotic health claim criteria for natural health products.

Species	Eligible Claims/Purposes	Conditions
Bifidobacteria adolescentis *Bifidobacteria animalis* subsp. *animalis* *Bifidobacteria animalis* subsp. *lactis* *Bifidobacteria bifidum* *Bifidobacteria breve* *Bifidobacteria infantis* *Bifidobacteria longum* *Lactobacillus acidophilus* *Lactobacillus casei* *Lactobacillus fermentum* *Lactobacillus gasseri* *Lactobacillus johnsonii* *Lactobacillus paracasei* *Lactobacillus plantarum* *Lactobacillus rhamnosus* *Lactobacillus salivarius*	"Probiotic that forms/contributes to a natural healthy gut flora" "Probiotic to benefit health and/or to confer a health benefit" "Provides live microorganisms that form part of a natural healthy gut flora/that contribute to a natural healthy gut flora/benefit health/confer a health benefit"	1.0×10^7 to 1.0×10^{11} colony forming units of one or more eligible microorganisms per day
Lactobacillus Rhamnosus GG	"Helps to manage acute infectious diarrhea"	6.0×10^9 to 1.2×10^{10} colony forming units/day
	"Helps to manage/reduce antibiotic-associated diarrhea"	1.0×10^{10} to 2.0×10^{10} colony forming units/day
Lactobacillus johnsonii La1/Lj1/NCC533	"An adjunct to physician-supervised antibiotic therapy in patients with *Helicobacter pylori* infections	1.25×10^8 to 3.6×10^9 colony forming units/day
Saccharomyces boulardii *Saccharomyces cerevisiae*	"Helps to reduce the risk of antibiotic-associated diarrhea"	1.0×10^{10} to 3.0×10^{10} colony forming units/day

Table 1-4. FDA authorized health claims regarding dietary fiber and disease.

Target of Claim	Product Requirements	Required Terms	Model Statement
Soluble Fiber and Risk of Coronary Heart Disease	1. Low Saturated Fat 2. Low Cholesterol 3. Low Fat, and Must Contain Whole Oat/Barley Foods, or Oatrim that contains 0.75 g Beta-glucan/RACC, or Psyllium husk that contains 1.7 g of soluble fiber/RACC[1]	"Heart Disease" or "Coronary Heart Disease" "Saturated Fat" "Cholesterol" Specify type of soluble fiber Specify amount of soluble fiber/serving	"Soluble fiber from foods such as [product], as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [product] supplies [g] of the soluble fiber necessary per day to have this effect."
Beta-Glucan Soluble Fiber (Oat and Barley Sources)	1. Oat bran, or 2. Rolled oats, or 3. Whole oat flour, or 4. Oatrim, or 5. Whole grain barley and dry milled barley, or 6. Barley beta fiber, or 7. Soluble fiber from psyllium husk with purity of <95% The amount of soluble fiber/RACC must be declared on nutrition label.	"Heart Disease" or "Coronary Heart Disease" "Saturated Fat" "Cholesterol" Specify type of soluble fiber Specify amount of soluble fiber/serving	"Soluble fiber from foods such as [product], as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [product] supplies [g] of the soluble fiber necessary per day to have this effect."

[1] Foods bearing a psyllium seed husk health claim must also bear a label statement concerning the need to consume them with adequate amounts of fluids.

Table 1-5. Current EFSA requirements for health claims in the European Union.

Claim Type	Nutrient	Claim Summary	Health Relationship
Article 13(1) [139]	Arabinoxylan from wheat endosperm	"Consumption of arabinoxylan as part of a meal contributes to a reduction of the blood glucose rise after that meal"	Reduction of post-prandial glycaemic responses
Article 13(5) (EUR-Lex, 2014)	Sugar beet fibre	"Sugar beet fibre and increasing faecal bulk"	Increasing faecal bulk
Article 13(1) [139]	Wheat bran fibre	"Wheat bran fibre contributes to an increase in faecal bulk"	Increase in faecal bulk
Article 13(1) [139]	Wheat bran fibre	"Wheat bran fibre contributes to an acceleration of intestinal transit"	Reduction in intestinal transit time
Article 13(1) [139]	Barley grain fibre	"Barley grain fibre contributes to an increase in fecal bulk"	Increase in faecal bulk
Article 13(1) [139]	Oat grain fibre	"Oat grain fibre contributes to an increase in faecal bulk"	Increase in faecal bulk
Article 13(1) [139]	Rye fibre	"Rye contributes to normal bowel function"	Changes in bowel function
Article 13(5) (EFSA, 2015)	Chicory Root Inulin	"Chicory root inulin contributes to maintenance of normal defecation"	Increasing stool frequency

CHAPTER TWO

Prebiotic Effects and Fermentation Kinetics of Wheat Dextrin and Partially Hydrolyzed Guar Gum In An In Vitro Batch Fermentation System

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Executive Summary

Scientific research demonstrates that two indigenous gut bacteria, *Lactobacillus and Bifidobacterium* can contribute to human health. The primary objective of this *in vitro* study was to quantitatively analyze at the genus level how wheat dextrin (WD) and partially hydrolyzed guar gum (PHGG) increased the levels of these two gut bacteria at 12 h and 24 h, via real time quantitative polymerase chain reaction (qPCR). Secondary objectives were changes in fecal pH, short chain fatty acids (SCFAs) and total gas volume produced. At 12 h WD was significantly more bifidogenic (9.50 CFU $\log_{10}$/mL) than PHGG (9.30 CFU $\log_{10}$/mL) ($p=0.052$), and also at 24 h WD (9.41 CFU $\log_{10}$/mL) compared with PHGG (9.27 CFU $\log_{10}$/mL) ($p=0.043$). WD produced less total SCFAs at both 12 h and 24 h than PHGG, and produced significantly lower amounts of gas at 12 h

and 24 h ($p<0.001$, $p<0.001$). Both PHGG and WD also promoted growth of *Lactobacilli* when measured at 12 h and 24 h compared with the 0 h analysis, indicating that both fibers are lactogenic. These results demonstrate the prebiotic effect of WD and PHGG. Based on fermentation kinetics, PHGG is more rapidly fermented than WD, and both fibers show prebiotic effects as early as 12 h.

Introduction

Dietary fiber is generally defined as nondigestible carbohydrates and lignin that are intrinsic and intact in plants, with functional fiber showing beneficial physiological effects in humans.[19] Currently the U.S. recommendations for dietary fiber, expressed as a Dietary Reference Intake, are 25 g/d for adult females and 38 g/d for adult males, although the typical daily intake for U.S. consumers is approximately 17g/d.[140,141] Generally accepted health benefits associated with regular fiber consumption include maintaining a healthy digestive system, increased satiety, decreased caloric intake, and fermentation that results in beneficial changes in the gut microflora.[6,37] Daily supplementation of beneficial dietary fibers may be an effective way to help consumers get the recommended amount of dietary fiber, and its associated health benefits.

Wheat dextrin (WD) and partially hydrolyzed guar gum (PHGG) are dietary fibers evaluated in this study. WD is a soluble, fermentable fiber composed of a glucose polymer formed by the polymerization and hydrolysis of wheat starch that resists digestion in the small intestine due to its glucoside linkages.[142] PHGG is a hydrolyzed

product of guar gum, composed of mannose and galactose monomers. Clinical studies have shown that 45g/d of WD is well tolerated in the gastrointestinal tract.[143] WD also has been shown to decrease hunger and increase satiety in a randomized, double-blind, placebo-controlled study.[144–146] PHGG has been shown in randomized, cross-over clinical studies to reduce hunger and increase satiety.[147,148]

Two different soluble fibers were selected in this study to help understand the mechanisms of action and subsequent changes in gut microflora through the use of an *in vitro* batch fermentation model. *In vitro* fermentation methods are a representative model of colonic fermentation, and work well to predict substrate availability and ability for fermentation in the gut.[149] Previous preliminary studies have shown that WD and PHGG ferment in the large intestine producing measurable levels of beneficial *Bifidobacteria* and *Lactobacillus* at 24 h, indicating that both dietary fibers demonstrate a prebiotic effect.[150]

The primary objective of this study was to measure changes in two beneficial genera of microbes, *Lactobacillus* and *Bifidobacterium*, to better understand the prebiotic effects of WD and PHGG and their mechanisms of action. Secondary objectives included measurements of common fermentation markers, such as pH, total gas volume and short-chain fatty acids (SCFA).

Materials

Fibers investigated in this study included wheat dextrin (Benefiber®, Novartis Consumer Health Inc., USA) and partially hydrolyzed guar gum (Benefibra™, Novartis Consumer Health Spa Origgio, Italy). A substrate blank was employed for all baseline measurements in fecal inoculum. Chemical reagents used in this study were provided by ThermoFisher Scientific (ThermoFisher Scientific Inc., Waltham, MN), Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and Oxyrase (Oxyrase Inc., Mansfield, OH).

Methods

Fecal Collection

Fecal samples were collected from three healthy volunteers (2 males, 1 female) under anaerobic conditions from three individuals (ages 18-28) consuming non-specific Western diets, free of antibiotic treatments in the last 6 months, not affected by any GI diseases and not consuming any prebiotic or probiotic supplements. Fecal samples were anaerobically collected within 1 hour of the start of the batch fermentation and homogenized, and combined, immediately upon collection.

Fermentation

Fiber samples (0.5 g) were hydrated in 40 mL of prepared sterile tricase peptone fermentation media in 100 mL serum bottles, capped to avoid contamination, and incubated for 12 hours at 4°C. Following incubation, serum bottles were transferred to a

circulating water bath at 37°C and allowed to incubate for 2 hours. Post-collection, fecal samples were homogenized using a 6:1 ratio of phosphate buffer solution to fecal matter. After mixing, obtained fecal slurry was combined with prepared reduction solution (2.52 g cysteine hydrochloride, 16 mL 1N NaOH, 2.56 g sodium sulfide nonanhydride, 380 mL DD H_2O) at a 2:15 ratio. 10 mL of the prepared fecal inoculum was added to each of the serum bottles, 0.8 mL Oxyrase® added, flushed with CO_2, sealed, and then immediately placed in the 37°C circulating water bath. Samples were prepared in triplicate and analyzed at 0, 4, 8, 12 and 24 hours. Triplicate positive controls (dextrose) and negative controls (substrate blank) were also analyzed at the same time points. Upon removal at appropriate time point, pH and total gas volume were measured. Then, 1 mL of copper sulfate (200 g/L) was added to cease fermentation. Lastly, 2 mL aliquots were frozen at -80°C for further analysis.

pH Analysis

2 mL aliquots were removed from serum bottles immediately following total gas measurement and measured with an Orion PerpHect LogR Meter – Model 350 (Orion Research, Inc. Boston, MA).

Gas Analysis

Total gas production (volume) was measured by syringe difference analysis. Gas was measured by piercing cap of serum bottle with syringe needle and measuring gas released from system.

SCFA Analysis

SCFA extraction methods were adapted and modified from Schneider *et al.*[151] 2 mL aliquots were removed from the -80°C freezer and placed in 4°C cooler for 12 hours to thaw prior to SCFA analysis. Tubes were then gently vortexed for 5 seconds. Then, 1.6 mL of DI H_20, 400µL H_2SO_4 (50% vol/vol), and 2 mL diethyl ether (premixed with 2-ethyl butyric acid as internal standard) were all added to tubes and vortexed again for 5 seconds. Tubes were then placed in an orbital shaker for 45 minutes at 100 RPM. Tubes were removed and then centrifuged for 5 minutes at 3000 RPM. Supernatant was removed from tube and placed in 10 mL plastic tubes containing $CaCl_2$ to remove any residual water. Solution was then filtered using a BD 1 mL syringe (Becton, Dickinson and Company Franklin Lakes, NJ) and a Millex 13 mm nylon membrane filter with a 0.20 µm pore size (Merck Millipore Ltd Tullagreen, Carrigtwohill, Co. Cork, Ireland). Extractions were then analyzed using a HP 5890 series gas chromatograph (Helwitt Packard, Palo Alto, CA) with a 30 m x 0.250 mm x 0.25 µm polyethylene glycol (PEG) column (Agilent Technologies, Santa Clara, CA), with a 110°C oven temperature. Samples were injected using an automated HP 7673 GC/SFC injector (Helwitt Packard, Palo Alto, CA). Injector and detector temperatures were 220°C and 240°C, respectively. Flow rates for air, helium and hydrogen were 26, 28 and 315 mL/min, respectively. All samples were analyzed with a 50:1 split ratio.

Microbiota Analysis – Quantitative polymerase chain reaction (qPCR)

Bifidobacterium genus and *Lactobacillus* genus were quantified by DNA extraction from fermented samples, followed by qPCR using specific primers, described in Hernot et al. Amplification was performed in a set of triplicate reactions for each bacterial group within each sample according to the procedures of Hernot et al.[152] For amplification, 10 µl final volume containing 2X SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA), 15 pmol of each primer, and 10 ng of template DNA were used. Standard curves were obtained by harvesting pure cultures of each bacterium in the logarithmic growth phase in triplicate to create a five-fold dilution series. DNA from each serial dilution was extracted using a PowerSoil DNA Isolation Kit (MO Bio Laboratories, Inc) and amplified along with fecal DNA samples using a Taqman ABI PRISM 7900HT Sequence Detection System (Applied BioSystems). The colony forming units (CFU) of each standard curve serial dilution was determined by plating the *Lactobacillus* genus on Difco *Lactobacilli* MRS broth (Becton, Dickenson, and Company, Sparks, MD), and *Bifidobacterium* genus on Difco Reinforced Clostridial Medium (Becton, Dickenson, and Company). Cycle threshold (Ct) values were plotted against standard curves for quantification (CFU/mL) of the target bacterial DNA from fermentation samples.

Statistical Analysis

Statistical analysis was conducted using SAS statistical program software version 9.3 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) with Tukey pair-wise was used for all tests measuring differences of means. Statistical significance was achieved for p-values less than 0.05. Log transformations were implemented as needed for analysis.

Results

Gas Production and pH Shift

Within the first four hours of analysis, neither WD or PHGG produced detectable amounts of gas. At 8 hours, PHGG produced significantly more gas ($p<0.001$) than WD, as well as at 12 and 24 hours of fermentation (Figure 2-1). Each fiber produced a significant decrease in pH (Table 2-1), with WD decreasing more consistently over 24 hours, while PHGG decreased quickly between 4 and 8 hours of fermentation, which is simultaneously reflected by the large increase in gas production at 8 hours for PHGG.

SCFA Production

Levels of acetate, propionate and butyrate indicate that increased amounts of SCFAs can be seen with time in the batch culture fermentation system for both WD and PHGG. Levels for PHGG remained constant or declined after 12 h. All levels expressed (Table 2-2) are expressed as μmol SCFA/mL of system media.

Prebiotic Effects

Shifts in *Bifidobacteria* and *Lactobacilli* were used to demonstrate the prebiotic effects measured in the batch culture fermentation system. For both genera of analysis, all three samples had similar baseline concentrations. At 12 h WD was slightly more bifidogenic than PHGG ($p=0.052$), and also at 24 h ($p=0.043$) (Table 2-3). Changes in *Lactobacilli* at 12 h and 24 h for both WD and PHGG show that both fibers demonstrate lactogenic prebiotic properties. Based on fermentation kinetics, PHGG is more rapidly fermented than WD, and both fibers show prebiotic effects, based on changes in *Bifidobacteria* and *Lactobacilli*, as early as 12 h.

Discussion

The human gut microflora is a diverse population, with many different genera of bacteria having very different influences on the host. Both *Lactobacillus* and *Bifidobacterium* are generally accepted as two beneficial genera of bacteria, and contribute to a myriad of health benefits to the host.[6] Mechanisms underlying these benefits are thought to be through modulating the immune response, and antagonizing pathogens either by production of antimicrobial compounds or through competition for mucosal binding sites.[153] Additionally, these gut microbes faciliate nutrient and energy extraction from the diet.[154] Therefore, increasing dietary fiber intake while modulating the gut microbiota through supplementation with dietary fibers with prebiotic activity may aid in promoting the gut health of the host. Long-term studies have shown that fiber supplementation can

alter the makeup of the intestinal microflora with soluble fibers,[155] while the purpose of this study was to evaluate the early prebiotic effects employing an *in vitro* model.

Quantitative PCR analysis of WD and PHGG display that both are prebiotic dietary fibers, with both fibers displaying bifidogenic and lactogenic properties as early as 12 h when compared to baseline measurements. Previous studies have also shown bifidogenic and lactogenic properties for WD and PHGG at 24 h, while this is the first study to show rapid prebiotic activity at 12 h. Between fibers, WD is more bifidogenic at both 12 h ($p=0.052$) and 24 h ($p=0.043$) when compared to PHGG, although PHGG still shows bifidogenic and lactogenic growth at both 12 h and 24 h. WD has also been shown to increase other genera of beneficial bacteria, including non pathogenic *Clostridium* and *Roseburia*, in similar models.[156]

Secondary measurements of fermentation kinetics, including change in pH, total gas production and development of SCFAs were also key components to modeling the mechanism of action for these fibers. For WD and PHGG, pH decreased from baseline until the 24 h time point. It is postulated that a decrease in gut pH allows for more efficient absorption of specific minerals,[157,158] and may provide protective effects in the colon. Both fibers did not produce detectable amounts of gas until 8 h, and for the 12 h and 24 h measurements, PHGG produced significantly more total gas. Acetate, propionate and butyrate are commonly measured SCFAs and are representative end-products of colonic fermentation and are typically influenced by both microbes present

and substrates utilized. Acetate is commonly metabolized by muscles for energy,[159] propionate used as a gluconeogenic substrate[160] and butyrate used as an oxidative fuel for colonocytes.[161] Production of these acidic metabolites also promotes a decrease in colonic pH,[162] which promotes the growth of many lactic acid bacteria that thrive in more acidic environments. Clinical studies have also shown that this decrease in pH promotes growth of beneficial bacteria such as *Bacteroides* and inhibits growth of *Clostridum perfringens.*[163] Based on secondary fermentation measurements, PHGG is more rapidly fermented than WD.

Because of the rapid formation and absorption of SCFAs *in vivo, in vitro* models are typically employed to accurately understand the kinetics of colonic fermentation. Although pooled fecal homogenates were used in this study, stimulation of bifidogenic and lactogenic bacteria are consistent with other clinical studies, demonstrating the prebiotic effects of WD and PHGG. The current study demonstrates that WD and PHGG act as prebiotic fibers and the prebiotic changes can occur as early as 12 hours. Future research should be conducted using individual fecal sampling to analyze specie-specific stimulation of WD and PHGG, and doing so will allow for accurate comparisons between individuals in the population and help us to better understand the relationship between fiber consumption, the host's microbiome and overall digestive health.

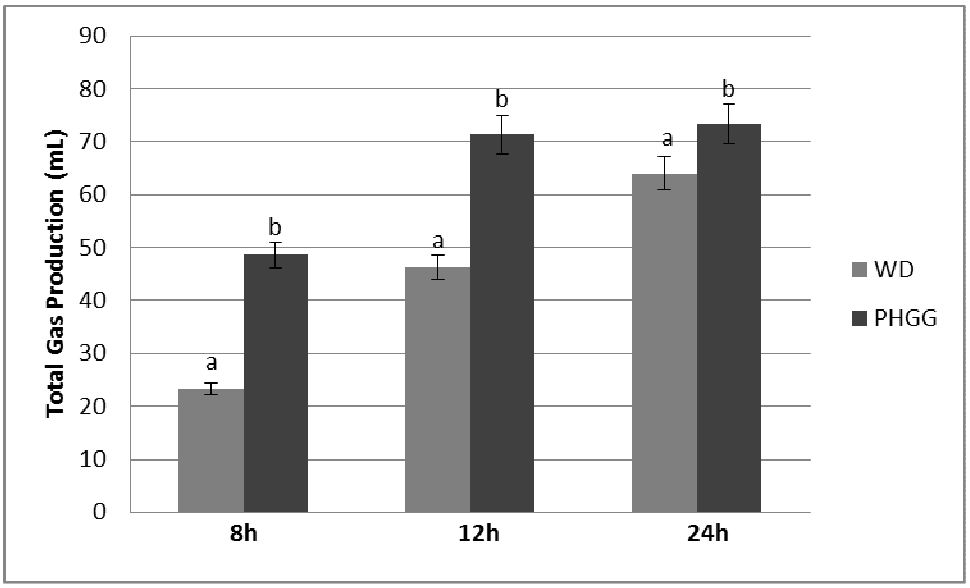
Table 2-1. pH shift in batch culture fermentation system with WD and PHGG across 24 h of analysis.

Time (h)	pH		
	WD	PHGG	Blank
0	6.96 (0.02) a	6.97 (0.01) a	7.06 (0.01) b
4	6.51 (0.00) a	6.59 (0.02) b	6.96 (0.01) c
8	6.34 (0.00) b	5.93 (0.00) a	6.92 (0.00) c
12	6.07 (0.01) b	5.90 (0.00) a	6.92 (0.01) c
24	5.78 (0.02) a	5.86 (0.00) b	6.92 (0.01) c

* Values are mean (n=3) followed by (SE).

** Values with different letters are statistically different from each other within rows ($p<0.05$).

Figure 2-1. Total gas volume produced in batch culture fermentation system with WD and PHGG across 24 h of analyis.



*Columns with different letters are significantly different from one another within the same time of measurement ($p<0.05$), at 8, 12 and 24 h.

**Values displayed are mean values (n=3) ± SE.

***No measurable amounts of gas were detected in the substrate blank, or at 0 h or 4 h for WH and PHGG.

Table 2-2. SCFAs of exposed human fecal inoculum across 24 h of analysis.

Time	Acetate (µmol/mL)		Propionate (µmol/mL)		Butyrate (µmol/mL)	
(Hours)	WD	PHGG	WD	PHGG	WD	PHGG
0	1.20 (0.05)	1.03 (0.09)	0.69(0.01)	0.68(0.02)	0.98(0.03)	0.95(0.03)
4	5.02 (0.38)	4.30 (0.14)	2.45(0.17)	2.36(0.03)	3.70(0.29)	3.22(0.05)
8	7.56 (0.36)	9.83(0.01)	7.37(0.19)	7.47(0.29)	7.77(0.39)	10.48(0.77)
12	9.71 (0.53)	10.85(0.23)	8.94(0.66)	10.62(0.10)	8.35(0.78)	10.76(0.18)
24	11.60(0.71)	10.74(0.13)	10.59(0.51)	10.62(0.44)	8.32(0.15)	9.85 (0.62)

*Values are mean (n=3) followed by (SE).

Table 2-3. Alterations in the microbiota concentration in batch culture fermentation with WD And PHGG after 0, 12 and 24 h of fermentation with human fecal inoculum.

Time (Hours)	Bifidobacteria (CFU log_{10}/mL)			Lactobacilli (CFU log_{10}/mL)		
	WD	PHGG	Blank	WD	PHGG	Blank
0	9.24(0.03)a	9.14(0.07)a	9.08(0.00)a	10.39(0.04)a	10.40(0.09)a	10.35(0.02)a
12	9.50(0.03)b	9.30(0.04)b	9.05(0.06)a	10.79(0.04)b	10.86(0.07)b	10.35(0.02)a
24	9.41(0.07)b	9.27(0.04)c	8.96(0.07)a	10.76(0.09)b	10.68(0.04)b	10.23(0.03)a

*Values displayed are mean (n=2) (standard error).

**Values with different letters are statistically different from each other within rows of three columns displaying data for each respective genus ($p<0.05$).

CHAPTER THREE

In Vitro Analysis of Partially Hydrolyzed Guar Gum Fermentation Differences Between Six Individuals

The original version of this article can be found at Food & Function 7.4(2016):1833-1838. The reprint of this article has been used with permission from the publisher, as published in agreed format.

Executive Summary

Partially hydrolyzed guar gum (PHGG) is a fermentable, soluble, non-gelling fiber consumed as both a supplement and ingredient. PHGG supports bifidogenic and lactogenic growth, and increases the concentration of SCFAs in the distal intestine due to its fermentability. Changes in SCFA development due to the fermentation of dietary fibers in the colon has been widely studied, but there are limited studies analyzing the differences in SCFA development across multiple individuals (ages 23-68) exposed to the same dietary fiber (PHGG). With the six donors analyzed in this study, gas production varied from 59-80 mL/0.5g fiber at 12 h and 85-93 mL/0.5g fiber at 24 h between the six donors. At 12 h butyrate concentrations varied from 6.99 μmol/mL to 23.84 μmol/mL and from 8.78 μmol/mL to 22.84μmol/mL at 24 h. Total SCFA concentration at 24 h ranged from 42.85 μmol/mL to 91.17 μmol/mL. The overall average ratio for the six fecal donors was 30:45:25 (acetate:propionate:butyrate), which is similar to other

fermentable fibers analyzed using *in vitro* systems. SCFA development in the distal intestine increases the amount of metabolizable energy from the diet, but varies greatly between people based primarily on the composition and changes of their gut microflora. With over a 2-fold difference in SCFA production, significant differences were found between healthy individuals fecal microflora when exposed to PHGG. Donor 6 SCFA concentrations decreased at 24 h, indicating a quicker fermentation process than the other five donors. All SCFAs measured fluctuated greatly between the six individuals within 24 h of analysis.

Introduction

Dietary fiber consumption in the U.S. is approximately 17 g/d for adults,[141] far below the recommended 38 g/d and 25 g/d for males and females, respectively.[140] The health benefits of consuming adequate fiber are quite extensive, including the ability to help maintain a healthy body weight,[164] increased satiety,[165] improved cardiovascular health,[166,167,168] digestive system health,[169] and support beneficial growth of the gut microflora.[170] An under-researched area is individual variation of fermentation dynamics, depending largely on the composition of the host's gut microflora. Many studies have demonstrated that changes in SCFA concentrations are primarily due to fluctuations in the host's bacterial makeup.[171–173]

Partially hydrolyzed guar gum (PHGG) is a commonly consumed fiber formed from the controlled hydrolysis of guar gum, and is composed of mannose and galactose

monomers. PHGG has been shown in randomized, cross-over clinical studies to reduce hunger while increasing satiety.[147,148] PHGG has also been shown to increase levels of bifidobacteria and lactobacilli,[150,174] two beneficial genera of bacteria. In a clinical feeding study, subjects that consumed 20 g/d of PHGG for four weeks showed decreased total serum cholesterol, increased fecal weight and output frequency and lower fecal pH without influencing fat, protein or mineral absorption.[175] PHGG has also been shown to alleviate irritable bowel syndrome (IBS) due to its non-gelling capacity and therapeutic effects.[176]

Short-chain fatty acids (SCFA) are commonly measured end-products of colonic fermentation. SCFA can contribute between 1.5-2.5 kcal/g,[177] contributing up to 10% of metabolizable energy (ME) to the diet. Schwiertz et al found that there was a higher concentration of SCFAs in overweight and obese individuals.[178] Similar studies have correlated higher ratios of *Firmicutes* to *Bacteroidetes* and increased concentrations of SCFAs with obesity.[179–181] This increase in metabolizable energy also has many other beneficial effects to the consumer.[182]

SCFAs can act as anti-diarrheal agents by their stimulation of water and sodium absorption in the distal intestine, which may be one of the reasons why diarrhea is sometimes a consequence of impaired fermentation in the distal intestine. Antibiotics sometimes cause diarrhea and have been shown to drastically decrease SCFA

concentrations *in vitro*.[183] Concentrations and oxidation rates of SCFAs may also play on important role in the pathogenesis of colitis.[184]

The objective of this study was to compare SCFA development among six donor's fecal microflora after exposure to PHGG in an *in vitro* fermentation system, with the secondary measurement of total gas production to analyze the differences in fermentation rates within the first 24 h among six individuals. To our knowledge, this is the first study to analyze inter-individual fermentation differences among six individuals exposed to PHGG within 24 h.

Materials

Fiber analyzed in this study was partially hydrolyzed guar gum (Benefibra™, Novartis Consumer Health Spa Origgio, Varese, Lombardy, Italy). Chemical reagents used were provided by ThermoFisher Scientific (ThermoFisher Scientific Inc., Waltham, MN, USA), Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Oxyrase (Oxyrase Inc., Mansfield, OH, USA).

Methods

Donor Information

Demographic characteristics of the six fecal donors (Table 3-1).

Fecal Collection

Fecal samples were collected from six healthy volunteers (5 males, 1 female) under anaerobic conditions from individuals (ages 21-68) consuming non-specific Western diets, free of any antibiotic treatments in the last year, not affected by any GI diseases and not consuming any probiotic or prebiotic supplements. Fecal samples were anaerobically collected within 30 minutes of the start of the fermentation, and homogenized immediately upon collection. All data and samples collected were done in accordance with University of Minnesota policies and procedures.

Fermentation

Fiber samples (0.5 g) were hydrated in 40 mL of prepared sterile tricase peptone fermentation media in 100 mL serum bottles, capped, and incubated for 12 hours at 4°C. Following incubation, serum bottles were transferred to a circulating water bath at 37°C and allowed to incubate for 2 hours. Post-collection, fecal samples were mixed using a 6:1 ratio of phosphate buffer solution to fecal sample. After mixing, obtained fecal slurry was combined with prepared reducing solution (2.52 g cysteine hydrochloride, 16 mL 1N NaOH, 2.56 g sodium sulfide nonanhydride, 380 mL DD H_2O) at a 2:15 ratio. 10 mL of the prepared fecal inoculum was added to each of the serum bottles, 0.8 mL Oxyrase® was added, flushed with CO_2, sealed, and then immediately placed in a 37°C circulating water bath. Samples were prepared in triplicate and analyzed at 0, 12 and 24 h. Upon removal at each time point, total gas volume was measured. Then, 1 mL of copper sulfate

(200 g/L) was added to cease fermentation. Lastly, 2 mL aliquots were frozen at -80°C for SCFA analysis.

Gas Analysis

Total gas production was measured by syringe difference analysis. Gas was measured by piercing cap of serum bottle with syringe needle and measuring gas released from each individual sealed serum bottle.

SCFA Analysis

SCFA extraction methods were adapted and slightly modified from Schneider et al.[151] 2 mL aliquots were removed from the -80°C freezer and placed in a 4°C cooler for 12 hours prior to analysis. Tubes were then gently vortexed for 5 seconds. Then, 1.6 mL of DI H_20, 400µL H_2SO_4 (50% vol/vol), and 2 mL diethyl ether (premixed with 2-ethyl butyric acid as internal standard) were all added to tubes and vortexed again for 5 seconds. Tubes were then placed in an orbital shaker for 45 minutes at 100 RPM. Tubes were removed and then centrifuged for 5 minutes at 3000 RPM. The supernatant was removed from tube and placed in 10 mL tubes containing $CaCl_2$ to remove residual water. The solution was then filtered using a BD 1 mL syringe (Becton, Dickinson and Company Franklin Lakes, NJ) and a Millex 13 mm nylon membrane filter with a 0.20 µm pore size (Merck Millipore Ltd Tullagreen, Carrigtwohill, Co. Cork, IRL). Extractions were then analyzed using a HP 5890 series gas chromatograph (Hewlett Packard, Palo Alto, CA) with a 30 m x 0.250 mm x 0.25 µm polyethylene glycol (PEG) column

(Agilent Technologies, USA), with a 110°C oven temperature. Samples were injected using an automated HP 7673 GC/SFC injector (Hewlett Packard, Palo Alto, CA). Injector and detector temperatures were 220°C and 240°C, respectively. Flow rates for air, helium and hydrogen were 26, 28 and 315 mL/min, respectively. All samples were analyzed utilizing a 50:1 split ratio.

Statistical Analysis

All statistical analysis was conducted using SPSS (SPSS Chicago, IL). Analysis of variance (ANOVA) with Tukey HSD was used for all tests measuring differences among means. Log transformations were applied where necessary. Statistical significance was achieved for p-values less than 0.05.

Results

Gas Production

At 12 h post-inoculation, gas production ranged from 59 mL to 80 mL (Figure 3-1), with an overall average production of 74 mL, similar to previously published data.[174] At 24 h, gas production ranged from 85 mL to 93 mL, with an overall average gas production of 90.2 mL for the six individuals. Between 12 h and 24 h of analysis the average increase in gas production was 16.3 mL, but ranged between 5 mL to 34 mL increases, with all individuals having higher gas production at 24 h compared to 12 h.

SCFA Production

Acetate production varied greatly among the six donors, with concentrations increasing at 24 h compared to 12 h for 5 of the six donors (Figure 3-2). Donor 4 had similar concentrations to donor 5 and 6, $p=0.343$ and $p=0.803$, respectively, but had the highest concentration at 24 h. Although donors 4, 5 and 6 had similar concentrations at 12 h, they were all statistically different at 24 h (4 vs. 5, $p=0.047$; 4 vs. 6, $p<0.001$; 5 vs. 6, $p=0.024$). At 12 h, donors 1, 2 and 3 had similar concentrations (1 vs. 2, $p=0.580$; 1 vs. 3, $p=0.239$; 2 vs. 3, $p=0.524$), and at 24 h both donor 1 and 3 had similar concentrations ($p=0.305$), while donor 2 was significantly lower than both (1 vs. 2, $p=0.003$; 2 vs. 3, $p=0.033$).

Propionate concentrations (Figure 3-3) closely resemble the acetate concentrations (Figure 3-2) in that donors 1, 2 and 3 had the lowest concentrations at 12 and 24 h, and donor 4 had the highest concentration at 24 h. At 12 h donor 2 had the lowest concentration (2 vs. 1, $p<0.001$; 2 vs. 3, $p=0.012$). Donor 2 also had the lowest concentration at 24 h, but statistically similar to donor 1 and donor 3, $p=0.115$ and $p=0.161$, respectively. At 24 h of exposure, donor 4 had the highest concentration (4 vs. 5, $p=0.001$; 4 vs. 6, $p=0.003$).

Butyrate concentrations and changes in concentrations varied greatly among the six donors (Figure 3-4). Donor 3 had the lowest butyrate concentration at 12 h (3 vs. 1, $p=0.048$; 3 vs. 2, $p=0.003$), and at 24 h had statistically similar concentration compared to

donor 2 ($p=0.455$) and a lower concentration than donor 1 ($p<0.001$). Donor 6 had the highest concentration at 12 h (6 vs 5, $p=0.009$) and had similar concentrations to donors 4 and 5 at 24 h (6 vs. 5, $p=0.288$; 6 vs. 4, $p=0.166$).

Between donor 4 and donor 2 at 24 h of exposure there was over a 2-fold difference in total SCFA production (Table 3-2). Donor 6 was the only subject to have a decrease in total SCFA at 24 h compared to 12 h. This is likely due to quicker ability to ferment PHGG prior to the 12 h measurement. Donor 5 had similar concentrations to donor 6 ($p=0.352$) at 12 h and had similar concentrations to donor 6 ($p=0.717$) at 24 h, but less than donor 4 ($p=0.028$).

Discussion

In vivo, total SCFA production by humans is usually between 100-200 mM per day, but is highly dependent on the host's environment and availability of substrate for fermentation.[185] The average total SCFA concentration after 24 h of analysis was 60.3 mM/L for all six fecal donors in this study. Once produced, over 95% of all SCFAs are immediately absorbed, often making them hard to accurately measure *in vivo*. The three most abundant SCFAs (acetate, propionate and butyrate) are commonly formed due to the fermentation of non-digestible carbohydrates and proteins. Other acids that escape digestion are typically formed due to the breakdown of branched-chain amino acids that surpass digestion in the upper gastrointestinal tract typically include: valerate, isovalerate, isobutyrate, 2-methyl-butyrate, formate and caproate.[186] Acetate is primarily metabolized

for energy in the muscles,[159] propionate used as a gluconeogenic substrate outside of the colon,[160] and butyrate as a fuel for colonocytes.[161] Typical ratios for acetate:propionate:butyrate range from 40:40:20 to 75:15:10, depending on substrate that is available for colonic fermentation.[186,187] Many studies show that the order of concentration typically follows acetate > propionate > butyrate, but actual concentrations vary between studies depending on study design.[175,188,189] The average approximate ratio for this study was 30:45:25, but varied greatly amount the six fecal donors. Although it is well accepted that PHGG is extensively fermented in the gut, little data on SCFA production with PHGG have been published.

Many studies have analyzed the impact of different fibers and other macronutrients and how they affect SCFA production in many *in vitro* models,[190–192] but to our knowledge, this is the first that addresses differences among six individuals within 24 h of exposure. One of the first *in vitro* studies to analyze differences for both inter-individual and intra-individual relationships between SCFA ratios was conducted by Mortensen et al[193], and showed that there was a significant correlation between substrate analyzed and resulting SCFA production, and no significant differences in inter-individual or intra-individual comparisons with the three similar donors used in the study. However, six drastically different substrates (glucose, wheat bran, pectin, ispaghula, cellulose and albumin) were analyzed with only three fecal donors.

Total gas production measures gas produced during fermentation, primarily composed of CO_2, H_2, and CH_4.[194] Previous studies have shown that breath hydrogen and methane poorly represent fiber digestion.[195] Total gas production potentially indicates overall fermentation rates likely to be seen in the gut. Excessive gas production may lead to undesirable flatus, abdominal pain and bloating.

Overall, the SCFA profiles for each of the six donors were quite different at 12 and 24 h. With an average overall ratio of 30:45:25 (acetate:propionate:butyrate), acetate production was slightly less compared to other fermentable fibers in similar *in vitro* models.[196] With over a 2-fold change in total SCFA among donors, ratios fluctuated greatly among individuals. Donor 6 had concentrations of acetate, propionate and butyrate that were lower at 24 h than 12 h for each respective SCFA, and was the only donor to have decreased levels for multiple SCFA. With the highest concentrations at 12 h of propionate and butyrate, and the second highest acetate concentration at 12 h it is clear that the PHGG was fermented primarily before the 12 h measurement. Compared to the two other males with similar ages and BMI (donors 4 and 5), differences in fermentation rates are likely due to differences in the fecal microflora.

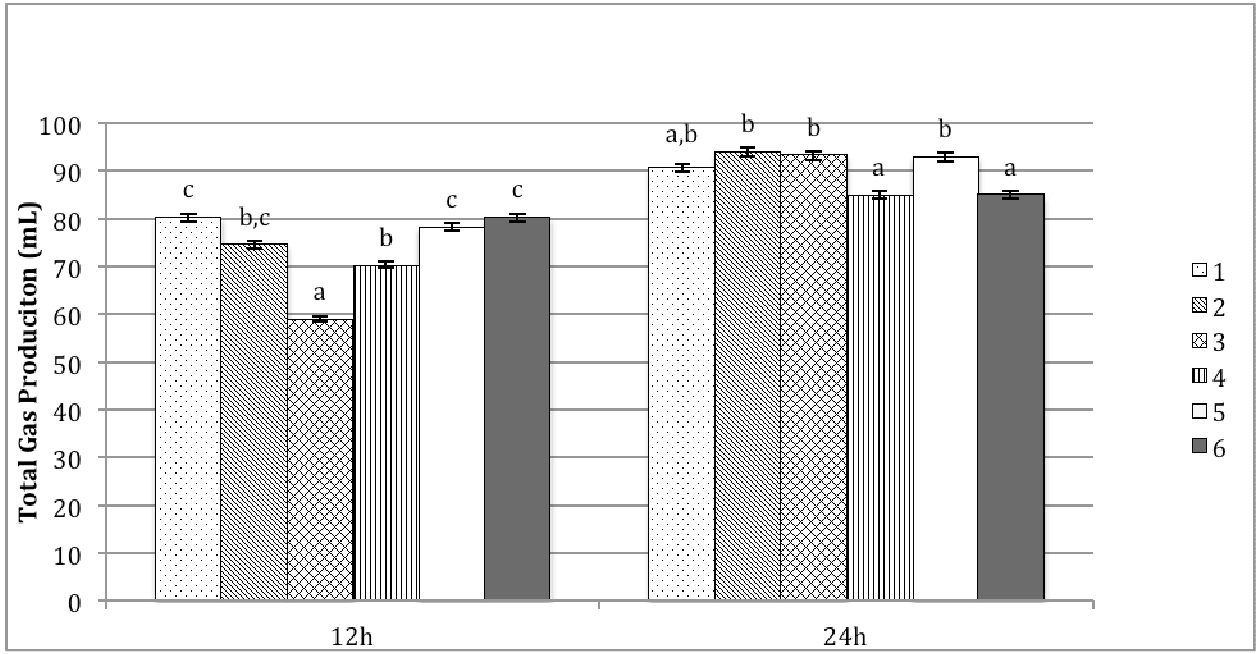
In conclusion, the overall average SCFA ratio for the six fecal donors was 30:45:25 (acetate:propionate:butyrate), which is similar to other fermentable fibers analyzed using *in vitro* systems. At 24 h there was over a 2-fold difference among individuals, indicating significant differences among different individuals exposed to PHGG. With one donor

displaying decreased concentrations of all SCFA at 24 h compared to 12 h, fecal microbiota from select individuals ferment the digestible components of PHGG completely within the first 12 h of exposure. Further studies should quantify those bacteria that ferment PHGG quicker than others, and correlations between SCFA concentration and targeted gut microbiota should be established.

Table 3-1. Demographic characteristics of six fecal donors.

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Age	31	68	60	24	22	21
Sex	Male	Male	Female	Male	Male	Male
BMI	23.7	33.6	19.5	26.3	24.7	23.0

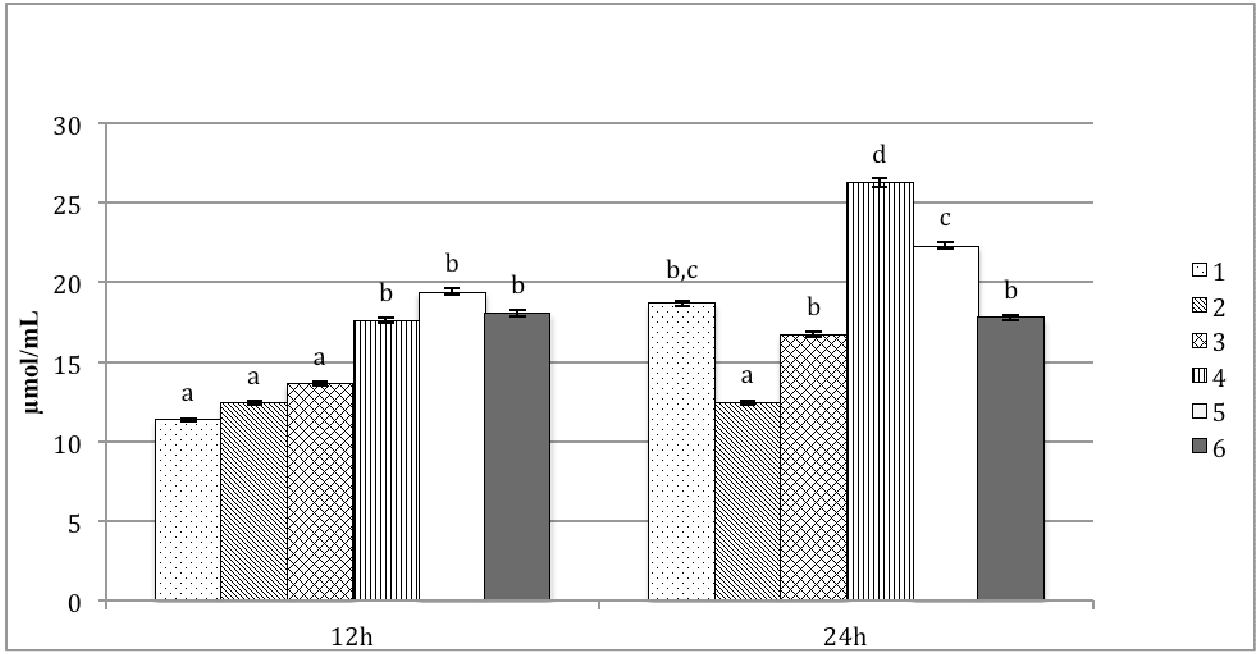
Figure 3-1. Total gas production comparing differences among six individuals at 12 h and 24 h post-exposure to PHGG.



* Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

** Columns with different letters are significantly different from one another within each time of measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

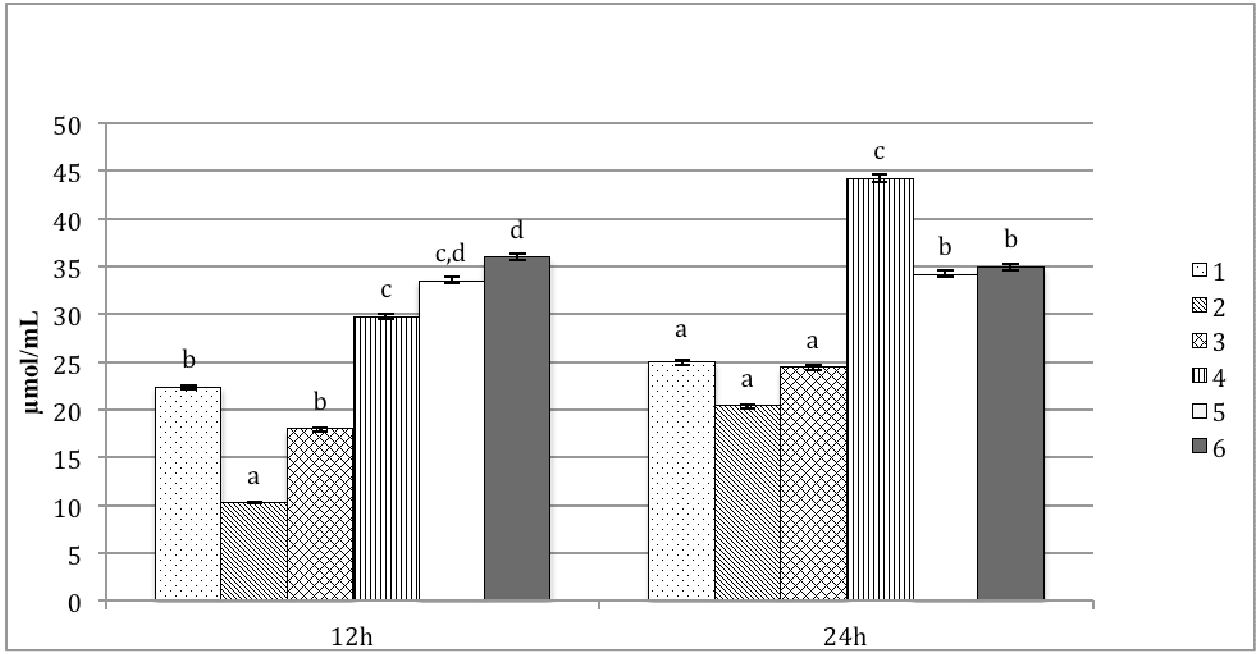
Figure 3-2. Acetate production at 12 h and 24 h of fermentation of PHGG by six individuals.



* Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

** Columns with different letters are significantly different from one another within each time of measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

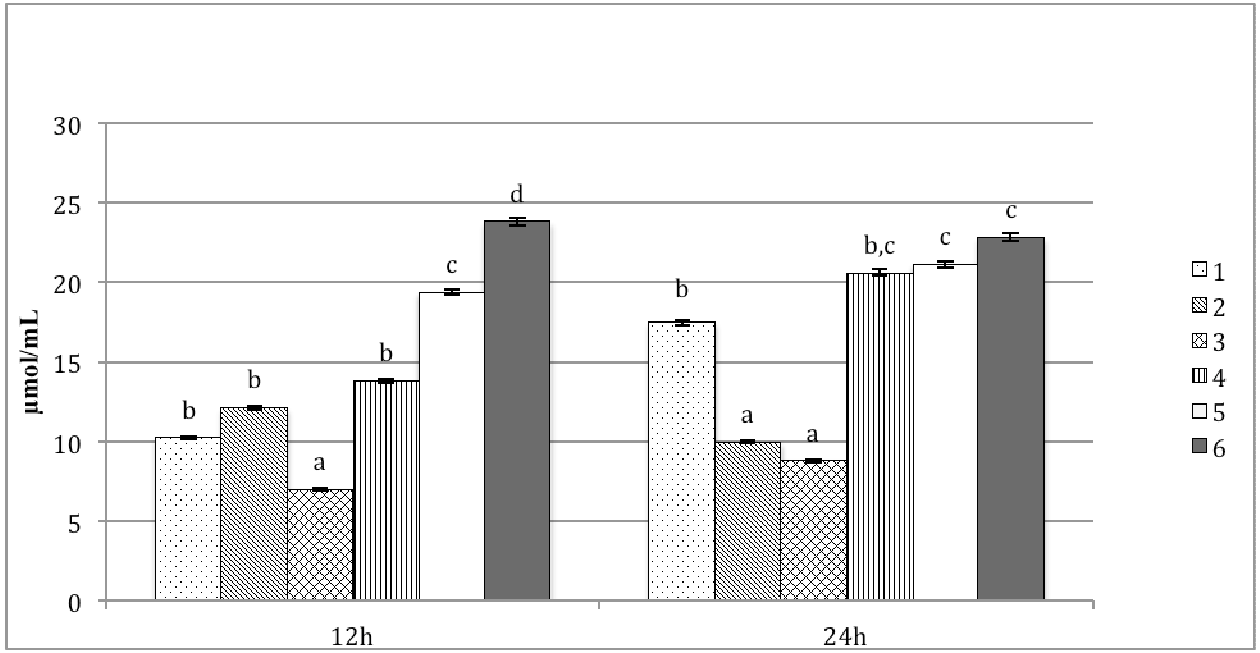
Figure 3-3. Propionate production at 12 h and 24 h of fermentation of PHGG by six individuals.



* Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

** Columns with different letters are significantly different from one another within each time of measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

Figure 3-4. Butyrate production at 12 h and 24 h of fermentation of PHGG by six individuals.



* Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

** Columns with different letters are significantly different from one another within each time of measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

Table 3-2. Average total SCFA profiles (μmol/mL) for six donors at 12 h and 24 h post-exposure to PHGG treatment.

Donor	12 h	24 h
1	43.98(6.21)[a]	61.17(4.81)[b]
2	34.84(0.88)[a]	42.85(4.71)[a]
3	38.57(2.53)[a]	49.97(3.54)[a,b]
4	61.23(4.34)[b]	91.17(4.47)[d]
5	72.43(3.47)[b,c]	77.73(4.32)[c]
6	77.89(2.37)[c]	75.62(4.36)[c]

*Values are means of triplicate determinations (SEM). Means within columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

**Total SCFA include: acetate, propionate and butyrate.

CHAPTER FOUR

In Vitro Analysis of Partially Hydrolyzed Guar Gum Fermentation on Identified Gut Microbiota.

The original version of this article can be found at Anaerobe 42(2016):60-66. The reprint of this article has been used with permission from the publisher, as published in agreed format.

Executive Summary

Prebiotic dietary fibers resist digestion in the upper gastrointestinal tract and allow for stimulation of bacteria in the distal intestine and colon. Stimulation of bacteria among different individuals varies greatly, depending on a wide range of variables. To determine the range of differences in response between individuals, a preclinical *in vitro* fermentation was conducted with six fecal donors. The primary objective was to compare the fecal microbiota of six individuals at baseline, 12 h and 24 h post-exposure to partially hydrolyzed guar gum (PHGG). Fecal donations were collected from six healthy individuals consuming a non-specific Western diet, free of antibiotic treatments in the past year, not affected by any GI diseases and not consuming any probiotic or prebiotic supplements. Fecal samples were exposed to 0.5 g of PHGG and measured for bacterial

changes at 0, 12 and 24 h base on 16S rRNA sequencing. *Parabacteroides* increased from 3.48% of sequence reads to 10.62% of sequence reads after 24 h ($p = 0.0181$) and Bacteroidetes increased from 45.89% of sequence reads to 50.29% of sequence reads ($p = 0.0008$). PHGG stimulates growth of *Parabacteroides*, a genus of bacteria that have been inversely associated with IBS and ulcerative colitis. PHGG provides stimulation of beneficial Bacteroidetes (*Bacteroides* and *Parabacteroides*), which may be correlated with many positive health markers and outcomes. PHGG is a prebiotic dietary fiber that is readily fermentable.

Introduction

Fiber consumption in the U.S. is approximately half of the recommended intake, with the average U.S. individual only consuming about 17 g/d.[141] Dietary fiber with prebiotic capacity offers a healthful, practical way to bridge the gap between consumption and recommended intake. Dietary fiber supplements can offer some of the same physiological benefits as dietary fiber found intact in foods.[197] When consumed in adequate levels, dietary fiber has been shown to help maintain a healthy body weight,[164] improve cardiovascular health,[166–168] support overall digestive health,[169] and support the overall growth of the intestinal microbiota.[170]

The intestinal microbiota have a tremendous impact on overall health, and have been recently shown to have a significant impact on the host's metabolism, immune system capacity and many other pathways affecting overall host health.[198–200] Recent studies

have also found many associations between many diseases and the host's microbial composition, including: metabolic syndrome, diabetes, and many gastrointestinal diseases.[201–204] The gut microbiome and it's correlation to health and disease is a quickly revolving, dynamic area of research. Next-generation sequencing and other advanced sequencing technologies have provided new insight into this rapidly expanding field, and have allowed efficient and effective ways to analyze the thousands of diverse taxa within the human gastrointestinal tract.[205,206]

Partially hydrolyze guar gum (PHGG) is a dietary fiber made from the controlled hydrolysis of guar gum, composed of both mannose and galactose monomers, and is commonly consumed as both a dietary fiber supplement, as well as in foods. PHGG has been shown to alleviate irritable bowel syndrome (IBS) due to its non-gelling effects,[176] and has been shown in randomized, cross-over clinical studies to increase satiety.[147,148] In a four-week clinical feeding study where participants consumed 20 g/d of PHGG researchers found an overall decrease in total serum cholesterol, an increase in fecal weight and a lower fecal pH without influencing key nutrient absorption. *In vitro* models have shown that PHGG supports the growth of *Bifidobacterium* and *Lactobacillus*, two genera of bacteria that have been associated with many health outcomes.[174,196]

This paper is a follow-up of previously published work from our laboratory that evaluated the differences in fermentation rates between six individuals' fecal microbiota all exposed to PHGG in an *in vitro* fermentation model, with the primary objective of

showing the differences in short chain fatty acid (SCFA) synthesis and total gas production, and found over a 2-fold difference in total SCFA production in 24 h.[207] The current study has the objective of determining key changes and individual differences among six individual's fecal microbiota exposed to PHGG in an *in vitro* fermentation model. To the authors' knowledge, this is the first *in vitro* model with six individuals to show the short-term effects of PHGG on the fecal microbiota.

Materials

Fiber analyzed in this study was partially hydrolyzed guar gum (Benefibra™, Novartis Consumer Health Spa Origgio, Varese, Lombardy, Italy). PHGG is a dietary fiber made from the controlled hydrolysis of guar gum from the guar plant *Cyamoposis tetragonolobus*, composed of both mannose and galactose monomers. PHGG is a soluble, non-viscous fiber often incorporated into both foods and beverages. Chemical reagents used were provided by ThermoFisher Scientific (ThermoFisher Scientific Inc., Waltham, MN, USA), Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Oxyrase (Oxyrase Inc., Mansfield, OH, USA).

Methods

Donor Information

Healthy fecal donors (male and female) were selected to represent a wide range in age (21-68) and BMI (19.5-33.6) for representative samples from a population (Table 4-1). Donors were selected based on screening questionnaire and previous participation.

Fecal Collection

Fecal samples were collected from six healthy volunteers (5 males, 1 female) under anaerobic conditions from individuals (ages 21-68) consuming non-specific Western diets, free of any antibiotic treatments in the last year, not affected by any GI diseases and not consuming any probiotic or prebiotic supplements. Fecal samples were anaerobically collected within 10 minutes of the start of the fermentation, and homogenized immediately upon collection. All data and samples collected were done in accordance with University of Minnesota policies and procedures.

Fermentation

Fiber samples (0.5 g) were hydrated in 40 mL of prepared sterile tricase peptone fermentation media in 100 mL serum bottles, capped, and incubated for 12 hours at 4°C. Following incubation, serum bottles were transferred to a circulating water bath at 37°C and allowed to incubate for 2 hours. Post-collection, fecal samples were mixed using a 6:1 ratio of phosphate buffer solution to fecal sample. After mixing, obtained fecal slurry was combined with prepared reducing solution (2.52 g cysteine hydrochloride, 16 mL 1N NaOH, 2.56 g sodium sulfide nonanhydride, 380 mL DD H_2O) at a 2:15 ratio. 10 mL of the prepared fecal inoculum was added to each of the serum bottles, 0.8 mL Oxyrase® was added, flushed with CO_2, sealed, and then immediately placed in a 37°C circulating water bath. Samples were prepared in triplicate and analyzed at 0, 12 and 24 h. Upon removal at each time point, total gas volume was measured. Then samples were divided

into aliquots for analysis and 1 mL of copper sulfate (200 g/L) was added to cease fermentation. All samples were immediately frozen and stored at -80°C for further analysis.

DNA Extractions

Fecal bacteria DNA from the *in vitro* system were extracted using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) following provided operating instruction, including bead beating for 20 min.

Primary/Secondary PCR Amplification

The V1-V3 region of the 16S rRNA was amplified using a two-step PCR protocol. The primary amplification was done using an ABI7900 qPCR machine. The following recipe was used: 3 µl template DNA, 0.48 µl nuclease-free water, 1.2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 µl DMSO (Fisher Scientific, Waltham, MA), 0.12 µl ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl 1000x SYBR Green, 0.12 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (V1_27F and V3_V34R), as well as adapter tails for adding indices and Illumina flow cell adapters in a secondary amplification. The following primers were used (16S-specific sequences in

bold): Meta_V1_27F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCMTGGCTCAG)** and Meta_V3_534R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG).**

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and indices. The secondary amplification was done on a fixed block BioRad Tetrad PCR machine using the following recipe: 5 µl template DNA, 1 µl nuclease-free water, 2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 µl DMSO (Fisher Scientific, Waltham, MA) 0.2 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The following indexing primers were used (X indicates the positions of the 8 bp indices): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC and Reverse indexing primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG

Normalization and Sequencing

The samples were normalized using a SequalPrep capture-resin bead plate (Life Technologies, Carlsbad, CA) and pooled using equal volume. The final pools were quantified via PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA) and diluted to 2nM. 10 µl of the 2 nM pool was denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% phiX, heat denatured at 96°C for 2 minutes, and sequenced using a MiSeq 600 cycle v3 kit (Illumina, San Diego, CA).

Sequence Processing and Analysis

Generated sequence data was processed and analyzed using QIIME.[208] Fastq sequence data was processed with the University of Minnesota's gopher-pipeline for metagenomics, and automated pipeline for high-throughput sequence data sets.[209] Sequence data had adapters removed and sliding quality trimming window by Trimmomatic[210]; primers removed and overlapping reads merged by Pandaseq.[211] Within QIIME, chimera checking done by chimera slayer, Open reference OTU picking completed with Usearch61, taxonomic identification using GreenGenes (Version 13.8) reference database, rarefied to 14,393 sequences per sample. Analysis was performed using R (R Development Core Team, 2012).

Statistical Analysis

All statistical analysis was performed using R software (R Development Core Team, 2012). Differences in means were determined using the Kruskal-Wallis ANOVA test

(data determined non-parametric with QIIME), testing the null hypothesis that the location parameter of the groups of abundances for a given operational taxonomic units (OTU) is the same. Multiple comparisons were corrected using the FDR procedure. Significance was set for p-values <0.05.

Consent

Voluntary informed consent was obtained from all fecal donors prior to this study according to University of Minnesota policies and procedures.

Results

Sequencing of the 16S rRNA gene was conducting using Illumina MiSeq 2000 sequencing platforms at the University of Minnesota Genomics Center. From the 6 fecal samples analyzed from the *in vitro* system across 24 h of fermentation (6 fecal samples x 3 time points x 3 technical replicates = 54 samples analyzed), there were >30 million total sequences generated, representing 11 bacteria phyla and 416 OTUs at a 97% similarity threshold. Changes in fecal microbiota were analyzed for all six fecal donors in the *in vitro* fermentation system at baseline, 12 and 24 h. Taxa with greater than 0.05% overall abundance were summarized (Table 4-2). At baseline, Bacteroidetes and Firmicutes were the most abundant phyla of bacteria, consisting of approximately 90% of all sample reads. Proteobacteria was the third most abundant phyla identified, consisting of approximately 4% of the sample reads. Actinobacteria and Verrucomicrobia accounted for less than 1% of all sequence reads.

Across the 24 h of analysis, overall abundance of sequences of Bacteroidetes increased significantly ($p<0.001$) from approximately 45.9% of sequence reads at baseline to approximately 50.3% of sequences at 24 h. Firmicutes accounted for approximately 44.9% of total sequences at baseline, which decreased to 40.4% of sequence reads at 24 h ($p = 0.0527$), trending towards significance. Verrucomicrobia accounted for 0.51% of sequence reads at baseline, and increased significantly over 24 h to 0.82% of sequence reads at 24 h ($p<0.001$), although still accounted for less than 1% of all sequence reads. Similar shifts in abundant phyla identified were similar for all fecal donors (Figure 4-1).

At the genus level, *Bacteroides* was the most abundant genus in all samples, consisting of 27.1% of sequence reads at baseline, which increased to 33.05% of all reads at 24 h ($p = 0.14$) (Table 4-2). *Parabacteroides* had the greatest stimulation, increasing from 3.48% of sequence reads at baseline to 10.18% of sequence reads at 12 h and 10.62% at 24 h ($p = 0.0181$). *Odoribacter* decreased significantly from baseline compared to 24 h of fermentation, decreasing from 0.54% at baseline to 0.18% after 24 h of fermentation ($p = 0.002$). *Phascolarctobacterium* increased significantly from 0.51% at baseline to 2.31% after 24 h ($p = 0.0011$). The 19 most abundant genera of bacteria composed >99% of all sequences analyzed (Figure 4-2), which we identified in all 6 fecal donors.

Comparing ratios of Bacteroidetes:Firmucutes, each individual's fecal microbiota responded much differently to PHGG after 12 h and 24 h post-exposure (Figure 4-3).

The average percent reads of Firmicutes at baseline was 44.87% of total reads, although this varied greatly from donor to donor, from as low as 29% of total reads, to as high as 47%. After 12 h of fermentation, some donors' microbiota populations of Firmicutes increased, while some decreased as much as 10% and some were unaffected. Firmicutes and Bacteroidetes population fluctuations were primarily specific to each donor, with some overall similar trends.

Differences in analyzed microbiota were analyzed between donors and across reference fermentation time points. The Unweighted Unifrac beta diversity PCoA plot, measuring dissimilarity between all sequence reads based their generated 99% OTU composition, showed that across all measured time points that samples were most similar to each donor, and not their time of analysis (Figure 4-4). Donors 1-3 (ages 31-68) were also more similar to one another than donors 4-6 (ages 21-24).

Discussion

The increase of *Parabacteroides* was the most significant increase among all identified taxa, increasing from 3.48% of sequence reads at baseline to 10.62% of sequence reads after 24 h of fermentation. In a clinical study, oral administration of *P. distasonis* has been shown to reduce severity of intestinal inflammation in induced acute and chronic colitis in murine models due to the modulation of both immunity and microbiota factors.[212] *Parabacteroides* has been identified as one of the most abundant genera of bacteria in the human gastrointestinal tract.[213] *Parabacteroides* has also been shown to be

present in higher concentrations in healthy controls compared to patients with IBS or ulcerative colitis, and it has been speculated that this genera of bacteria may play an influential role in the pathogenesis of both diseases.[214] *Parabacteroides* stimulation has been considered to have a prebiotic effect due to their preference to perform saccharolytic activity instead of proteolytic activity.[170,215]

PHGG has been extensively reviewed for the treatment of IBS.[216,217] Briefly, PHGG has been shown to offer prebiotic activity due to its increase in colonic SCFA production and stimulation of *Lactobacillus* and *Bifidobacterium* [218,219] in two studies utilizing culture-dependent and florescent in situ hybridization (FISH) methods. Baseline *Bifidobacterium* and *Lactobacillus* sequence reads were less than 0.01% of all sequence reads for each respective genus in all six donors. Low bifidobacteria and lactobacilli counts could be due to a wide variety of influential factors (age, stress, environmental components)[106–110] or potentially due to the age of fecal donors.[110] Other studies have found bifidobacteria to be found in very low quantities, if found at all.[121,122] Metagenomic 16S rRNA studies have often found under representation of bifidobacteria due to potential biases in PCR primers and amplification, and have found potential solutions using *cpn60*-based methods.[220] Because of the low concentrations of these genera of bacteria in most populations, it is imperative to analyze other abundant taxa of bacteria using next-generation sequencing methods. PHGG fermentation often doesn't support growth of these genera of bacteria in *in vitro* models because it is the degraded products of PHGG that support growth of these bacteria, which aren't formed in *in vitro* models.[219] PHGG is

enzymatically broken down into low molecular weight galactomannan, which is the primary carbon source for intestinal bacteria.[221–223] Galactomannan formation from PHGG consists of a mannose backbone with galactose side groups (mannose:galactose ~ 2:1).[224]

Odoribacter, Butyricimonas, Faecalibacterium, Lachnospira, Turicibacter, Bilophila and *Akkermansia* populations all significantly decreased after 24 h of exposure to PHGG ($p<0.05$). *Parabacteroides* and *Phascolarctobacterium* populations significantly increased after exposure to PHGG ($p<0.05$). These shifts in targeted genera allowed for a significant change in the ratio of Bacteroidetes:Firmicutes. The overall abundance of Firmicutes decreased from 44.87% of sequence reads at baseline to 40.43% of sequence reads after 24 h ($p = 0.0527$). Bacteroidetes increased from 44.89% of sequence reads at baseline to 50.29% of sequence reads after 24 h ($p = 0.0008$).

Various mouse models have shown lean mice to have increased ratios of Bacteroidetes:Firmicutes compared to obese mice.[225,226] In human intervention studies, similar increased ratios of Bacteroidetes:Firmicutes have been seen following weight loss,[227] although have been contradicted elsewhere.[228] A recent study has also shown increased proportions of Bacteroidetes:Firmicutes ratios with fiber supplementation, independent of caloric restriction, which was associated with total fiber intake and not BMI.[229] PHGG, along with many other types of dietary fiber, may offer feasible ways to

increase Bacteroidetes in healthy individuals, although the precise function of these bacteria still remains largely unclear.

In conclusion, PHGG offers similar bacterial stimulation as other dietary fibers, most notably similar in the stimulation of *Parabacteroides* and Bacteroidetes. PHGG is a fermentable, versatile fiber that can be used in many applications as a way to help consumers bridge the gap between recommended intake and actual fiber intake, especially for consumers with IBS or related issues, as PHGG supplementation has been shown to alleviate IBS symptoms. PHGG offers stimulation of beneficial bacteria and produces significant amounts of SCFA within 24 h of exposure to microbiota, thus displaying effective prebiotic properties, and potentially therapeutic effects.

Table 4-1. Demographic characteristics of six fecal donors.

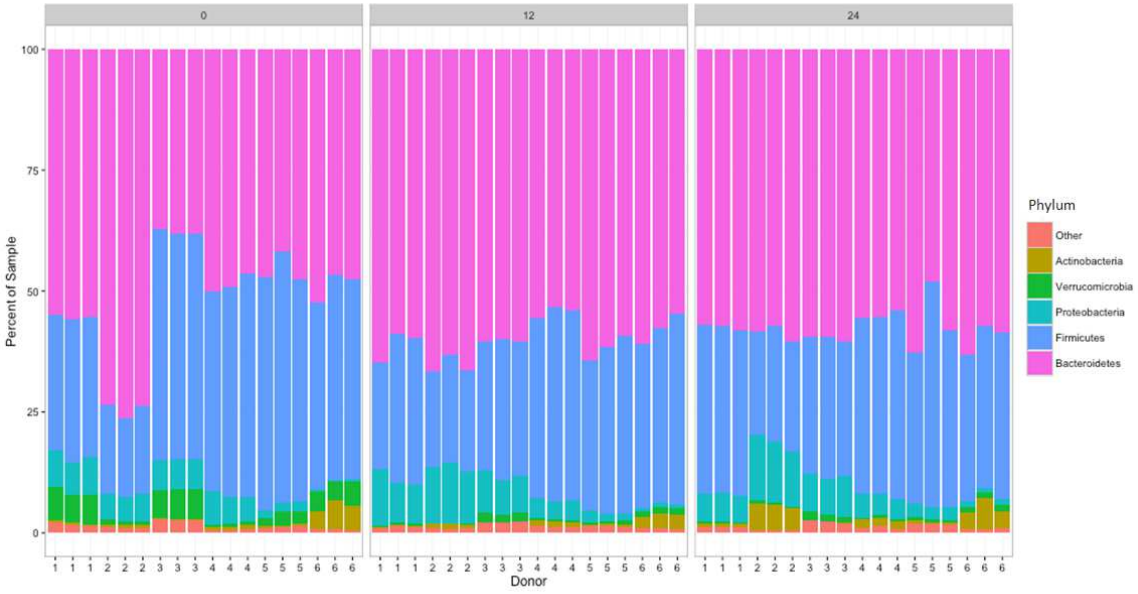
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Age	31	68	60	24	22	21
Sex	Male	Male	Female	Male	Male	Male
BMI	23.7	33.6	19.5	26.3	24.7	23.0

Table 4-2. Combined changes across 24 h of fermentation for identified abundant taxa. Samples were analyzed between differentially represented OTUs for significant overall changes, not between any two time points.

Targeted Taxa (Phlyum and Genus)	**Baseline**	**12 h**	**24 h**	***p*-value**
Actinobacteria	0.97	0.79	1.77	0.1294
Collinsella	0.78	0.68	1.64	0.0986
Adlercreutzia	0.03	0.02	0.02	0.6104
Bacteroidetes	45.89	52.32	50.29	0.0008
Bacteroides	27.12	36.13	33.05	0.1455
Parabacteroides	3.48	10.18	10.62	0.0181
Prevotella	0.83	0.30	0.18	0.6385
Odoribacter	0.53	0.14	0.18	0.0002
Paraprevotella	0.21	0.04	0.01	0.9877
YRC22	0.18	0.03	0.02	0.6477
Butyricimonas	0.13	0.03	0.04	0.0323
Firmicutes	44.87	39.33	40.43	0.0527
Blautia	2.48	1.34	1.32	0.1763
Faecalibacterium	1.59	1.10	0.93	0.0043
Ruminococcus	1.52	1.78	2.12	0.9829
Coprococcus	0.77	0.78	0.55	0.2631
Oscillospira	0.74	0.57	0.63	0.2303
Lachnospira	0.55	0.08	0.09	<0.0001
Phascolarctobacterium	0.51	2.32	2.31	0.0011
Dorea	0.27	0.13	0.27	0.0535
Streptococcus	0.19	0.06	0.07	0.0590
Dialister	0.18	0.14	0.16	0.5368
Clostridium	0.16	0.06	0.06	0.1235
Eubacterium	0.13	0.45	0.68	0.2619
Veillonella	0.12	0.07	0.08	0.6247
Ruminococcus	0.11	0.08	0.11	0.4163
Catenibacterium	0.08	0.17	0.16	0.5981
Roseburia	0.08	0.12	0.06	0.0906
Turicibacter	0.08	0.01	0.02	0.0239
SMB53	0.07	0.02	0.03	0.1632
Anaerostipes	0.06	0.75	0.94	0.9189
Proteobacteria	4.09	5.64	5.62	0.5850
Sutterella	2.36	3.97	3.60	0.6508

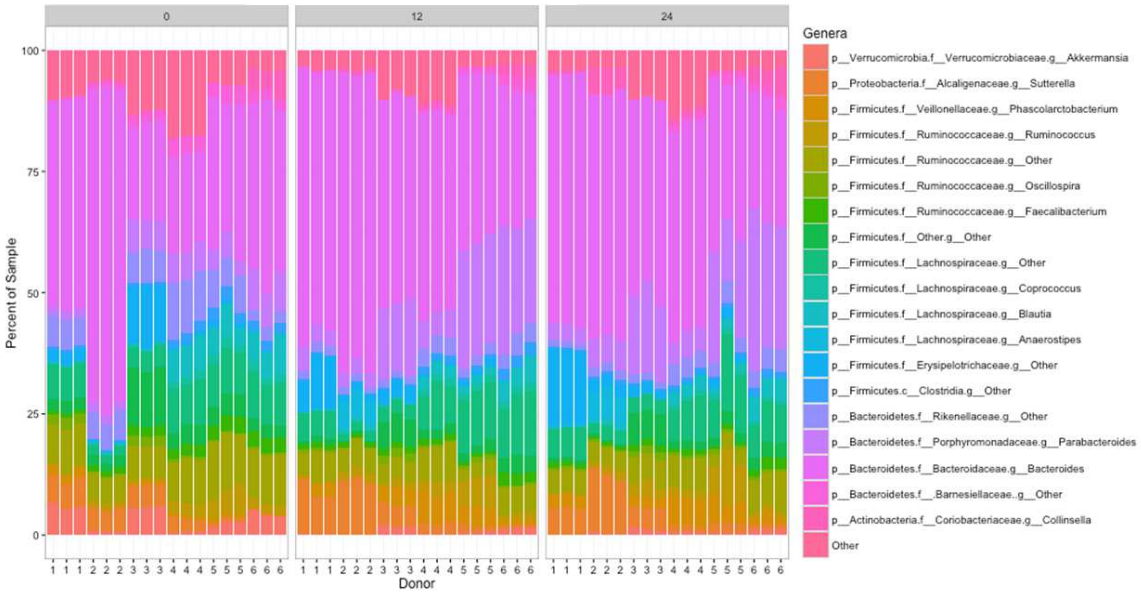
Bilophila	0.14	0.05	0.08	<0.0001
Haemophilus	0.10	0.05	0.03	0.0744
Oxalobacter	0.06	0.009	0.01	0.2984
Verrucomicrobia	0.51	0.90	0.81	<0.0001
Akkermansia	2.52	0.58	0.56	<0.0001

Figure 4-1. Identified abundant phyla based on percent of sequence reads at 0, 12 and 24 h of fermentation of PHGG for six fecal donors. Specified genera consisted of >99% of all sample reads for each individual donor. Technical replicates listed for each fecal donor at each specified time of analysis.



*PHGG (partially hydrolyzed guar gum)

Figure 4-2. Identified abundant genera at 0, 12 and 24 h of fermentation of PHGG for six fecal donors. Specified genera consisted of >99% of all sample reads for each individual donor. Technical replicates listed for each fecal donor.



*PHGG (partially hydrolyzed guar gum)

Figure 4-3. Fluctuations in ratios of Bacteroidetes:Firmicutes at 0, 12 and 24 h of analysis based on percentage of total sequence reads for six fecal donors analyzed. Red (0 h), green (12 h) and blue (24 h) indicate length of sample fermentation in vitro.

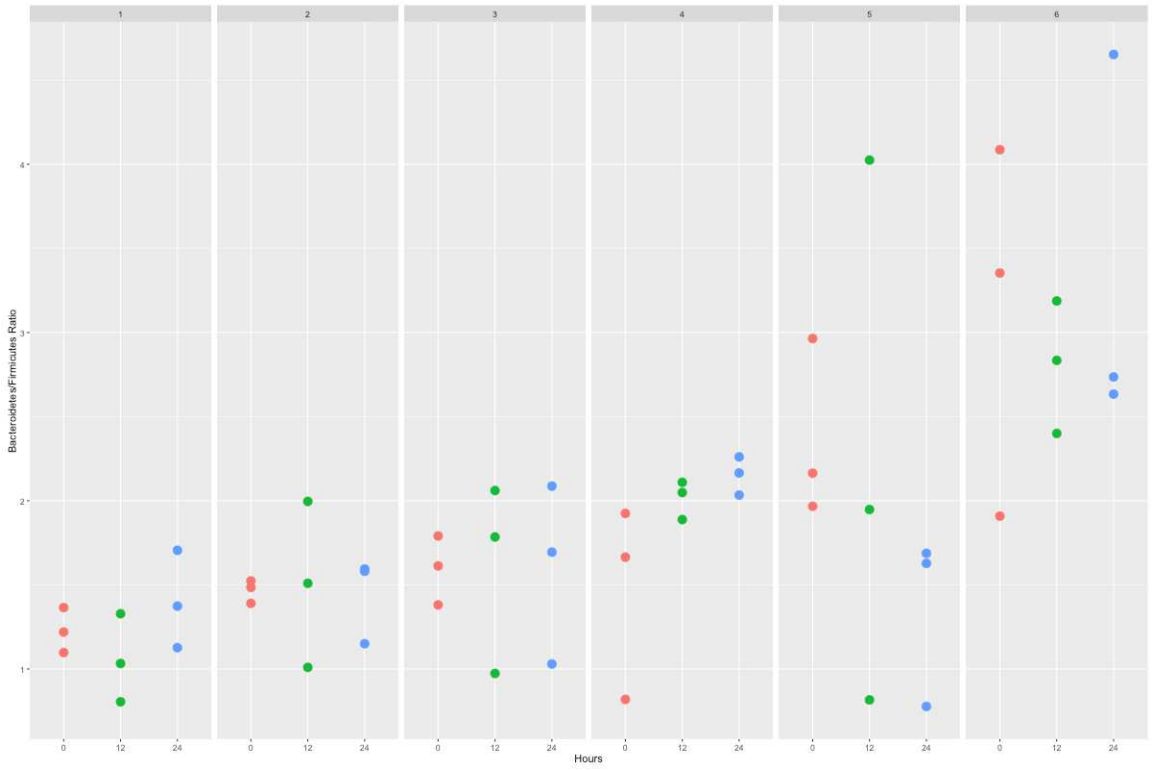
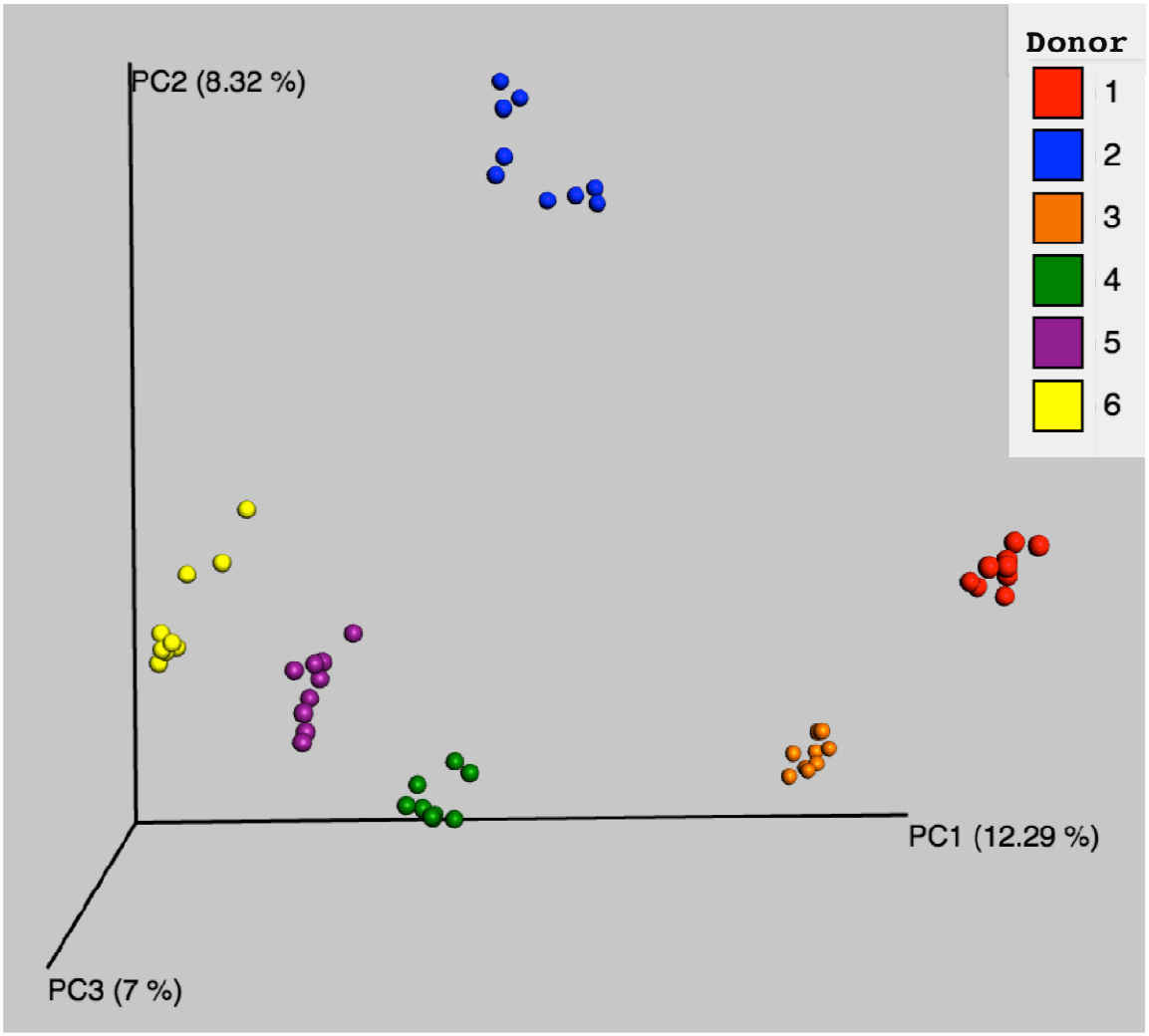


Figure 4-4. Principal coordinates analysis (PCoA) of analyzed bacterial composition of donor samples, including 0, 12 and 24 h samples for all donors. Identifications displayed as Unweighted Unifrac distances among all samples for respective donors displayed based on their generated 99% OTU composition.



CHAPTER FIVE

Comparing The Prebiotic Effects of Five Common Dietary Fibers.

Executive Summary

Prebiotic dietary fiber supplements are commonly consumed to help meet fiber recommendations and improve gastrointestinal health. Stimulation of beneficial bacteria varies greatly between individuals and between products. Prebiotic dietary fibers also produce short-chain fatty acids, molecules beneficial to host health. The objective of this research project was to compare potential prebiotic effects between six commonly consumed fibers using an *in vitro* fermentation system. The primary objective was to measure changes in identified fecal microbiota, with secondary objectives measuring total gas production and formation of common short-chain fatty acids. Fecal donations were collected from 3 healthy volunteers consuming non-specific Western diets, free of antibiotic treatment and supplements. Fibers analyzed included: pure beta-glucan, Oatwell (a commercially available oat-bran containing 22% high molecular weight oat β-glucan, xylooligosaccharides (XOS), WholeFiber (a dried Chicory Root containing inulin, pectin, and hemi/celluloses), and pure inulin. Oatwell stimulated the highest production of propionate at 12 h (4.76 μmol/mL) compared to inulin, WholeFiber and XOS samples ($p<0.03$). Its effect were similar to those of the pure beta-glucan samples. Oatwell and pure beta glucan samples promoted the highest mean propionate production at 24 h. XOS resulted in a significant increase in the genus *Bifidobacterium* after 24 h of fermentation (0 h: 0.67 OTUs; 24 h: 5.22 OTUs; $p = 0.038$). Inulin and WholeFiber

increased the beneficial genus *Collinsella*, consistent with findings in clinical studies. All studied prebiotic dietary fibers studied promoted the formation of beneficial markers due to fermentation of each specific compound. All compounds provided different, significant fermentation patterns, and all provided beneficial effects that would promote host health *in vivo*.

Introduction

Prebiotic definitions vary among different scientific and political arenas across the world.[230] Depending on the local definition, nearly all prebiotics can be classified as dietary fiber, but not all fibers are considered prebiotics.[16] The most recent definition describes a prebiotic as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health".[15] Functional characteristics of prebiotics include the ability to: resist the low pH of the stomach, resist hydrolysis by mammalian enzymes, resist absorption in the upper gastrointestinal tract, the ability to be fermented by intestinal microbiota and selectively stimulate the growth and/or activity of intestinal bacteria associated with host health and overall well-being.[13,14] Inulin, beta-glucans, and xylooligosaccharides (XOS) all provide health benefits to consumers that are related to the fermentation of these compounds in the distal gastrointestinal tract, and are also considered functional fibers with many other benefits.[6] As the definition of "prebiotic" broadens to include the overall impact from the metabolism from these compounds, the

category of prebiotics will expand.[231] The importance of displaying direct health benefits due to bacterial fermentation is still the driving mechanism for all prebiotics.

As our awareness and understanding of the importance of the gut microbiome and gut microbiota increases, it's imperative for consumers to understand the key differences between different forms of prebiotics, and where they can be found in various foods and food products. XOS is an emerging prebiotic with well-displayed, consistent health benefits[232] and is composed of sugar oligomers composed of xylose units,[233] found naturally in fruits, vegetables, milk, honey and bamboo shoots. XOS is commonly produced from xylan containing lignocellulosic materials through various chemical methods, direct enzymatic hydrolysis, or a combination of both treatments.[234–238] Inulin is a heterogeneous blend of fructose polymers (DP<10)[239] which occurs naturally in thousands of plant species, including wheat, onion, bananas, garlic and chicory.[240] Beta-glucan is a polysaccharide composed of D-glucose monomers with beta-glycosidic linkages, present in either linear chains in grains, such as oat and barley (up to 7%), or in branched structures in fungi, yeast and certain bacteria.[241] These prebiotics, or prebiotic mixtures, each provide a unique carbon source for selective stimulation of different bacterial taxa and are important microbiota-shaping compounds.

Because no analytical method currently exists to measure the prebiotic capacity of foods in terms of their influence on gastrointestinal taxa, this field relies heavily on fecalbiotics (living or once living fecal microbial populations) to quantify the effects of these

compounds. *In vitro* fermentation models allow for quantitative analysis of specific materials and are semi-representative models of colonic fermentation.[242] Although not a complete substitute for human studies, when paired with *in vivo* models, *in vitro* analysis can be an accurate systematic approach to analyze different parameters and end points in colonic fermentation.[243]

The objective of this project was to compare currently available prebiotics by their ability to change specific taxa as well as compare differences in the production of gas and common short chain fatty acids (SCFA) between these products. Inulin, XOS and beta-glucan based products were chosen for this experiment because they are established and emerging prebiotics that are commonly consumed, and offer well-demonstrated health benefits to their consumers.

Methods

Prebiotic Dietary Fibers Analyzed

The established and emerging prebiotic dietary fibers with well-demonstrated health benefits included in this study are all shown in Table 5-1.

Fecal Collection & Donor Information

Fecal samples were collected from three healthy volunteers (2 males, 1 female) under anaerobic conditions from individuals (ages 22-28) consuming non-specific Western diets, free of any antibiotic treatments in the last year, non-smokers, not affected by any

known GI diseases and not consuming any supplements (Table 5-2). Fecal samples were anaerobically collected within 5 minutes of the start of the fermentation, and homogenized immediately upon collection. All data and samples collected were done in accordance with University of Minnesota policies and procedures.

Fermentation

Fiber samples (0.5 g) were hydrated in 40 mL of prepared sterile tricase peptone fermentation media in 100 mL serum bottles, capped, and incubated for 12 hours at 4°C. Following incubation, serum bottles were transferred to a circulating water bath at 37°C and allowed to incubate for 2 hours. Post-collection, fecal samples were mixed using a 6:1 ratio of phosphate buffer solution to fecal sample. After mixing, obtained fecal slurry was combined with prepared reducing solution (2.52 g cysteine hydrochloride, 16 mL 1N NaOH, 2.56 g sodium sulfide nonanhydride, 380 mL DD H_2O) at a 2:15 ratio. 10 mL of the prepared fecal inoculum was added to each of the serum bottles, 0.8 mL Oxyrase® was added, flushed with CO_2, sealed, and then immediately placed in a 37°C circulating water bath. Samples were prepared in triplicate and analyzed at 0, 12 and 24 h. Upon removal at each time point, total gas volume was measured (by syringe difference analysis). Then samples were divided into aliquots for analysis and 1 mL of copper sulfate (200 g/L) was added to cease fermentation. All samples were immediately frozen and stored at -80°C for further analysis.

SCFA Analysis

SCFA samples were extracted according to Schneider et al[151] with minor modifications, and analyzed with previously described methods.[244]

DNA Extractions

Fecal bacteria DNA from the *in vitro* system were extracted using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) following the provided operating instruction, including bead beating for 20 min.

Primary/Secondary Amplification

The V1-V3 region of the 16S rRNA was amplified using a two-step PCR protocol. The primary amplification was done using an ABI7900 qPCR machine. The following recipe was used: 3 µl template DNA, 0.48 µl nuclease-free water, 1.2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 µl DMSO (Fisher Scientific, Waltham, MA), 0.12 µl ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl 1000x SYBR Green, 0.12 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (V1_27F and V3_V34R), as well as adapter tails for adding indices and Illumina flow cell adapters in a secondary amplification. The following primers were used (16S-specific sequences in

bold): Meta_V1_27F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCMTGGCTCAG)** and Meta_V3_534R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG).**

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and indices. The secondary amplification was done on a fixed block BioRad Tetrad PCR machine using the following recipe: 5 µl template DNA, 1 µl nuclease-free water, 2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 µl DMSO (Fisher Scientific, Waltham, MA) 0.2 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The following indexing primers were used (X indicates the positions of the 8 bp indices): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC and Reverse indexing primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG

Normalization and Sequencing

The samples were normalized using a SequalPrep capture-resin bead plate (Life Technologies, Carlsbad, CA) and pooled using equal volume. The final pools were quantified via PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA) and diluted to 2nM. 10 µl of the 2 nM pool was denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% phiX, heat denatured at 96°C for 2 minutes, and sequenced using a MiSeq 600 cycle v3 kit (Illumina, San Diego, CA).

Sequence Processing and Analysis

Generated sequence data was processed and analyzed using QIIME[208]. Fastq sequence data was processed with the University of Minnesota's gopher-pipeline for metagenomics.[209] Sequence data had adapters removed and sliding quality trimming window by Trimmomatic[210]; primers removed and overlapping reads merged by Pandaseq.[211] Within QIIME, chimera checking done by chimera slayer, Open reference OTU picking completed with Usearch61, taxonomic identification using GreenGenes (Version 13.8) reference database, rarefied to 14,393 sequences per sample. Analysis was performed using R (R Development Core Team, 2012).

Statistical Analysis

All statistical analysis was performed using R software (R Development Core Team, 2012). Differences in means were determined using the Kruskal-Wallis ANOVA test, testing the null hypothesis that the location parameter of the groups of abundancies for a given OTU is the same. Multiple comparisons were corrected using the FDR procedure.

For gas and SCFA data, ANOVA with Tukey HSD was used to compare means. Significance was set for p-values < 0.05 for all statistical tests. Replicates (3) for donors (3) within treatments groups were separate *in vitro* fermentations, resulting in a n=9 for each treatment group, at each time point of analysis.

Consent

Voluntary informed consent was obtained from all fecal donors prior to this study according to University of Minnesota policies and procedures.

Results

Gas Production

At 12 h, the OatWell and the pure beta-glucan samples produced similar amounts of total gas (Figure 5-1). The XOS samples produced significantly more gas than the pure beta glucan samples ($p < 0.01$) or the OatWell samples ($p < 0.01$). The WholeFiber and pure inulin samples produced similar amounts of total gas ($p = 0.102$), and the total gas production for both of these prebiotic dietary fibers was significantly higher than for the XOS samples, ($p < 0.01$ and $p = 0.045$), respectively. At 24 h, the OatWell samples had the lowest gas production (46.2 mL) and were similar to the pure beta-glucan samples (63.7 mL; $p = 0.498$). The 24 h XOS samples (74.0 mL) were also similar to the beta-glucan samples ($p = 0.926$). However, the 24 h WholeFiber (109.6 mL) and pure inulin (107.1 mL) samples produced significantly more gas than XOS, beta-glucan and Oatwell samples ($p < 0.01$).

SCFA Production

For all SCFA analysis, analyses at 12 and 24 h were for production only, from baseline corrected samples. Acetate, propionate and butyrate production is shown as μmol/mL of fermentation media.

Acetate production at 12 h was similar for the Oatwell, WholeFiber and beta-glucan samples (Figure 5-2). The XOS samples produced significantly more acetate at 12 h than the Oatwell, WholeFiber or beta-glucan samples ($p<0.05$). The inulin samples had similar amounts of acetate compared to the WholeFiber and XOS samples, and significantly more than the Oatwell ($p=0.024$) and beta-glucan ($p=0.013$) samples at 12 h. After 24 h of fermentation (Figure 5-3), the inulin samples contained less acetate than the XOS samples ($p=0.038$), while the Oatwell, WholeFiber and beta-glucan samples were similar to both the XOS and inulin samples.

Propionate production at 12 h of fermentation was highest for the OatWell samples (4.76 μmol/mL) and was significantly greater than the WholeFiber ($p=0.029$), XOS ($p=0.005$) and inulin samples ($p=0.004$), and similar to the beta-glucan samples (Figure 5-4). At 24 h of fermentation, the Oatwell samples had the highest mean production 5.05 μmol/mL, which was significantly greater than the XOS samples (2.58 μmol/mL; $p=0.021$), and similar to WholeFiber, inulin and beta-glucan samples (Figure 5-5).

Butyrate production after 12 h of fermentation ranged from 7.30 μmol/mL for the beta-glucan samples to 16.76 μmol/mL for the inulin samples (Figure 5-6). The inulin samples had the highest average production, and were similar to the XOS (16.38 μmol/mL) and WholeFiber samples (12.89 μmol/mL). The XOS samples were significantly higher than the Oatwell (p=0.035) and beta-glucan samples (p=0.014). At 24 h of fermentation, all five prebiotic dietary fibers were statistically similar to one another, ranging from 7.93 – 14.08 μmol/mL due to a wide ranges in response differences between the three fecal donors used in this study (Figure 5-7).

Microbiota Analysis

DNA extracts from *in vitro* samples were sequenced using the MiSeq Illumina platforms, generating a total of 31,591,899 sequence reads. Sequencing parameters identified reads belonging to 11 bacterial phyla, 61 families and 97 genera.

For all three donors, the phyla Bacteroidetes and Firmicutes represented >80% of all sequence reads (Figure 5-8) across 24 h of fermentation. At the family level, 13 families represented 85% of all sequence reads (Figure 5-9), while 11 genera represented >75% of all sequence reads (Figure 5-10). Six metrics measuring α-diversity for all donors showed various degrees of grouping by donors (Figure 5-11), and by treatment group (Figure 5-12). Both Unifrac and Bray-Curtis β-diversity metrics (measuring pairwise dissimilarity between samples), showed similarity among technical replicates of treatment groups for each donor (Figure 5-13) as well as for all treatment groups for each respective donor (Figure 5-14).

After 24 h of fermentation, the Oatwell samples significantly decreased the population of the SMB53 genus of Clostridiaceae (0 h: 9.11 OTUs; 24 h: 2.11 OTUs; $p = 0.008$), *Lachnospira* and *Faecalibacterium* (0 h: 26.56 OTUs; 24 h: 4.44 OTUs; $p = 0.008$ and 0 h 136.44 OTUs; 24 h: 66 OTUs; $p = 0.022$, respectively) (Table 5-3). *In vivo* studies with inulin, short-chain fructooligosaccharides and resistant starch supplementation have all resulted in decreases in the SMB53 genus.[245,246] No genera analyzed showed significant increases in 24 h for the Oatwell samples measured for the three fecal donors in this study. The WholeFiber samples (Table 5-4) significantly increased the genus *Collinsella* at 24 h compared to 0 h (0 h: 68 OTUs; 24 h: 299.78 OTUs; $p = 0.011$). *Bifidobacterium* populations were only significantly increased at 24 h compared to 0 h for the XOS samples (0 h: 0.67 OTUs; 24 h: 5.22 OTUs; $p = 0.038$), while the same samples showed a significant decrease in *Lachnospira* and *Faecalibacterium* ($p = 0.038$ and $p = 0.03$) (Table 5-5). The inulin samples (Table 5-6) increased *Collinsella* (0 h: 55.11 OTUs; 24 h: 291.44 OTUs; $p = 0.016$). The pure beta-glucan samples significantly decreased *Lachnospira* and *Faecalibacterium* ($p = 0.008$).

Discussion

The aim of this study was to investigate the beneficial effects of commonly consumed prebiotic dietary fibers, including their ability to influence the growth of identified bacterial populations, form beneficial SCFAs, and the amount of gas they produce due to fermentation. Total gas production due to fiber fermentation depends on a wide range of

factors. The inulin samples and the WholeFiber samples (mixture of dried chicory root inulin, pectin and hemicellulose) resulted in the highest gas production at both 12 and 24 h. These results are consistent with results from both clinical feeding studies and other *in vitro* experiments, in which fermentation of inulin products resulted in high amounts of gas production, sometimes resulting in mild negative GI symptoms, depending on the dosage.[247,248] Similar *in vitro* studies have found inulin to be much more fermentable than beta-glucan products, for both barley and oat-derived beta-glucans.[249] XOS fermentation results in less gas production than the inulin products, and more gas than beta-glucan products. Because of these findings, previous studies based on digestive tolerance and parameters have established a tolerated daily dosage of approximately 12 g/d.[250]

SCFA production due to the fermentation of prebiotic dietary fibers promotes many beneficial health outcomes to the host. SCFA production may contribute to up to 10% of the host's daily metabolizable energy, with production of total SCFAs usually between 100-200 mM/d, but is highly dependent on the donor and availability of substrates for fermentation.[177,185] At 12 h of fermentation, the OatWell and beta-glucan samples had significantly higher concentrations of propionate, and the highest mean concentration at 24 h compared to the other prebiotic dietary fibers analyzed. Similar *in vitro* studies with beta-glucan based products have also shown similar preference for these products to result in propionate formation.[249] Although no mechanism has been identified, and studies show conflicting results,[251] elevated serum propionate concentrations have been shown to have a hypocholesterolaemic effect.[252] Propionate may also play an influential

role in satiety, although mechanisms still remain unclear.[253,254] Cholesterol-lowering properties of beta-glucans may be limited to effects from the upper-GI, although many propionate-producing bacteria have a preference for fermenting various types of beta-glucans (*Bacteroides, Prevotella, Clostridium*) based on the presence of genes responsible for endo-β-glucanase enzyme production.[255]

Microbial diversity among fecal donors complicates the identification of trends among the five treatment groups (Figures 5-15 and Figure 5-16). In terms of taxonomic shift, the inulin-based products were fermented nearly identically by all three fecal donors. Both pure inulin and WholeFiber promoted the growth of *Collinsella* comparing the 24 h samples to 0 h samples. Inulin-type fructans have been shown in clinical studies to promote substantial growth of *Collinsella*, paralleled with increased urinary hippurate levels.[256] Hippurate is a metabolite derived from various fermentation processes in the gut that has been found in decreased concentrations in obese individuals compared to lean individuals, and also between diabetics and non-diabetics.[257–259] The genus *Collinsella* has been found in lower concentrations in individuals with IBD compared to healthy controls,[260] while *Collinsella aerofaciens* has been associated with low risk of colorectal cancer.[261] Increases in *Collinsella* and increased urinary hippurate levels are considered a beneficial effect of inulin consumption due to its prebiotic capacity.[256]
A significant increase in the genus *Bifidobacterium* was observed only with the XOS treatment. Increases in *Bifidobacterium* have been heavily studied and reviewed, and are considered a beneficial effect due to their correlation with many positive health

outcomes.[230] *Bifidobacteria* reside naturally in the gastrointestinal tract of healthy human adults and have a strong affinity to ferment oligosaccharides, making them a common marker for prebiotic capacity. *Bifidobacterium* is a unique genus of bacteria in that no gas is formed as an end product of metabolism.[126] Like *Lactobacillus*, these bacteria are saccharolytic, often considered a beneficial trait.[102] *Bifidobacteria* also do not produce any known carcinogenic substances *in vivo*. *Bifidobacteria* concentrations have been negatively associated with obesity and weight gain.[127–130] Increases in *Bifidobacteria* have also been correlated with a decrease in blood lipopolysaccharides (LPS), inflammatory reagents that play a role in the development of inflammatory metabolic disorders and conditions, and are primarily found in gram-negative bacteria.[262] LPS induce the activation of Toll-like receptor 4, which leads to inflammation due to release of pro-inflammatory cytokines and chemokines.[263]

In vitro fermentations are semi-representative models of colonic fermentation, but have limitations.[242] *In vivo*, formed gases are continually absorbed and colonic absorption is rapid. Because SCFAs are rapidly absorbed and difficult to measure, *in vitro* models help to understand the kinetics of colonic fermentation. However, *in vitro* models must be paired with similar *in vivo* models to better understand the full mechanisms of action resulting from colonic fermentation of prebiotic dietary fibers.

All five prebiotics measured in this study offer specific health benefits that can be attributed to their fermentation. Depending on their structure, each compound offers a

specific carbon source for fermentation by different bacterial populations, yielding changes in beneficial taxa and production of various amounts of SCFAs and gas *in vitro*. For instance, while OatWell and beta-glucans promoted propionate production, XOS increased concentrations of *Bifodobacterium,* and WholeFiber and pure inulin promoted *Collinsella* growth. Findings in this study are consistent with other *in vitro* studies on similar prebiotic dietary fibers, as well as numerous clinical feeding studies.

Table 5-1. Comparison prebiotic dietary fibers analyzed with *in vitro* fermentation system.

Prebiotic Dietary Fibers	Supplier Information
OatWell (Oatbran containing 28% beta-glucan)	DSM Nutritional Products, Ltd.
Dried Chicory Root (containing: inulin, pectin, hemi/cellulose)	WholeFiber, Inc.
Xylooligosaccharide (XOS)	AIDP, Inc.
Pure Inulin	Cargill, Inc.
Pure Beta-glucan	Megazyme, Inc.

Table 5-2. Demographic characteristics of three fecal donors.

	Donor 1	Donor 2	Donor 3
Age	26	25	22
Sex	Female	Male	Male
BMI	28.1	26.3	23.0

Figure 5-1. Total gas production comparing fermentation differences among five prebiotic dietary fibers for three individuals at 12 h and 24 h post-exposure to fecal microbiota in an *in vitro* fermentation system. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another within each time measurement (lowercase: 12 h; uppercase: 24 h). Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

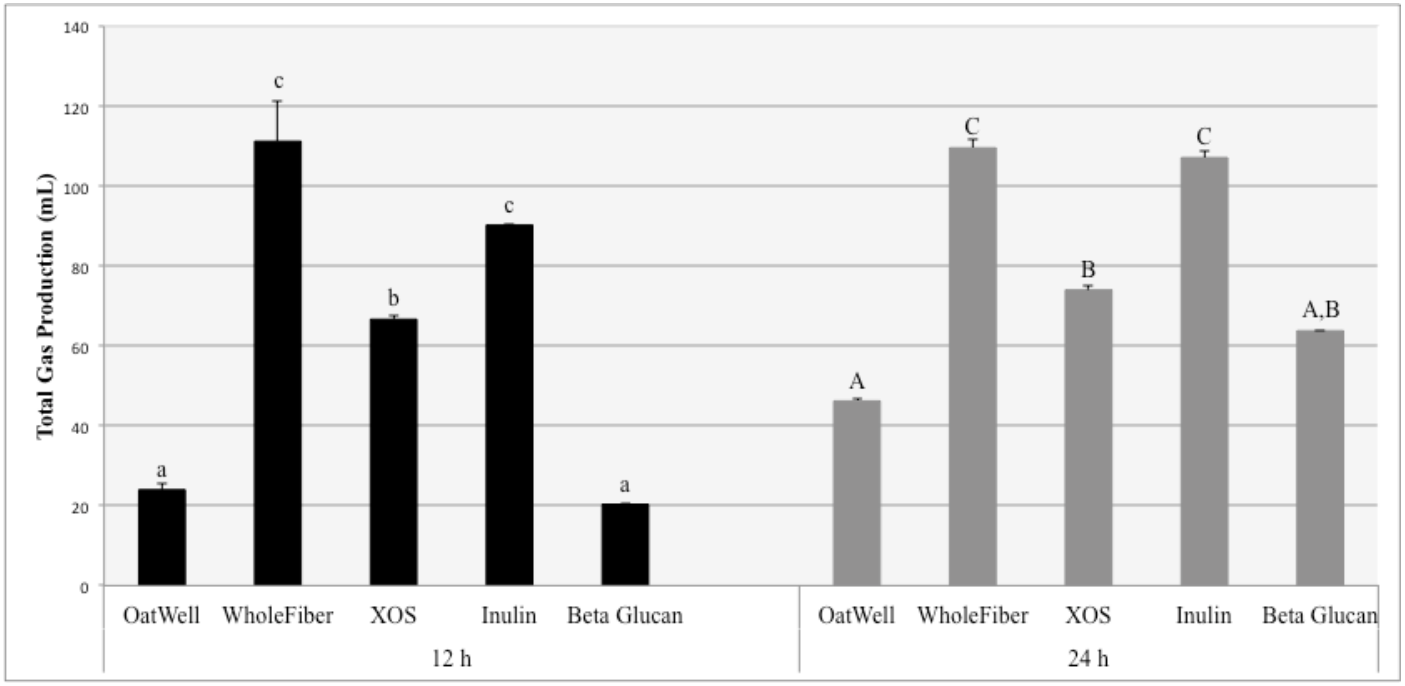


Figure 5-2. Acetate production at 12 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

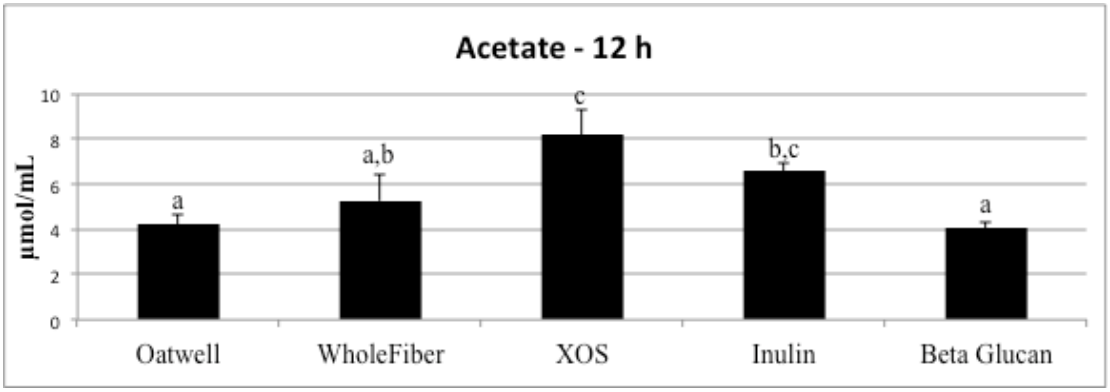


Figure 5-3. Acetate production at 24 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

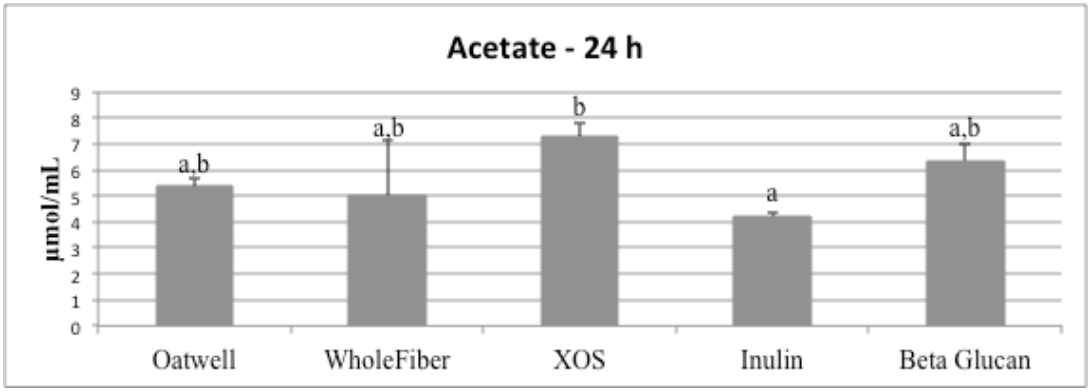


Figure 5-4. Propionate production at 12 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

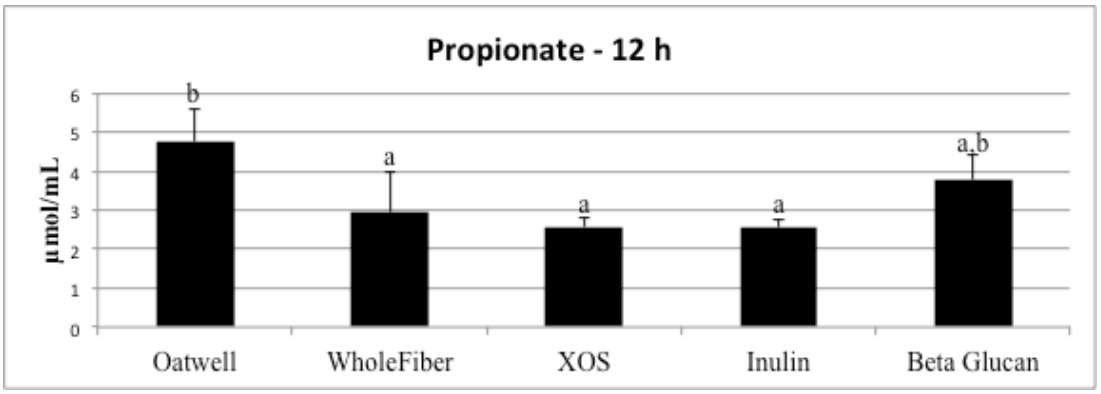


Figure 5-5. Propionate production at 24 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

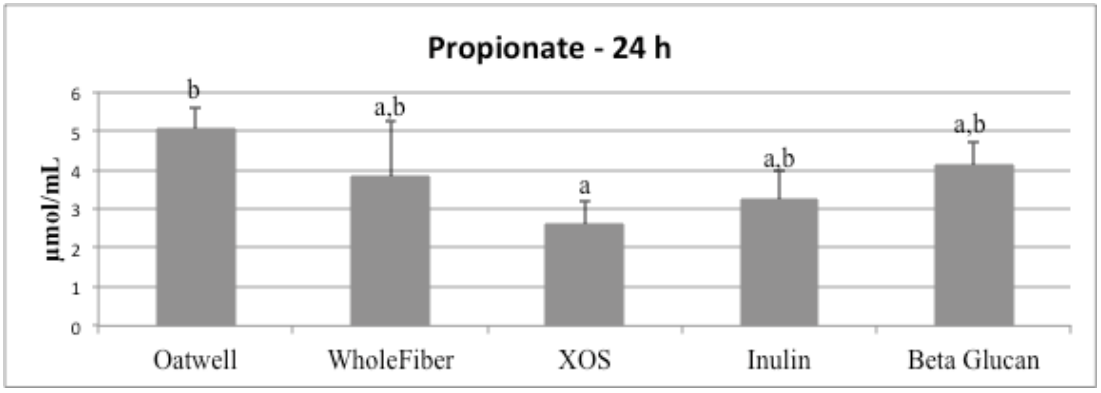


Figure 5-6. Butyrate production at 12 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

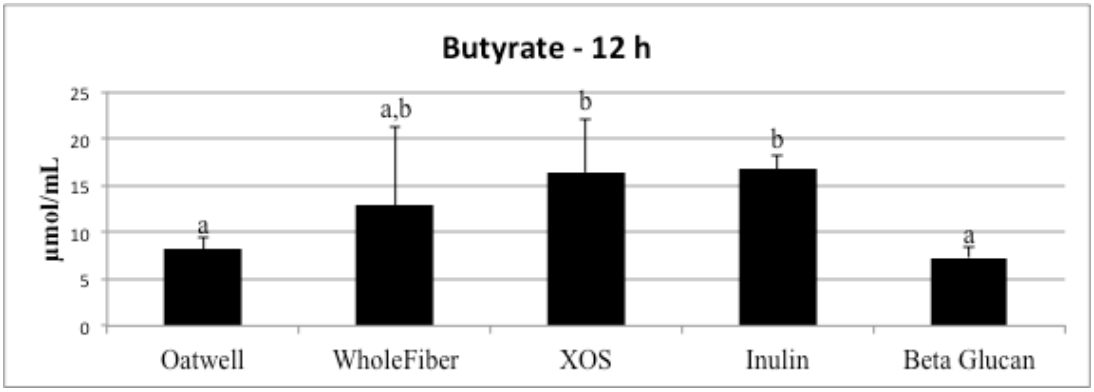


Figure 5-7. Butyrate production at 24 h of fermentation for five prebiotic dietary fibers displayed as μmol/mL of fermentation inoculum. Data displayed are means (3 donors x 3 replicates = 9) for each prebiotic dietary fiber ± SD. Columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p < 0.05$).

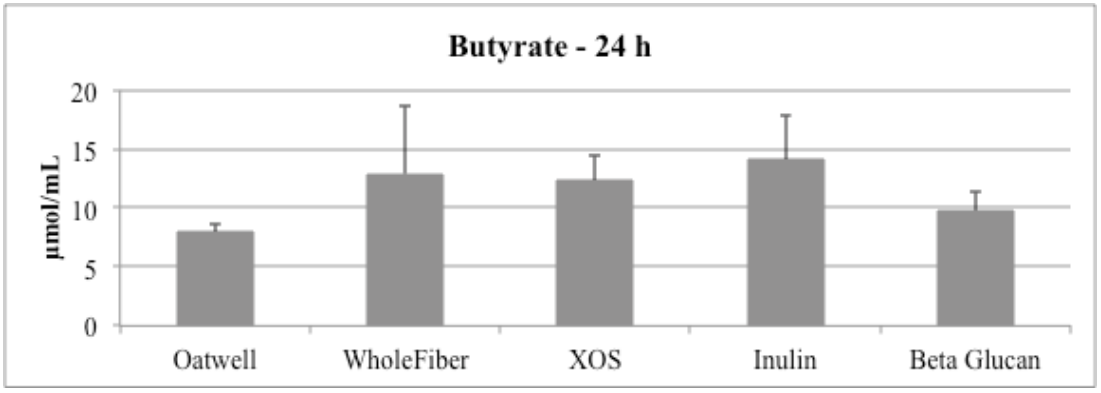


Table 5-3. Combined changes across 24 h of fermentation for Oatwell samples of identified phyla and genera. Replicate samples were pooled among donor at each respective time point (3 donors x 3 replicated = 9). Samples were analyzed between differentially represented OTUs for significant changes after 24 h of fermentation compared to 0 h samples. Data were analyzed using the Kruskal-Wallis ANOVA test, with the FDR multiple comparisons correction.

Phyla & Genera	0 h	24 h	Test-Statistic	P-value
Actinobacteria				
Bifidobacterium	1.22	0.89	0.63	0.660
Adlercreutzia	1.44	3.00	1.37	0.470
Collinsella	48.44	140.56	6.33	0.089
Bacteroidetes				
Alistipes	2.56	1.33	0.55	0.674
Parabacteroides	135.00	155.89	0.05	0.952
Bacteroides	755.67	865.00	3.95	0.192
Firmicutes				
Eubacterium	0.44	0.56	0.20	0.817
Veillonella	1.11	1.22	0.01	0.980
Dorea	2.33	3.56	1.08	0.516
Acidaminococcus	3.22	10.44	0.22	0.817
Clostridium	7.67	8.33	0.33	0.769
Anaerostipes	8.11	6.00	0.52	0.674
Turicibacter	8.67	1.22	2.54	0.286
SMB53	9.11	2.11	12.83	**0.008**
Ruminococcus	11.22	23.22	3.13	0.263
Lactococcus	11.67	10.67	0.00	0.980
Streptococcus	15.22	8.11	1.15	0.511
Roseburia	20.22	22.33	0.01	0.980
Oscillospira	21.78	36.67	4.90	0.121
Lachnospira	26.56	4.44	12.94	**0.008**
Phascolarctobacterium	27.78	173.33	3.03	0.263
Dialister	39.56	43.00	0.88	0.560
Blautia	41.89	53.11	1.32	0.470
Coprococcus	49.89	39.00	1.76	0.396
Ruminococcus	61.33	40.67	2.39	0.289

Faecalibacterium	136.44	66.00	9.58	**0.022**
Proteobacteria				
Escherichia	0.44	1.44	2.13	0.325
Haemophilus	10.22	0.67	2.49	0.286
Sutterella	10.78	14.44	0.00	0.980
Bilophila	13.67	14.78	0.28	0.788
Verrucomicrobia				
Akkermansia	5.00	12.00	0.01	0.980

Table 5-4. Combined changes across 24 h of fermentation for WholeFiber samples of identified phyla and genera. Replicate samples were pooled among donor at each respective time point (3 donors x 3 replicated = 9). Samples were analyzed between differentially represented OTUs for significant changes after 24 h of fermentation compared to 0 h samples. Data were analyzed using the Kruskal-Wallis ANOVA test, with the FDR multiple comparisons correction.

Phyla & Genera	0 h	24 h	Test-Statistic	P-value
Actinobacteria				
Adlercreutzia	0.89	3.89	3.61	0.239
Bifidobacterium	1.11	1.11	0.56	0.785
Collinsella	68.00	299.78	12.17	**0.011**
Bacteroidetes				
Alistipes	1.11	0.56	0.31	0.894
Parabacteroides	131.44	142.00	0.07	0.913
Bacteroides	743.56	776.56	0.56	0.785
Firmicutes				
Eubacterium	1.11	0.78	0.50	0.799
Veillonella	1.22	1.00	0.01	0.960
Dorea	2.00	5.00	0.66	0.785
Acidaminococcus	2.67	11.33	0.22	0.894
SMB53	5.67	4.00	3.58	0.239
Clostridium	7.33	13.22	0.10	0.896
Anaerostipes	10.22	1.22	3.60	0.239
Ruminococcus	10.89	19.67	0.03	0.943
Streptococcus	12.67	8.78	0.57	0.785
Turicibacter	14.22	2.44	0.01	0.960
Lachnospira	14.78	72.00	4.13	0.237
Oscillospira	17.22	14.89	1.13	0.647
Lactococcus	20.44	9.22	0.12	0.896
Phascolarctobacterium	24.67	60.44	1.82	0.501
Dialister	26.11	58.22	0.03	0.943
Roseburia	28.56	6.00	0.95	0.674
Blautia	32.44	49.44	5.08	0.156
Coprococcus	45.78	66.44	1.64	0.501
Ruminococcus	54.22	39.33	3.29	0.261

Faecalibacterium	154.89	93.11	7.75	0.080
Proteobacteria				
Escherichia	0.78	1.44	0.00	0.960
Sutterella	4.00	32.44	0.22	0.894
Haemophilus	10.67	0.56	6.72	0.107
Bilophila	10.67	7.67	0.10	0.896
Verrucomicrobia				
Akkermansia	17.00	3.33	1.67	0.501

Table 5-5. Combined changes across 24 h of fermentation for xylooligosaccharide samples of identified phyla and genera. Replicate samples were pooled among donor at each respective time point (3 donors x 3 replicated = 9). Samples were analyzed between differentially represented OTUs for significant changes after 24 h of fermentation compared to 0 h samples. Data were analyzed using the Kruskal-Wallis ANOVA test, with the FDR multiple comparisons correction.

Phyla & Genera	**0 h**	**24 h**	**Test-Statistic**	**P-value**
Actinobacteria				
Bifidobacterium	0.67	5.22	9.11	**0.038**
Adlercreutzia	1.33	1.78	0.08	0.972
Collinsella	58.44	154.00	1.87	0.413
Bacteroidetes				
Alistipes	1.44	0.56	1.85	0.413
Parabacteroides	147.33	133.33	0.02	0.972
Bacteroides	770.89	870.44	4.31	0.189
Firmicutes				
Eubacterium	0.33	1.67	2.75	0.364
Veillonella	0.67	0.00	4.78	0.162
Acidaminococcus	1.33	2.33	0.03	0.972
Dorea	2.11	3.67	1.70	0.423
SMB53	7.33	5.44	1.14	0.558
Anaerostipes	7.44	3.44	1.51	0.447
Turicibacter	8.00	8.56	0.02	0.972
Clostridium	8.44	4.00	6.51	0.087
Ruminococcus	12.78	26.11	6.58	0.087
Streptococcus	14.11	4.67	2.46	0.367
Lachnospira	21.11	5.33	9.37	**0.038**
Oscillospira	21.33	21.78	0.02	0.972
Phascolarctobacterium	23.44	16.33	0.10	0.972
Lactococcus	23.89	21.00	0.00	0.982
Roseburia	28.89	35.33	0.16	0.972
Dialister	33.89	41.56	0.42	0.831
Blautia	39.22	65.00	6.37	0.087
Ruminococcus	45.11	37.33	2.13	0.385
Coprococcus	47.11	48.67	0.78	0.705

Faecalibacterium	148.56	79.56	11.56	**0.030**
Proteobacteria				
Escherichia	0.89	0.44	0.07	0.972
Haemophilus	6.44	3.11	0.03	0.972
Bilophila	17.78	6.22	5.73	0.107
Sutterella	25.78	40.89	0.43	0.831
Verrucomicrobia				
Akkermansia	2.78	5.00	0.34	0.841

Table 5-6. Combined changes across 24 h of fermentation for pure inulin samples of identified phyla and genera. Replicate samples were pooled among donor at each respective time point (3 donors x 3 replicated = 9). Samples were analyzed between differentially represented OTUs for significant changes after 24 h of fermentation compared to 0 h samples. Data were analyzed using the Kruskal-Wallis ANOVA test, with the FDR multiple comparisons correction.

Phyla & Genera	0 h	24 h	Test-Statistic	P-value
Actinobacteria				
Bifidobacterium	1.33	5.44	3.94	0.304
Adlercreutzia	1.33	2.00	0.47	0.845
Collinsella	55.11	291.44	12.79	**0.016**
Bacteroidetes				
Alistipes	1.56	0.89	0.36	0.878
Parabacteroides	147.44	164.78	0.28	0.887
Bacteroides	726.78	644.44	1.64	0.652
Firmicutes				
Veillonella	0.78	0.56	0.03	0.908
Eubacterium	0.89	1.56	0.02	0.908
Dorea	1.78	7.00	1.96	0.640
Acidaminococcus	3.11	18.67	0.22	0.887
SMB53	7.44	9.11	0.00	0.965
Turicibacter	7.78	4.89	1.06	0.652
Clostridium	8.22	7.11	0.51	0.845
Ruminococcus	9.56	34.11	3.61	0.309
Anaerostipes	11.22	4.67	1.17	0.652
Streptococcus	13.00	12.44	0.16	0.887
Lactococcus	19.11	9.67	0.02	0.908
Lachnospira	21.00	4.89	10.85	**0.022**
Phascolarctobacterium	26.22	21.00	0.16	0.887
Oscillospira	26.33	10.11	9.30	**0.034**
Roseburia	26.78	14.11	0.28	0.887
Dialister	32.67	95.11	0.16	0.887
Blautia	38.22	50.22	0.86	0.690
Coprococcus	48.11	60.89	1.88	0.640
Ruminococcus	52.33	43.00	0.10	0.908

Faecalibacterium	148.11	187.33	1.22	0.652
Proteobacteria				
Escherichia	1.00	1.22	0.04	0.908
Haemophilus	9.11	2.67	1.45	0.652
Sutterella	14.00	31.22	0.02	0.908
Bilophila	16.89	7.78	3.31	0.309
Akkermansia	7.78	7.44	4.02	0.304

Table 5-7. Combined changes across 24 h of fermentation for pure beta-glucan samples of identified phyla and genera. Replicate samples were pooled among donor at each respective time point (3 donors x 3 replicated = 9). Samples were analyzed between differentially represented OTUs for significant changes after 24 h of fermentation compared to 0 h samples. Data were analyzed using the Kruskal-Wallis ANOVA test, with the FDR multiple comparisons correction.

Phyla & Genera	**0 h**	**24 h**	**Test-Statistic**	**P-value**
Actinobacteria				
Bifidobacterium	0.33	0.33	0.00	1.000
Adlercreutzia	2.00	1.89	0.07	0.843
Collinsella	69.22	85.11	0.86	0.723
Bacteroidetes				
Alistipes	0.78	0.89	0.32	0.778
Parabacteroides	119.56	179.78	0.56	0.778
Bacteroides	776.11	854.33	1.03	0.664
Firmicutes				
Eubacterium	0.11	0.44	0.46	0.778
Veillonella	0.56	0.22	0.38	0.778
Dorea	0.89	3.11	7.06	0.110
Acidaminococcus	2.33	15.11	0.22	0.778
SMB53	6.11	4.89	0.39	0.778
Lactococcus	6.11	0.67	0.22	0.778
Anaerostipes	7.44	5.22	0.40	0.778
Turicibacter	8.11	3.00	0.13	0.803
Ruminococcus	9.44	18.67	4.35	0.166
Clostridium	10.11	3.33	6.38	0.110
Streptococcus	14.89	6.44	3.32	0.256
Roseburia	16.11	54.33	1.54	0.510
Lachnospira	21.22	3.89	12.88	**0.008**
Oscillospira	24.33	35.11	2.40	0.389
Phascolarctobacterium	29.00	125.33	3.03	0.283
Dialister	30.56	43.67	0.10	0.819
Coprococcus	44.11	20.78	3.79	0.211
Blautia	45.11	68.11	2.13	0.408
Ruminococcus	59.67	44.44	1.64	0.500

Faecalibacterium	152.11	62.67	12.82	**0.008**
Proteobacteria				
Escherichia	0.89	0.56	0.31	0.778
Haemophilus	11.00	0.78	5.45	0.110
Sutterella	14.00	35.44	0.22	0.778
Bilophila	14.44	13.89	0.20	0.778
Verrucomicrobia				
Akkermansia	9.11	15.89	0.45	0.778

Figure 5-8. Identified phyla from three fecal donors microbiota at 0, 12 and 24 h of fermentation for five prebiotic dietary fibers analyzed based on percent of sequence reads.

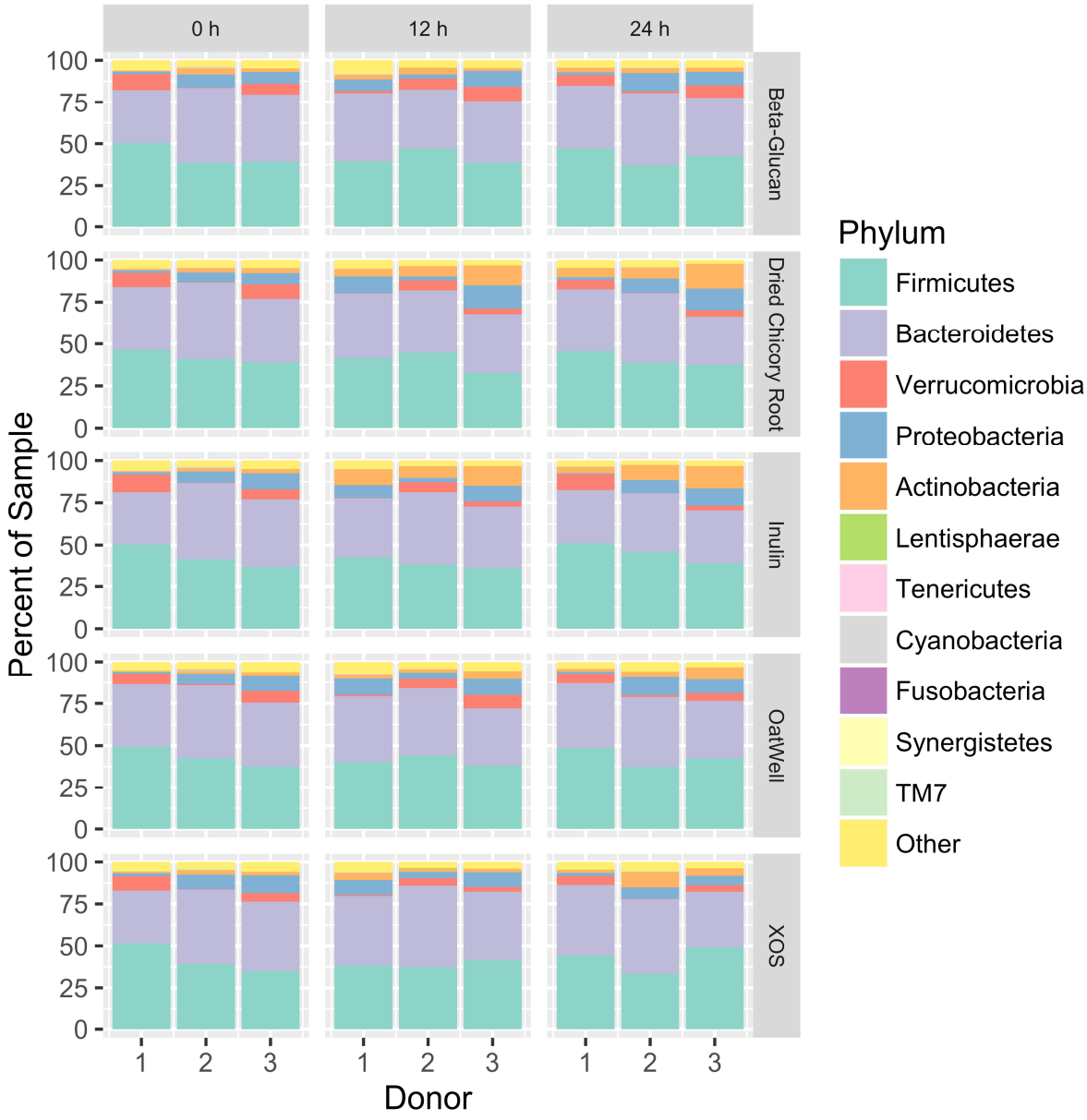


Figure 5-9. Identified abundant families for three fecal donors at 0, 12 and 24 h of fermentation for five prebiotic dietary fibers analyzed based on percent of sequence reads.

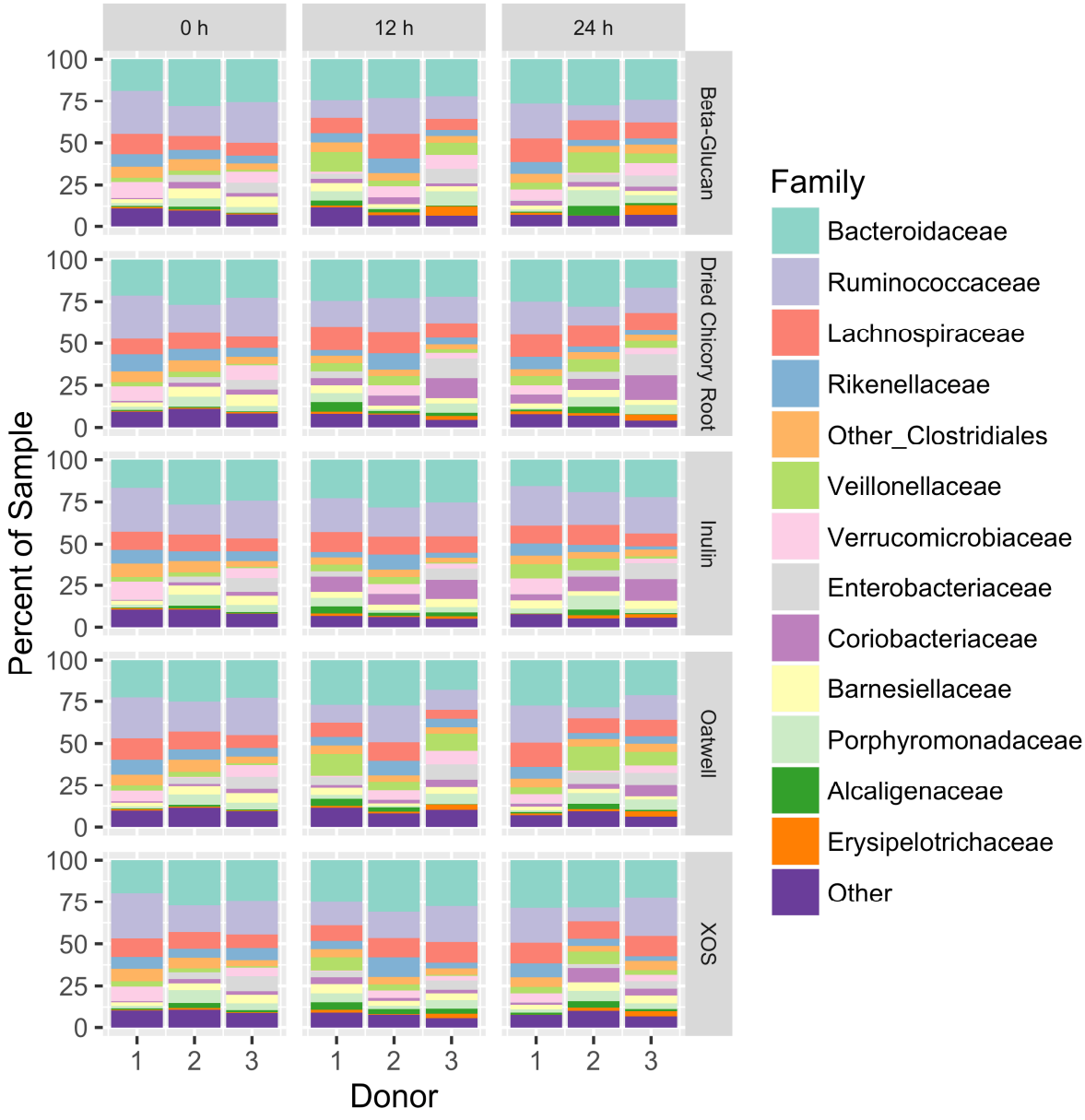


Figure 5-10. Identified abundant genera for three fecal donors at 0, 12 and 24 h of fermentation for five prebiotic dietary fibers analyzed based on percent of sequence reads.

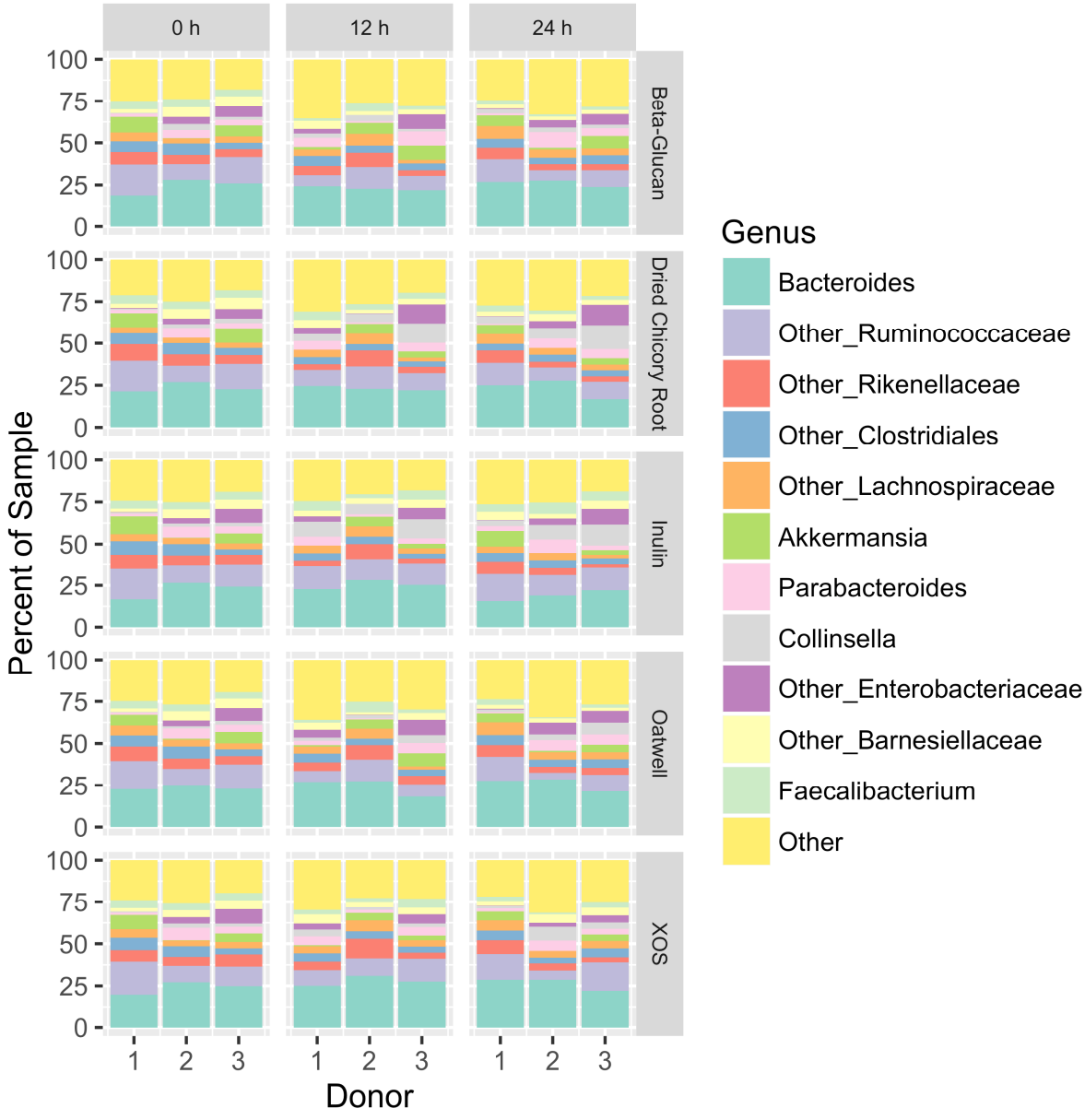


Figure 5-11. Six metrics of analysis for alpha-diversity among samples at 0, 12 and 24 h of analysis, grouped by donor for all five prebiotic dietary fibers analyzed.

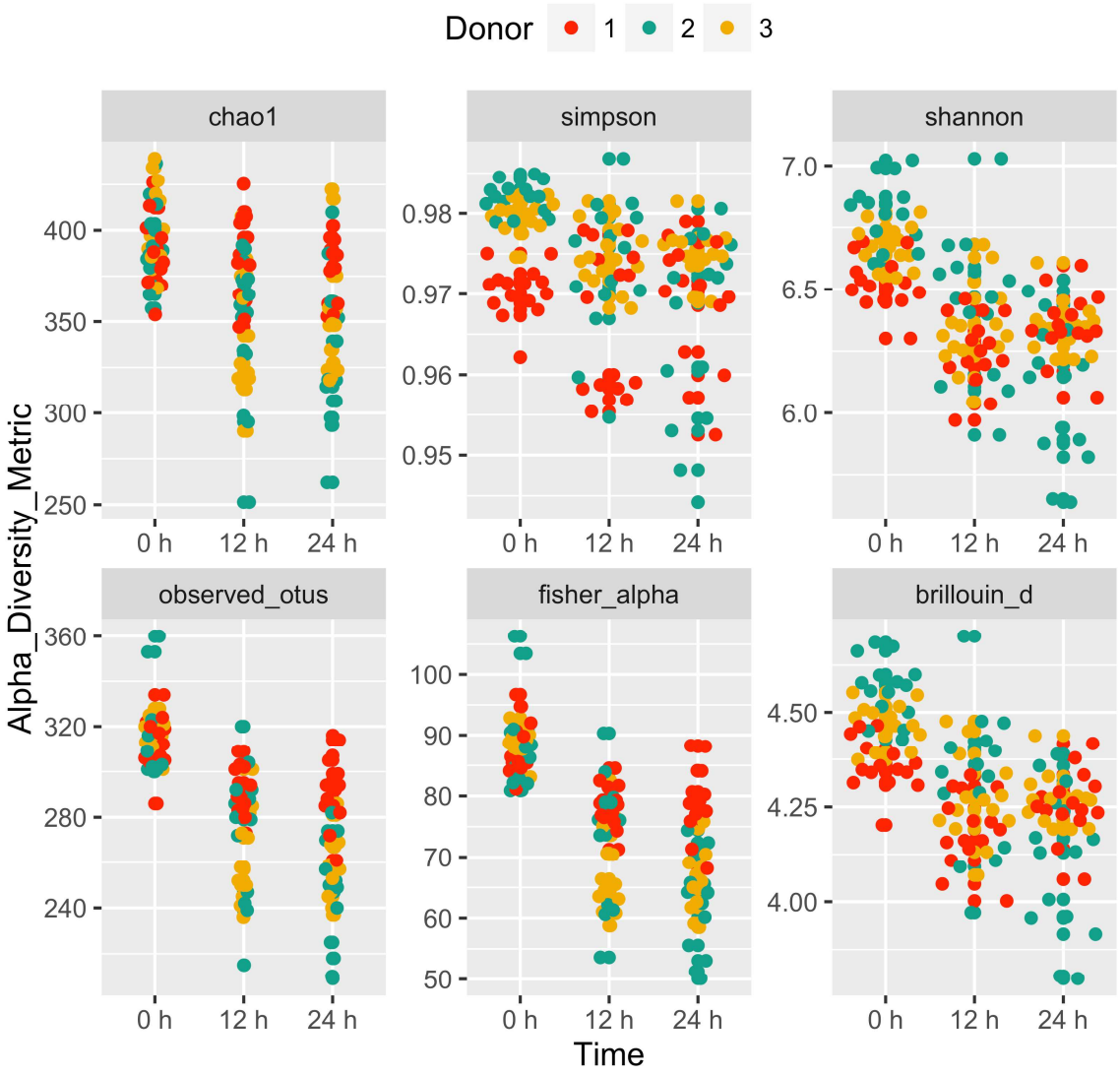


Figure 5-12. Six metrics of analysis for alpha-diversity among samples at 0, 12 and 24 h of analysis, grouped by treatment for all three fecal donors.

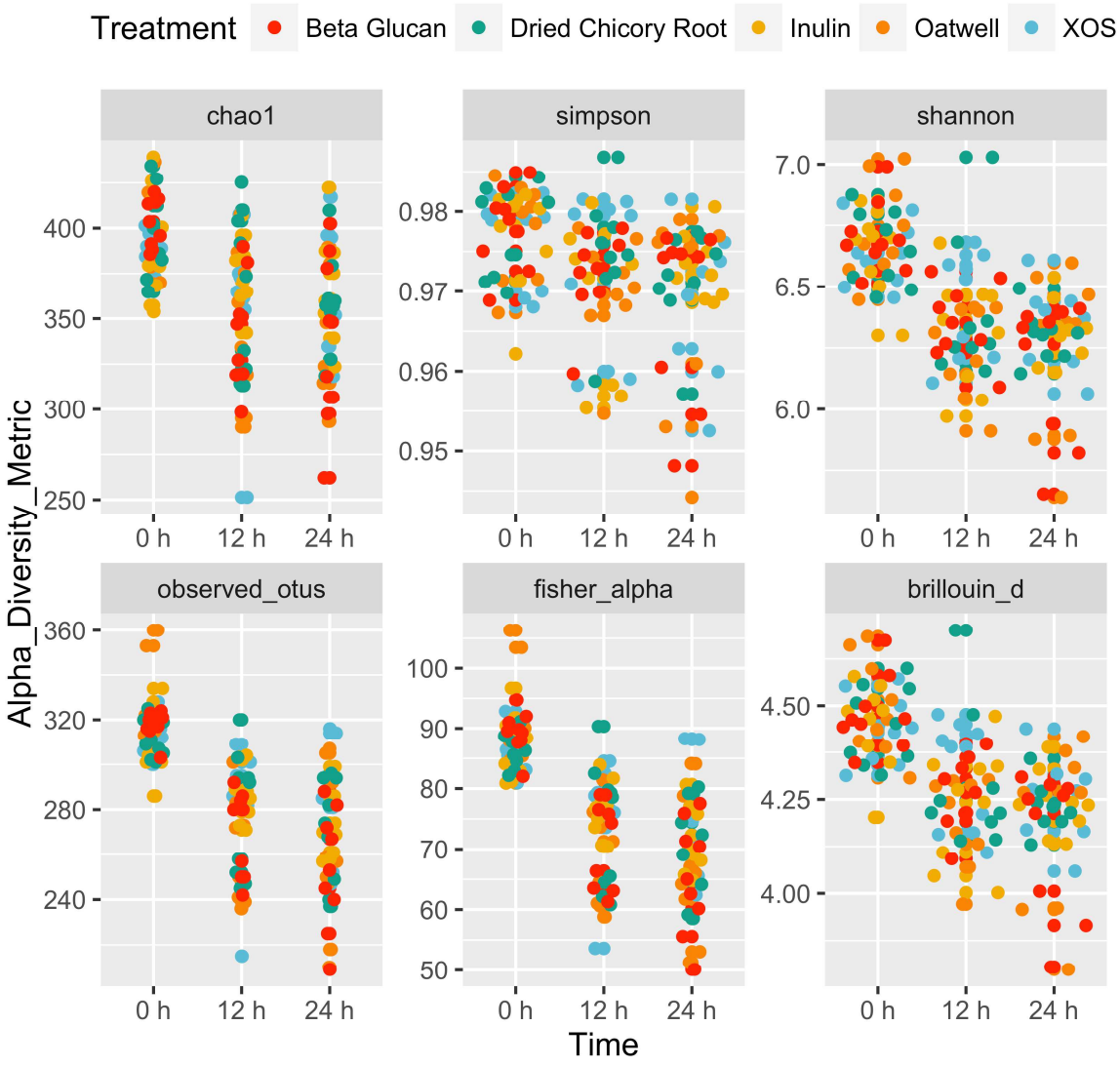


Figure 5-13. Bray-Curtis β-diversity principal component analysis of technical replicates among each treatment group between microbiota analysis of three fecal donors.

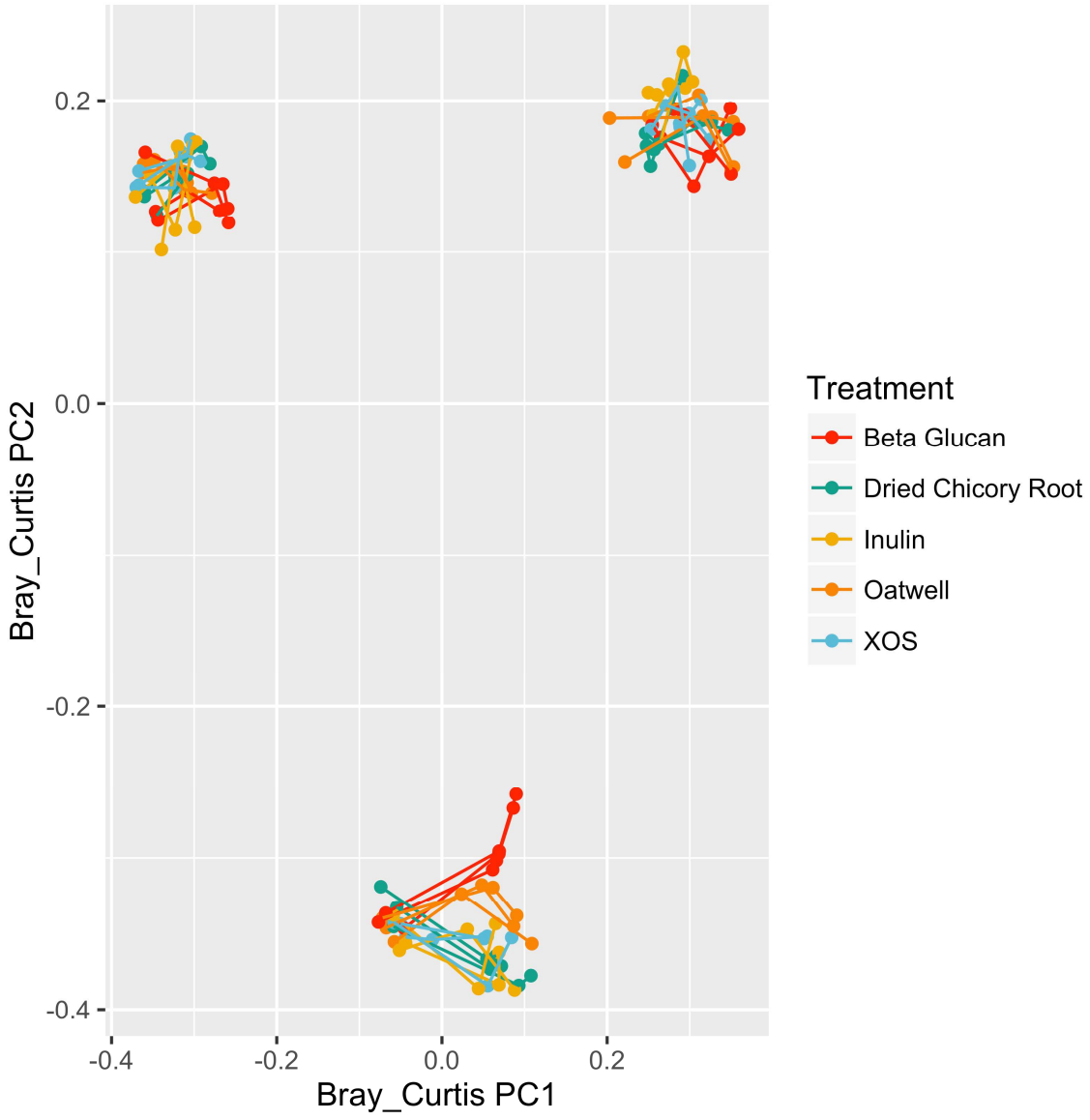


Figure 5-14. Bray-Curtis β-diversity principal component analysis among microbiota of three fecal donors at 0, 12 and 24 h of analysis.

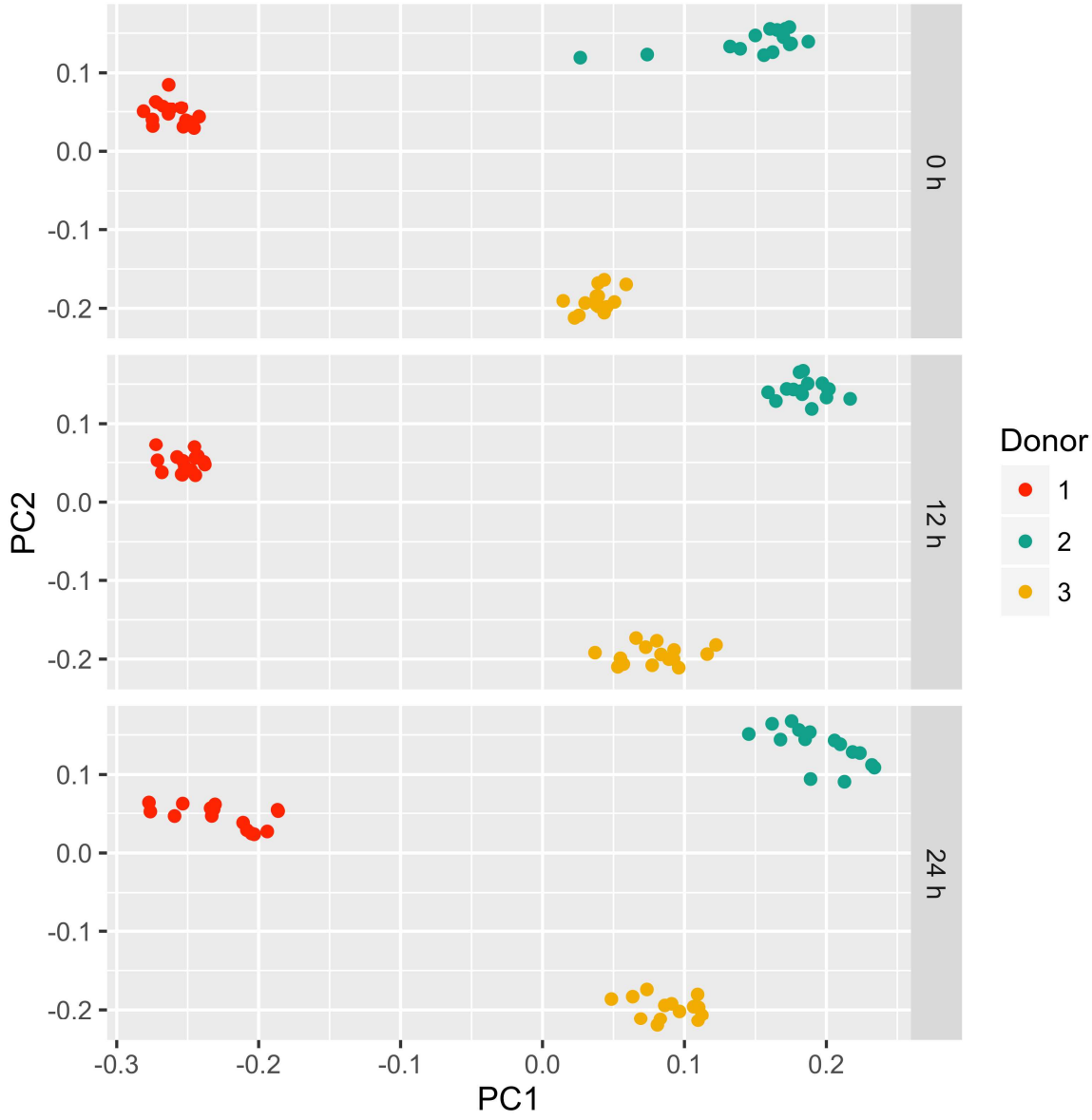


Figure 5-15. Variations in abundant phyla among three donors analyzed.

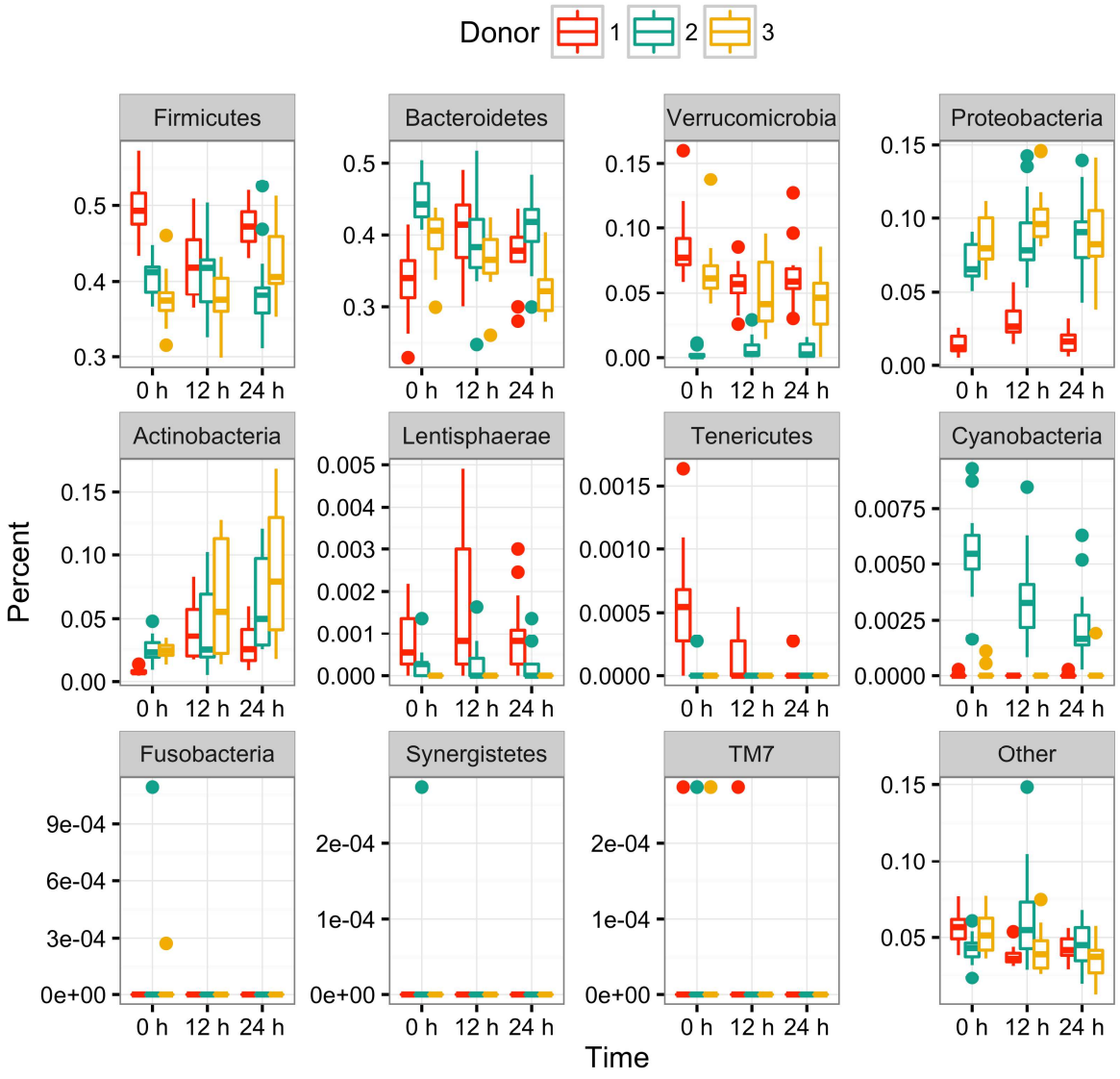
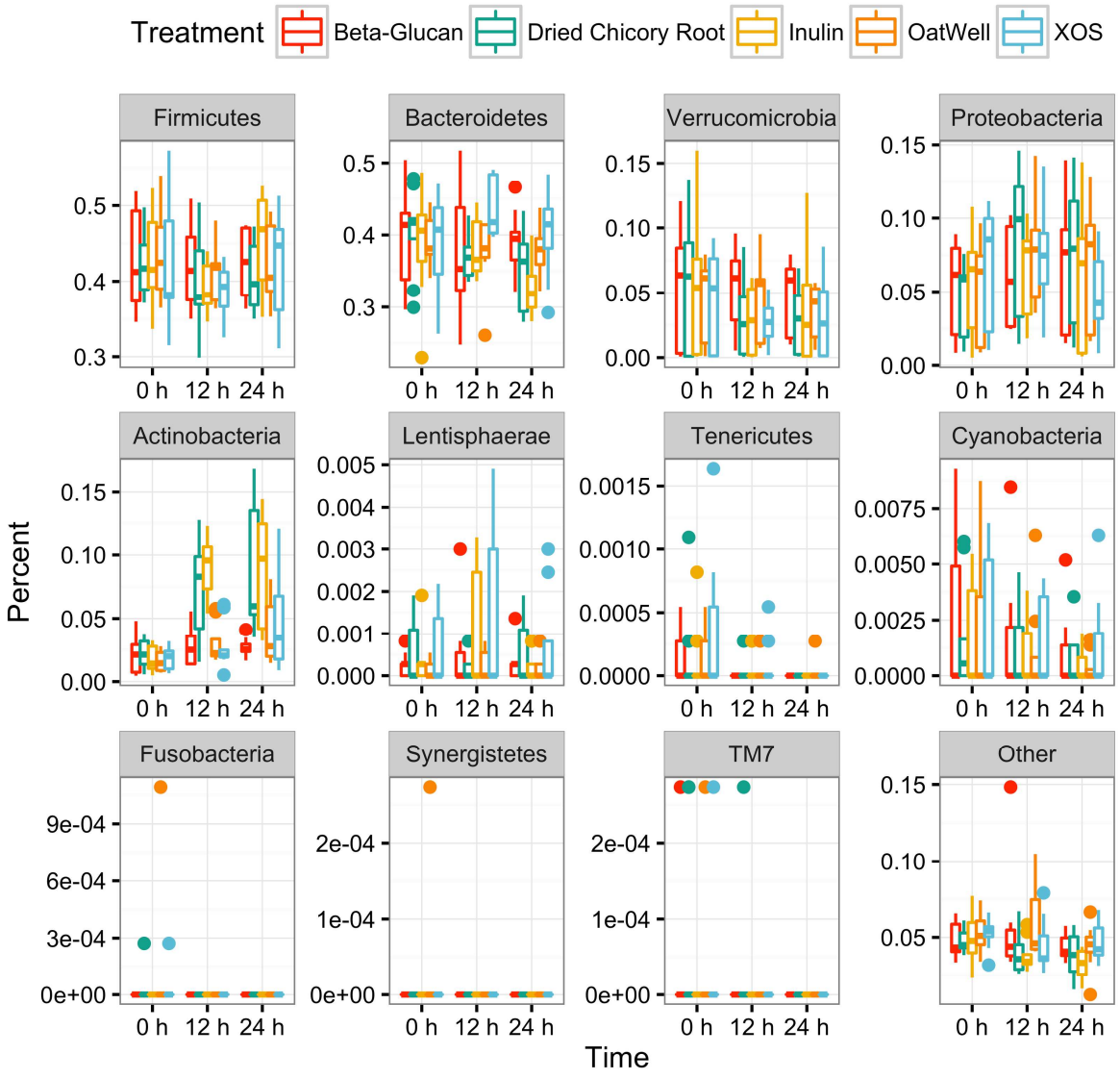


Figure 5-16. Variations in treatment groups and pooled donors analyzed.



CHAPTER SIX

Organic Fertility Treatment Effects on Shortfall Nutrients and Nitrate Accumulation in Leafy Greens

Executive Summary

Arugula (*Eruca sativa*), mizuna (*Brassica rapa* var. *nipponsinca*), red giant mustard (*Brassica juncea*) and spinach (*Spinacia oleracea* 'Tyee') are fresh produce crops high in nutritive value that provide shortfall and high interest nutrients addressed in the 2015 U.S. Dietary Guidelines.[264,265] The primary objective of this project was to evaluate fertility treatments unique to these crops that optimize their nutritional capacity. Measurements discussed include: vitamin C, dietary fiber, calcium, iron, potassium, sodium and nitrate. Plants were grown at the University of Minnesota St. Paul Campus (St. Paul, MN) in a greenhouse from November to April under an 18 h photoperiod and a 24/13C day/night temperature. Plants were grown using five different fertility treatments, including four organic treatments and one conventional control. The plant treatment combinations were replicated three times and the entire experiment was duplicated. Fertility treatments had a high impact on vitamin C (with over a 3-fold difference in treatments in the first experiment), nitrate (over 10-fold difference among fertility treatments in some species) and potassium concentrations (over 5-fold difference among fertility treatments in some species) in analyzed plant tissue. No consistent differences were found for fiber, calcium, iron and sodium concentrations in tissue analyzed. This is the first study to analyze the impact that different organic treatments can have on multiple

deficient nutrients and compounds addressed by the U.S. Dietary Guidelines for high-impact, highly-consumed produce crops.

Introduction

Consuming fresh produce offers a healthy way to introduce many shortfall nutrients into the diet of the consumer, while increasing their intake of wholesome, nutrient-dense foods.[266] The 2015 U.S. Dietary Guidelines suggests increasing vegetable intake, specifically green salad vegetables, as these are consumed in low quantities and have been associated with many health outcomes.[267] Arugula (*Eruca sativa*), mizuna (*Brassica rapa* var. *nipposinica*) and red giant mustard (*Brassica juncea*) are Mesclun mixture plants that are nutrient-dense and consumed in many regions.[268] Spinach (*Spinacia oleracea*) is commonly consumed as both a plate vegetable and salad green, and has quintupled in consumption since the early 1970's, with nearly 60% of current consumption coming from fresh-market spinach.[269]

Organic crops are produced in systems that do not utilize synthetic fertilizer, pesticides and growth regulators, and rely heavily on animal and plant manures and biological pest control for successful growth of crops.[270] Key fundamentals of traditional organic production encourage long-term fertility of soils, minimization of carbon footprints and maintenance of genetic diversity in current food systems.[271] In greenhouse systems in enclosed environments, key organic practices can be implemented, like the use of non-synthetic fertilizers and pesticides, although are not complete replacements of traditional

organic practices in an open field environment. Differences in organically and conventional produced foods have been extensively reviewed, based on their nutritive value, sensory qualities and overall safety.[272]

Minerals play many critical roles in human physiology, and are responsible for a wide range of activities in the body. In plants, iron plays a critical role as a cofactor in chloroplast biosynthesis.[273] Calcium is known for its ability to aid in the formation of stable cell walls and membranes, and regulates stimulus of cells.[274,275] Potassium primarily acts as a cofactor for protein synthesis and is a major solute in maintaining water balance and osmosis.[275] In the human body, iron aids in oxygen and electron transport, DNA synthesis and many other critical roles.[276] Calcium aids in bone formation and higher intake has been associated with higher rates of whole-body fat oxidation.[277] Potassium intake has been associated with protective effects against hypertension.[278]

In plants, vitamin C acts as a substrate for ascorbic acid peroxidase,[279,280] plays a fundamental role in photosynthesis,[281] acts as an enzyme cofactor in the synthesis of anthocyanins and ethylene[282] and helps keep alpha-tocopherol in a reduced state.[283] In the human body, vitamin C plays many roles, including the ability to act as a water-soluble antioxidant, aids in collagen synthesis, increases absorption of iron from the diet and plays other critical roles in the metabolism of folate and some amino acids.[284]

Dietary fiber is a critical shortfall nutrient in the United States. The typical U.S. individual consumes 17 g/d while the current Dietary Recommended Intake (DRI) is 25 g/d for adult women and 38 g/d for adult men, respectively.[140,141] The DRI is based on decreased risk for developing cardiovascular disease[19], but fiber has also been shown to decrease the risk for developing type 2 diabetes when more than 15 g/d[285] is consumed, increase satiety,[286] promote weight loss[37] and provide many other beneficial effects.[6,287,288]

Nitrate is generally considered safe to consume in moderate amounts, and is easily converted to nitrite through reduction.[289] Although nitrate and nitrite are not carcinogenic themselves, they can easily yield carcinogenic compounds. Nitrates react with secondary and tertiary amines endogenously, forming N-nitroso compounds.[289] N-nitroso compounds have been associated with higher risk of developing esophagus, stomach and liver carcinomas.[290–292] Nitrate accumulation is common in Brassica plants as well as other leafy vegetables such as spinach and lettuce, and is influenced heavily by nitrogen fertilization practices.[293]

Under the new FDA Nutrition & Supplements Facts Label Rule (2016), foods with nutrition facts panel will now be required to label potassium (a new addition) along with calcium and iron (previously required). The Daily Values (DV) for sodium decreased (2400 mg to 2300 mg), and increased for potassium (3500 mg to 4700 mg), calcium (1000 mg to 1300 mg) and dietary fiber (25 g to 28 g).[294] With these changes,

requirements for nutrient content claims will also be affected, changing the levels required in the product to make an "excellent source" or "good source" claim for any of these nutrients. Understanding the factors that influence concentrations of these key nutrients in produce is critical, especially as manufacturers reformulate nutrition facts panels and consumers are interested in new nutrient labels.

The purpose of this project was to address differences in shortfall nutrients and nutrients of interest in arugula, red giant mustard, mizuna and spinach and how they differ among organic nutrient amendments along with a conventional comparison. These four plants were chosen because they have a high nutrient density, are frequently consumed in many populations and have high amounts of common shortfall nutrients.

Materials and Methods

Plants

Arugula (*Eruca sativa*), mizuna (*Brassica rapa* var. *nipponsinca*), red giant mustard (*Brassica juncea*) and spinach (*Spinacia oleracea* 'Tyee') were grown on five different fertilizer/media combinations. All seed for this study was obtained from Johnny's Selected Seeds (Winslow, ME).

Arugula, mizuna, and red giant mustard seeds were sown into standard 1020 trays (28 cm x 54 cm x 6 cm) at a density of 11 mg per tray. Spinach was sown into 50 cell deep plug trays with two seeds per cell, plants were thinned to one plant per cell after germination.

All four species were grown on all five fertilizer/media combinations and replicated three times. The entire experiment was completed twice.

Fertility Treatments

Media and fertility treatments (Table 6-1) were mixed prior to planting. Fertility was scaled to meet field nitrogen requirements for crops on low organic matter soils according to University of Minnesota Extension materials.[295] Treatments were fertilized by converting the recommended kg N/ha to kg N/m3 using a depth of 15 cm, then calculating the weight of solid fertilizer or volume of liquid fertilizer to apply to the volume of media in each container using the guaranteed analysis of each fertilizer product. Both the custom mix and all-in-one potting mix exceeded nitrogen recommendations and so were not further amended. Full nitrogen recommendation was 112 kg/ha for leafy greens. Bulk densities of the compared media ranged from 0.086 - 0.307 g/cm^3 for the treatment groups (Table 6-2). Chemical analysis of media nutrients was conducted for complete comparison of soil nutrients (Table 6-3). Greens were grown to baby leaf lettuce size, and so did not require the second application.

Plant Harvest

All plants were grown on benches in a greenhouse (on campus) maintained with a 24°C day temperature and 13°C night temperature. Benches were blocked by treatment. Greens were harvested when the majority of leaves were 10 cm long by cutting with scissors. Greens were harvested between 1 to 3 times depending on plant vigor. All plant tissue and soil data were taken at time of first harvest. Harvest occurred between 4 and 9 weeks

after planting. Tissue samples for vitamin C and dietary fiber analysis were frozen immediately at -80°C. Tissue samples for mineral analysis were dried to completion at 60°C, and crosschecked to control for moisture removal completion.

Plants (Table 6-8) were also grown at three greenhouses in Minnesota that participated as volunteers in this study in order to provide comparisons to plants grown on campus. Growers were provided with packets of detailed instructions for the experiment and provided with all required materials. Regular visits were scheduled with research staff to ensure compliance and conduct analysis.

Media and Tissue Analysis

Exchangeable calcium was extracted from the media by mixing 10 mL or 1N, pH7, ammonium acetate with 10 g of air-dried sample, and then placed in a shaker for 5 minutes. The filtered extract was analyzed with an inductively coupled plasma atomc (DTPA) emission spectrometer (ICP-AES). Extractable iron was determined by treating a 10 g sample of air-dried media with 20 mL of diethylenetriamine-pentaacetic acid (DTPA) extracting solution. Samples were placed in a shaker for 2 h, and then filtered an analyzed with an ICP-AES for iron concentration of media. Available potassium was extracted from the media by mixing 10 mL of 1N ammonium acetate, pH7, with 1 g of air-dried media and then placed in a shaker for 5 minutes. Available potassium was then measured by analyzing the filtered extract on an ICP-AES set on emission mode at 776 nm. Nitrate-nitrogen was determined by adding 60 mL of KCl extracting solution to a 2 g sample of air-dried media, and then placed in a shaker for 15 minutes. The nitrate level in

the filtered extract was measured on a Lachat QuckChem 8500 Flow Injection Analyzer by the cadmium reduction method. Bulk densities of media were calculated according to Grafton et al with minor revisions.[296] Plant tissue minerals (calcium, iron, potassium and sodium) were analyzed by weighing 500 mg of air-dried tissue into a 20 mL high form silica crucible and dry ashed at 485°C for 12 h (covered). Ash was then equilibrated with 5 mL of 20% HCl at room temperature for 30 minutes, followed by an addition of 5 mL of deionized water, then allowed to settle for 3 hours prior to ICP-AES analysis. Plant tissue nitrate nitrogen was extracted by shaking 300 mg of dried sample with 30 mL 0.1 m CaSO4 solution for 30 minutes, followed by the addition of 0.85 cc of prewashed charcoal, followed by shaking for an additional 5 minutes. Samples were filtered through Whatman (No. 42) filter paper and nitrate concentrations in the filtrate were determined colorimetrically by the cadmium reduction method.

Vitamin C and Fiber Quantitation

Vitamin C was quantified using the AOAC 967.22 method from 50 g samples of frozen (-80°C) plant tissue. Total dietary fiber was quantified using the AOAC 991.43 method from 100 g samples of frozen (-80°C) plant tissue.

Statistical Analysis

All statistical analysis was conducted using SPSS (SPSS Chicago, IL). Analysis of variance (ANOVA) with Tukey HSD was used for all tests measuring differences among

means. Log transformations were applied where necessary based on regression fit. Statistical significance was achieved for *p*-values less than 0.05.

Comparison of Plants Grown in Experiment 1 and Experiment 2

ANOVA indicated that there were significant differences between experiment 1 and experiment 2 for fertility treatment interactions ($p<0.001$), so biomass, nitrate and nutrient levels were analyzed separately for each experiment.

Results and Discussion

Harvested Biomass

Harvested biomass for arugula varied among the five treatments in experiment 1 (Figure 6-1). CM had the highest harvested biomass (598.5 g/m^2), which was significantly more than the FE treatment (330.7 g/m^2; $p=0.039$). The CM, AO, CC and PL treatments produced similar results. In experiment 2, the CC and CM treatments resulted in significantly greater harvested biomass than the AO, FE and PL treatments ($p<0.01$).

The harvested biomass for mizuna (Figure 6-2) in experiment 1 varied among treatments, and also varied from experiment 2. In experiment 1, the CM, CC, FE and PL treatments all had similar harvested weights, and the AO treatment was significantly greater than the other four treatments (1719.6 g/m^2; $p<0.001$). In experiment 2, the CM treatment yielded a higher harvest biomass than the other four treatments (2087 g/m^2; $p<0.01$).

The harvested biomass for red giant mustard (Figure 6-3) was highest in experiment 1 for the AO treatment (1314.3 g/m^2) and was significantly higher than all other treatments in

experiment 1 (p<0.002). The CC, FE and PL treatments were all statistically similar to one another, while the CM treatment was the significantly lower than these three treatments (303.6 g/m^2; p<0.01). In experiment 2 the CM treatment had the highest harvested biomass (1747.3 g/m^2) and was statistically similar to the AO treatment (1435.2 g/m^2; p=0.22) and the CC treatment (1343.2 g/m^2; p=0.081). The PL treatment had the lowest harvested biomass (205.0g/m^2) and was significantly lower than all other treatments (p<0.001).

Harvested spinach biomass for experiment 1 (Figure 6-4) differed among treatments. The AO treatment (1161.5 g/m^2) was similar to the CC treatment (834.6 g/m^2; p=0.392), but significantly higher than the CM, FE and PL treatments (p<0.05). The CM treatment had the lowest overall harvested biomass (418.6 g/m^2), but was statistically similar to the CC, FE and PL treatments. In experiment 2, the AO resulted in the highest overall harvested biomass (1494.1 g/m^2) and was statistically similar to the CM treatment (1195.8 g/m^2; p=0.376) and the CC treatment (996.4 g/m^2; p=0.058).

Minerals

In experiment 1, the mineral concentration in the harvested plants varied greatly among treatments and species (Table 6-4). For red giant mustard, there was no difference between iron, potassium and sodium concentrations. For calcium, the CC treatment had the highest concentration (191.3 mg/100 g fw), which was statistically higher than the CM treatment (138.2 mg/100g fw; p=0.031), while the FE, AO and PL treatments were

all similar. For the mizuna plants the potassium and sodium concentrations were most affected by the treatments. The CM treatment (860.3 mg/100g fw) was significantly higher than the CC (393.1 mg/100g fw), FE (120.16 mg/100g fw) and PL (164.39 mg/100g fw) treatments for measured potassium ($p<0.001$). Sodium concentrations were also greatly affected by the treatments in the mizuna plants with nearly a 10-fold difference between the CM treatment (14.25 mg/100g fw) and the FE treatment (142.66 mg/100g fw; $p<0.001$). Arugula iron concentrations were unaffected by the fertility treatments. Arugula potassium varied greatly among the treatments, most notably with the CM treatment having the highest concentration (950.7 mg/100g fw; $p<0.001$). In spinach, concentrations of calcium, iron and sodium were unaffected by the fertility treatments, although the CC treatment was over 3-fold higher in potassium (995.12 mg/100g fw) than the FE treatment (287.43 mg/100g fw; $p<0.001$). Statistical relations between the collected soil and tissue mineral data in experiment 1 showed a significant correlation between potassium concentrations in the collected mizuna plants and soil at time of harvest ($p=0.028$), and a positive correlation for mizuna and red giant mustard (soil mineral data only collected for experiment 1).

In experiment 2, mineral concentrations followed some similar trends compared to experiment 1 (Table 6-5). For red giant mustard plants, iron was not affected by the fertility treatments. Potassium concentrations varied greatly, with the largest difference between the CM treatment (696.31 mg/100g fw) and the PL treatment (98.86 mg/100g fw; $p<0.001$). For the mizuna plants the iron also was not affected by the fertility

treatments, but the potassium concentration was over 8-fold higher in the CM treatment (666.67 mg/100g fw) compared to the FE treatment (81.49 mg/100g fw; $p<0.001$). For arugula, calcium concentrations were similar among all treatments, while there was over a 5-fold difference between the CM and FE potassium concentrations. The spinach iron concentrations were similar for all the treatments, while the FE treatment resulted in the highest sodium concentration compared to the other treatments (261.86 mg/100g fw; $p<0.001$).

Nutrient content claims can play a significant role in consumer perception of a product, and a consumers willingness to buy a product.[297] Under the new FDA requirements for labeling, the minimum requirement of calcium in a product needed to make a "good source" nutrient content claim is 130 mg per 100 g serving (10% of 1300 mg required by DV), nearly all of the plants under all the fertility treatments would qualify from both experiments. For potassium, a "good source" claim would require a minimum of 470 mg per 100 g serving. This claim could only be made for the CM and AO treatments (based on averages from both experiments) in the red giant mustard, mizuna and arugula plants. The use of different fertility treatments affects potassium and could have a significant impact on product sales and consumer preferences as new nutrient content claims and nutrition facts panels are made for these products. The FE treatment produced the highest average sodium concentrations in all plants analyzed in experiment 1 and 2 (with the exception of arugula in experiment 2). As a publicized nutrient of concern with a

decreasing DV, produce grown with similar FE treatments might produce less marketable products to consumers.

Vitamin C

Vitamin C is a highly unstable water-soluble vitamin known for its antioxidant properties, both in humans and plants. Key effects on vitamin C concentration include the growing conditions, plant stage at harvest, storage temperature and wide range of postharvest conditions.[298,299] Traditionally, accumulation of vitamin C is increased whenever plants are exposed to high oxidative stress, including full sunlight, low nitrogen availability in the soil and drought conditions.[300] For fresh produce, vitamin C losses can be enhanced when postharvest storage is extended, or at higher temperature, low relative humidity and freezing.[298] High nitrogen fertilizers have also been associated with decreased vitamin C concentrations in many fruits and vegetables.[298]

Mizuna vitamin C was analyzed for all five fertility treatments in experiment 1 and for four fertility treatments for experiment 2. In experiment 1, the PL treatment had the highest average vitamin C concentration (16.1 mg), but was statistically similar to all other treatments, except for the CM treatment ($p = 0.002$) (Figure 6-5). In experiment 2, the CC treatment had the highest concentration (48.13 mg) compared to all other treatments ($p<0.001$) (Figure 6-5). CM had a higher concentration than FE (16.86 mg; $p=0.018$) and AO (16.03 mg; $p=0.008$). Between experiments, fertility treatments resulted in a wide fluctuation in mizuna vitamin C concentrations, with all treatment averages lower in the first experiment. For the CC treatment, the average for experiment

1 was 11.94mg/100 g whereas in experiment 2 it was 48.13 mg/100 g. Because many pre-harvest factors (genotypic, climatic and environmental) influence vitamin C, variation between experiments and growing seasons is common.[301]

In experiment 1, the red giant mustard PL treatment had the highest mean vitamin C concentration (21.26 mg), but was statistically similar to the CC treatment (15.7 mg; p=0.116) (Figure 6-6). The PL treatment had significantly higher concentrations than the FE treatment (14.03 mg; p=0.049), AO treatment (12.48 mg; p=0.022) and CM treatment (8.45 mg; p=0.022). In experiment 2 (Figure 6-4), the CC treatment (32.4 mg) had similar concentrations to the FE treatment (27.53 mg; p=0.369), the AO treatment (43.23 mg; p=0.067) and CM treatment (23.36 mg; p=0.115).

Fiber

Dietary fiber is a mixture of complex organic substances that are non-digestible in the upper gastrointestinal tract, present as both soluble and insoluble compounds. Studies have analyzed dietary fiber differences between conventionally and organically grown plums, with little differences observed.[302] To our knowledge, dietary fiber has not been analyzed in nutrient dense crops that address shortfall nutrients, comparing either organic or conventional nutrient sources.

For mizuna plants in experiment 1 (Figure 6-7), the CC treatment (2.94 g) had statistically similar concentrations of total dietary fiber compared to the PL treatment (3.24 g; p=0.051) and also to the FE treatment (3.06 g; p=0.367). Both the CM treatment

(2.04 g) and the AO treatment (1.82 g) had similar total dietary fiber concentrations, $p<0.001$ and $p<0.001$, respectively. In experiment 2, the CC treatment (4.30 g) had significantly higher total dietary fiber than the CM treatment (2.47 g; $p<0.001$) and lower than the AO treatment (3.77 g; $p=0.02$) and the FE treatment (4.96 g; $p=0.007$).

For red giant mustard plants in experiment 1 (Figure 6-8), the CC treatment (2.88 g) had statistically similar concentrations to the PL treatment (2.86 g; $p=0.868$), while the CM treatment (2.03 g; $p<0.001$), the FE treatment (2.20 g; $p<0.001$) and AO treatment (1.44 g; $p<0.001$) were all significantly less than the CC treatment. In experiment 2, the CC treatment (4.79 g) was statistically similar to both the FE treatment (4.65 g; $p=0.588$) and the AO treatment (4.32 g; $p=0.091$), but was greater than the CM treatment (2.01 g; $p<0.001$) in total dietary fiber concentration.

Under the 2016 FDA Nutrition & Supplements Facts Label Revisions, the new DV for dietary fiber is increasing to 28 g/d. Based on this new rule, 2.8 g fiber/serving will be required to make a "good source" nutrient content claim. Based on the combined averages from both experiments, only the mizuna and red giant mustard with the CC and FE treatments would qualify for this claim. Based on a single experiment, the mizuna and red giant mustard with the PL treatment would also qualify. Products labeled as a "good source of fiber" have been perceived as being slightly healthier to consumers, and are often more marketable products.[297]

Nitrate (NO_3-N)

Nitrate-N concentrations varied greatly among treatments, species and experiments (Table 6-6). Compared to the four other treatments, the CM treatment had significantly higher nitrate-N concentrations for all the analyzed plants ($p<0.01$). For the arugula in experiment 1, the CM treatment (1244 ppm [mg/kg fresh weight]) and the AO treatment (1255 ppm) were significantly higher than the other three treatments ($p<0.001$). In the mizuna plants, the CM (1443 ppm) and AO (1656 ppm) treatments were significantly higher than the other treatments ($p<0.001$). In experiment 2, the arugula had the same nitrate-N concentration for the CC, FE and PL treatments, while the CM treatment (734 ppm) was significantly higher than the rest ($p<0.001$). For the spinach, mizuna and red giant mustard plants analyzed in experiment 2, the CM treatment had the highest nitrate-N concentration ($p<0.001$). Statistical analyses between the soil nitrate-N concentrations (taken at first harvest) and plant tissue nitrate-N concentrations showed a significant correlation ($p<0.05$) for arugula, mizuna and red giant mustard plants analyzed from Experiment 1 (Table 7), showing that high nitrate-N in soil leads to high nitrate-N in harvested plant tissue.

For nearly half of the produce analyzed in this study, an average consumer (68 kg) would exceed their recommended daily nitrate intake with only two servings (100 g/serving) of produce. The European Food Safety Authority (EFSA) and The World Health Association (WHO) recommend that daily nitrate intake is below 3.7 mg dietary nitrate/ lb of body weight, as expressed by the Acceptable Daily Intake (ADI). (When converted

from nitrate to NO_3-N as used in this study [multiplying by 0.22] this would be equal to a recommendation 130 mg, or less, of calculated NO_3-N for a 160 lb individual). Vegetables are the number one source of nitrates in the diet for many populations, so monitoring intake is crucial to minimize the potentially harmful effects of high nitrate diets.[303,304]

Careful considerations need to be made when applying nitrogen to both conventional and organic plants, as excessive nitrogen yields excessive amounts of nitrate accumulation in plant tissues. Both organic and conventional produce can have excessive amounts of nitrates, depending on growing and environmental conditions.

Comparison Plants from Commercial Opeations

Red giant mustard and mizuna plants were collected from three growers and greenhouses in Minnesota for nutrient and nitrate comparisons to campus-grown plants throughout experiment 1 and experiment 2 (Table 6-8). For the red giant mustard, the vitamin C concentrations in experiment 2 nearly all fell within range of the comparison produce, while experiment 1 was slightly lower. Calcium levels in the comparison produce were higher than both experiment 1 and 2 for red giant mustard. For mizuna, experiment 1 had slightly lower vitamin C concentrations than the comparison produce, while experiment 2 had similar concentrations. Similar to red giant mustard, the mizuna plants also had much lower calcium concentrations in experiment 1 and 2 (128 – 251 mg/100g) compared to the comparison mizuna (229-422 mg/100g). Nitrate levels varied greatly for both red

giant mustard and mizuna plants, similar to experiment 1 and 2. The average concentration in the comparison red giant mustard was 1599 ppm, which was less than the CM and AO treatments in experiment 1, but greater than all other analyzed red giant mustard plants. The comparison mizuna had an average concentration of 1175 ppm, which was only less than the CM and AO treatments in experiment 1, and greater than all other analyzed mizuna plants, which was the same trend for both species of plants.

Conclusions

For the arugula, red giant mustard, mizuna and spinach plants evaluated in these experiments, significant differences in iron, potassium, calcium, sodium, vitamin C and fiber found among the fertility treatments were dependent on the experiment. Depending on the season in which the plants are grown, this can result in changes in any of these shortfall nutrients or nutrients of high interest. For all treatments in both experiments, for both mizuna and red giant mustard plants, plants harvested in experiment 2 had significantly higher vitamin C concentrations than in experiment 1 ($p<0.001$). For vitamin C, concentrations in plants are dependent on the plants exposure to oxidative stress, and are also dependent on the amount of available nitrogen. Fluctuations in vitamin C concentrations between experiments was most likely due to changes in sunlight exposure, as the plants in experiment 2 were grown in early spring, whereas plants grown in experiment 1 were grown during the winter months. Among all the combined samples analyzed, the CC treatment had the highest vitamin C concentrations ($p=0.02$) compared to the other treatments, and equal or higher calcium concentrations in those plants,

although only in experiment 1. Calcium concentrations were most likely higher in the CC treatment plants because the CC treatment had the second highest calcium concentration out of all the experimental media.

Careful considerations need to be made when choosing fertility treatments for produce items that could be marketed with nutrient content claims, or labeled with nutrition facts panels. These values have been shown to influence consumers and therefore they could have potential positive or negative influences on consumer preference. Because the FDA will now require potassium to be labeled on nutrition facts panels, and because it is heavily influenced by the fertility treatment, growers will have to choose fertility treatments carefully in the future to maximize potassium concentrations in produce.

Table 6-1. Fertility Treatment Combinations of Media and Fertility Sources.

Treatment	Media Base	Fertilizer
All-in-One Potting Mix (AO)	Purple Cow Organic Potting Mix (Purple Cow Organics, Middleton, WI)	None
Custom Mix (CM)	Peat, vermiculite, leaf litter compost (3:2:3 mix by volume)	Greensand, rock phosphate, bloodmeal and lime. (1:1:1:0.5 mix by volume)
Conventional Comparison (CC)	SunGro LC8 (SunGro Horticulture, Agawam, MA)	Peter's Excel CalMag 15-5-15 (Everris, Dublin, OH)
Fish Emulsion (FE)	Sunshine Natural and Organic Planting Mix (SunGro Horticulture, Agawam, MA)	Dramatic 2-5-0.2 (Dramm, Manitowoc, WI)
Poultry Litter (PL)	Sunshine Natural and Organic Planting Mix (SunGro Horticulture, Agawam, MA)	SUSTANE 8-4-4 (Sustane, Cannon Falls, MN)

Table 6-2. Bulk Densities of Compared Media

Treatment	**Bulk Density (g/cm^3)**
All-in-One Potting Mix (AO)	0.307
Custom Mix (CM)	0.181
Conventional Comparison (CC)	0.138
Fish Emulsion (FE)	0.086
Poultry Litter (PL)	0.086

Table 6-3. Soil Nutrient Profile Comparisons For Five Fertility Treatments Analyzed.

	CC	AO	CM	FE	PL
	Mineral data below as mg/kg dry soil				
pH	5.90 (0.20)	6.75 (0.25)	6.40 (0.08)*	6.15 (0.05)	6.25 (0.05)
Soluble Salts (dS/m)	3.08 (0.50)	3.57 (1.05)	10.20 (1.18)*	1.78 (0.27)	1.39 (0.27)
NO_3-N	3734 (714)	3617 (2410)	1492 (1418)*	1010 (5.44)	Below detection limit
NH4-N	225(32)	13.74 (5.16)	352 (106)*	433 (26)	223 (71)
P	141 (13)	346 (27)	110 (15)*	614 (39)	140 (31)
K	1099 (157)	2970 (123)	10330 (684)*	421 (62)	457 (131)
Fe	3.23 (0.23)	1.59 (0.20)	3.62 (0.94)*	2.15 (0)**	1.23 (0)**
Ca	2051 (167)	1462 (117)	2333 (221)*	1155 (78)	928 (102)
Mg	1675 (69)	754 (120)	715 (62)*	1180 (175)	906 (124)
B	1.01 (0.15)	2.89 (0.25)	4.43 (0.60)*	Below detection limit	Below detection limit
Na	182 (10)	635 (24)	215 (16)*	674 (100)	279 (40)
Mn	5.60 (2.6)	2.70 (1.1)	7.38 (1.2)*	2.89 (0.26)	2.43 (0.26)
Mo	0.21 (0)**	0.22 (0.04)	0.22 (.08)	Below detection limit	0.25 (0)**
Zn	1.90 (0.24)	0.5 (0.07)	0.67 (0.05)*	0.72 (0)	0.59 (0.15)

Data shown are mean (SD). Data shown are n=2 unless otherwise noted (*n=3, **n=1). Conventional Control (CC); All-in-One Potting Mix (AO); Custom Mix (CM); Fish Emulsion (FE); Poultry Litter (PL).

Table 6-4. Mineral Comparison Among Fertility Treatments (mg/100 g fresh weight) – Experiment 1

		Fertility Treatment				
	Mineral	**CC**	**CM**	**FE**	**AO**	**PL**
Red Giant Mustard	**Ca**	191.3 b	138.2 a	166.1 a,b	188.3 a,b	189.4 a,b
	Fe	1.1	0.7	1.1	0.7	1.2
	K	416	600.54	347.93	813.76	262.60
	Na	32.3	43.7	144.9	55.9	75.8
Mizuna	**Ca**	239.3 b	128.9 a	201.6 b	229.6 b	205.9 b
	Fe	0.6 a	0.9 b	0.6 a	0.8 a,b	0.7 a
	K	393.1 b	860.2 c	120.1 a	750.4 c	164.4 a
	Na	26.4 a	14.3 a	142.7 d	70.7 b	105.2 c
Arugula	**Ca**	200.9 b	130.7 a,b	154.62 a,b	187.8 a,b	112.9 a
	Fe	0.9	0.8	0.8	0.7	0.9
	K	455.3 b	950.7 d	309.0 a,b	662.7 c	277.6 a
	Na	26.6 a	10.8 a	113.7 b	31.4 a	60.7 a,b
Spinach	**Ca**	83.9	*	144.3	*	76.3
	Fe	0.8	*	0.9	*	0.8
	K	995.1 b	*	287.4 a	*	820.2 b
	Na	42.8	*	137.9	*	92.1

Data shown are mean (n=3) for each respective species. Results analyzed using ANOVA Tukey HSD for significance testing ($p<0.05$). Significance analyzed for each species within each experiment. * Indicates missing samples due to space capacity of experiment in greenhouse.

Table 6-5. Mineral Comparison Among Fertility Treatments (mg/100g fresh weight) – Experiment 2 Results

		Fertility Treatment				
	Mineral	**CC**	**CM**	**FE**	**AO**	**PL**
Red Giant Mustard	**Ca**	154.1 a	191.8 b	139.7 a	144.6 a	134.1 a
	Fe	0.6	0.7	0.5	0.6	0.9
	K	130.9 a	696.3 c	104.6 a	388.6 b	98.9 a
	Na	41.2 b,c	16.5 a	75.7 d	29.7 a,b	50 c
Mizuna	**Ca**	176.0 a	215.2 b	258.3 c	177.8 a	251.8 b,c
	Fe	0.5	0.7	0.3	0.4	1.1
	K	134.6 a	666.7 c	81.5 a	464.7 b	107.1 a
	Na	28.7 a,b	16.2 a	58.4 c	30.2 b	57.5 b,c
Arugula	**Ca**	162.2	193.1	153.1	N/A	164.9
	Fe	0.6 b,c	0.7 c	0.5 a,b	N/A	0.5 a
	K	315.8 b,c	642.2 c	122.8 a	N/A	155.1 a,b
	Na	35.1 b	14.8 a	44.1 b	N/A	45.8 b
Spinach	**Ca**	94.9 c	88.3 b,c	71.2 a,b	62.6 a	66.9 a
	Fe	0.6	0.6	0.7	0.6	0.5
	K	332.9 a	1201.9 b	229.4 a	1147.1 b	285.6 a
	Na	119.8 c	20.3 a	261.9 e	60.2 b	184.8 d

Data shown are mean (n=3) for each respective species. Results analyzed using ANOVA Tukey HSD for significance testing ($p<0.05$). Significance analyzed for each species within each experiment.

Table 6-6. Nitrate-N Concentrations (NO_3-N ppm; mg/kg fresh weight) in Arugula, Spinach, Mizuna and Red Giant Mustard for Experiment 1 and Experiment 2.

		Fertility Treatments				
		CC	**CM**	**FE**	**AO**	**PL**
Experiment 1	**Arugula**	266 a	1244 b	218 a	1255 b	100 a
	Spinach	1988 b	N/A	1236 a,b	N/A	932 a
	Mizuna	171 a	1443 b	20 a	1656 b	12 a
	Red Giant Mustard	171 a	1673 b	194 a	2279 c	21 a
Experiment 2	**Arugula**	5 a	734 b	4 a	N/A	6 a
	Spinach	587 c	1251 d	241 a,b	473 b,c	48 a
	Mizuna	3 a	614 b	< 1 a	2 a	< 1 a
	Red Giant Mustard	19 a	877 b	3 a	16 a	3 a

Table 6-7. Soil Nitrate-N Concentrations (NO_3-N ppm; mg/kg dry weight) Correlated to Plant Tissue Nitrate-N Concentrations (NO_3-N ppm; mg/kg fresh weight) in Arugula, Mizuna and Red Giant Mustard in Experiment 1.

	Pearson's *r*	***p***
Arugula	0.813	<0.001
Mizuna	0.539	0.047
Red Giant Mustard	0.723	0.002

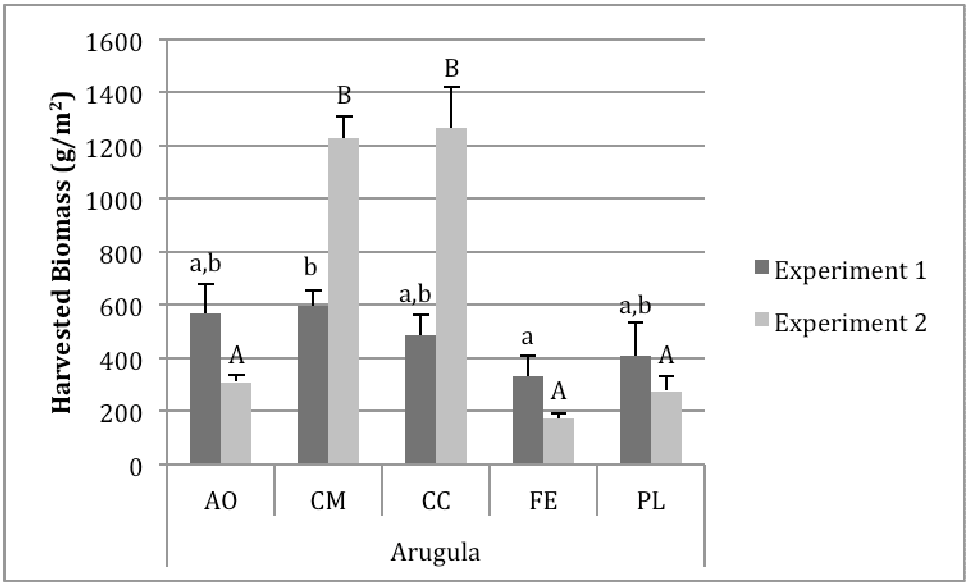
Statistical relations were determined with bivariate correlations (Pearson's *r*), and a $p < 0.05$ was accepted as statistically significant. Correlations were made between soil and tissue Nitrate-N concentrations for each individual plant species.

Table 6-8. Conventionally Grown Produce Collected Throughout Both Experiments From Three Minnesota Growers and Greenhouses.

	Vitamin C (mg/100g)	Fiber (g/100g)	Ca (mg/100g)	Fe (mg/100g)	K (mg/100g)	Na (mg/100g)	Nitrate-N (ppm)
Red Giant Mustard							
Mean	31.73	3.81	251.6	0.76	558.3	48.9	1599
Median	29	3.5	254.3	0.79	585.5	42.8	2218
Range	20.4-45.8	2.19-5.74	254-331	0.64-0.83	486-602	33-71	59.7-2520
Mizuna							
Mean	28.6	3.65	294.8	0.87	482.9	42.1	1175
Median	23.4	3.5	232.3	0.93	492.6	38.4	1577
Range	16.6-45.8	2.13-5.34	229-422	0.72-0.97	395-560	36-51	70-1878

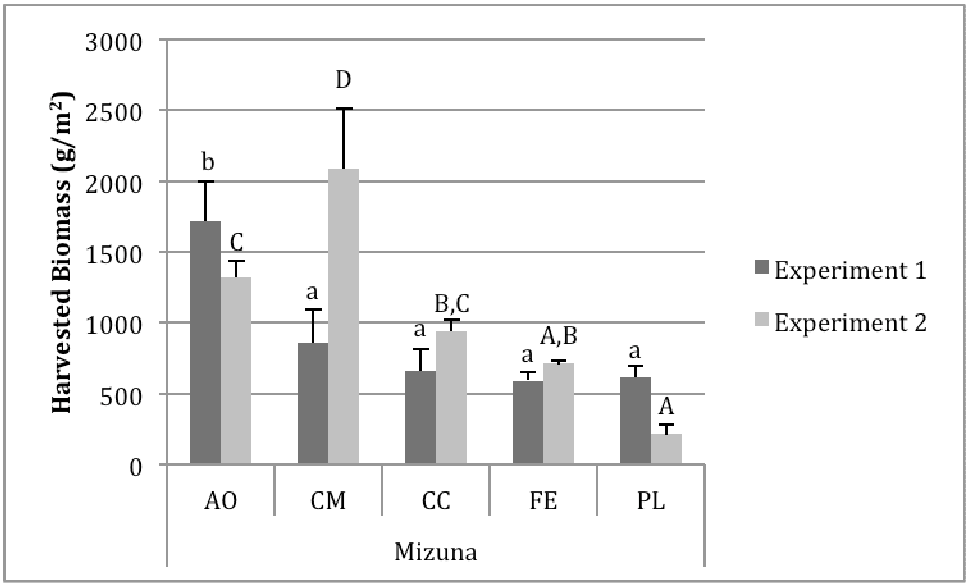
*Data displayed are three triplicate samples from three different greenhouses and growers.

Figure 6-1. Comparison of Arugula Harvested Biomass Among Treatments in Experiment 1 and Experiment 2.



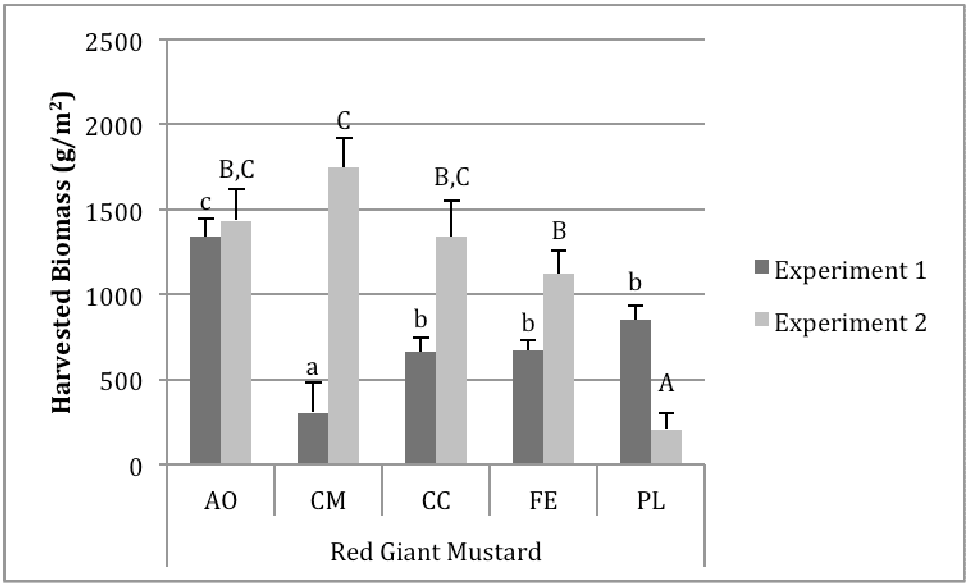
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-2. Comparison of Mizuna Harvested Biomass Among Treatments in Experiment 1 and Experiment 2.



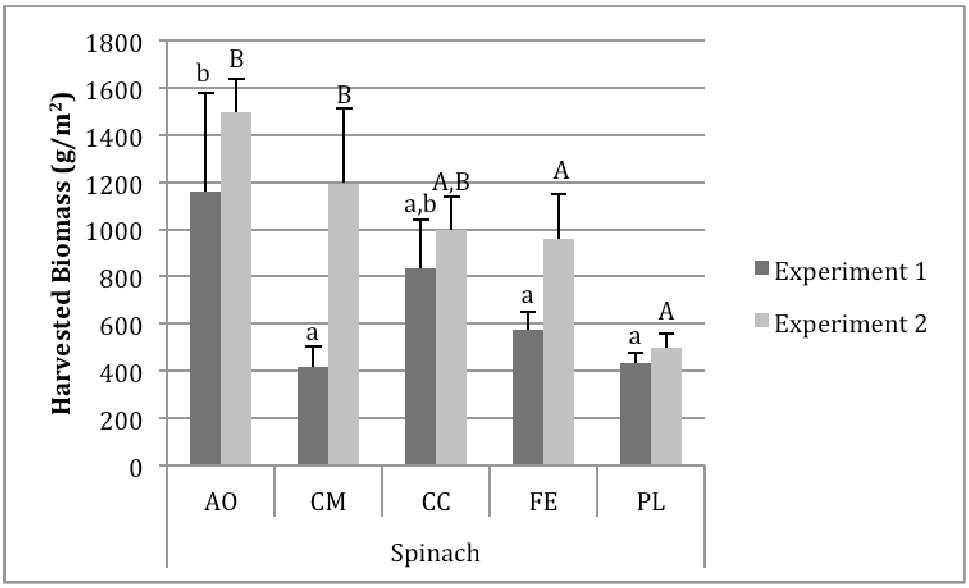
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-3. Comparison of Red Giant Mustard Harvested Biomass Among Treatments in Experiment 1 and Experiment 2.



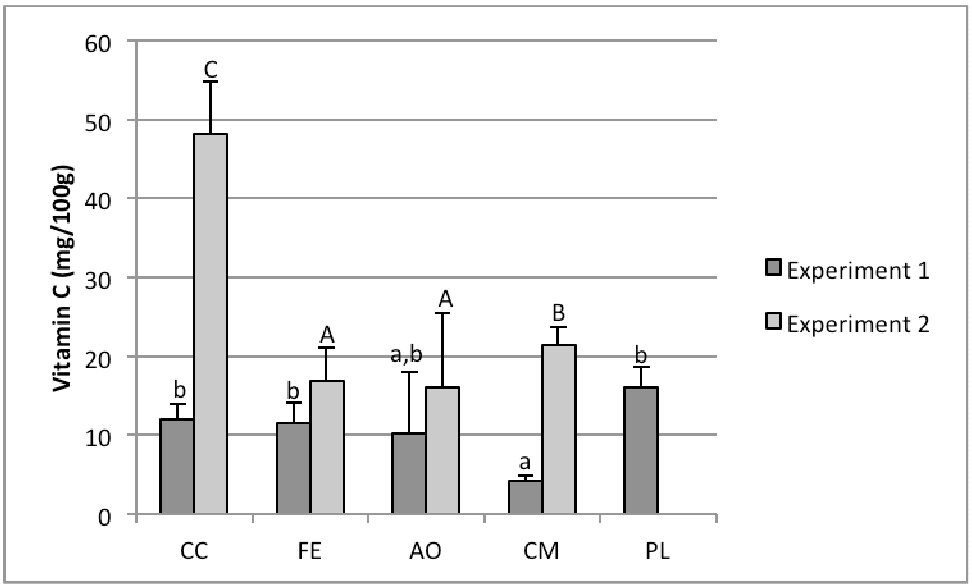
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-4. Comparison of Spinach Harvested Biomass Among Treatments in Experiment 1 and Experiment 2.



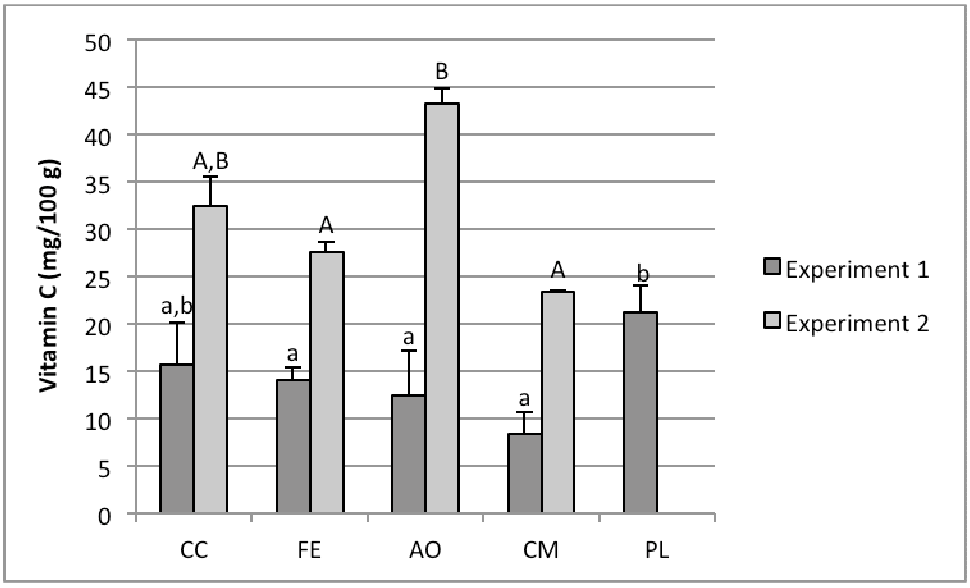
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-5. Mizuna Vitamin C Concentration (mg/100g Fresh Weight) in Harvested Biomass – Experiment 1 and Experiment 2.



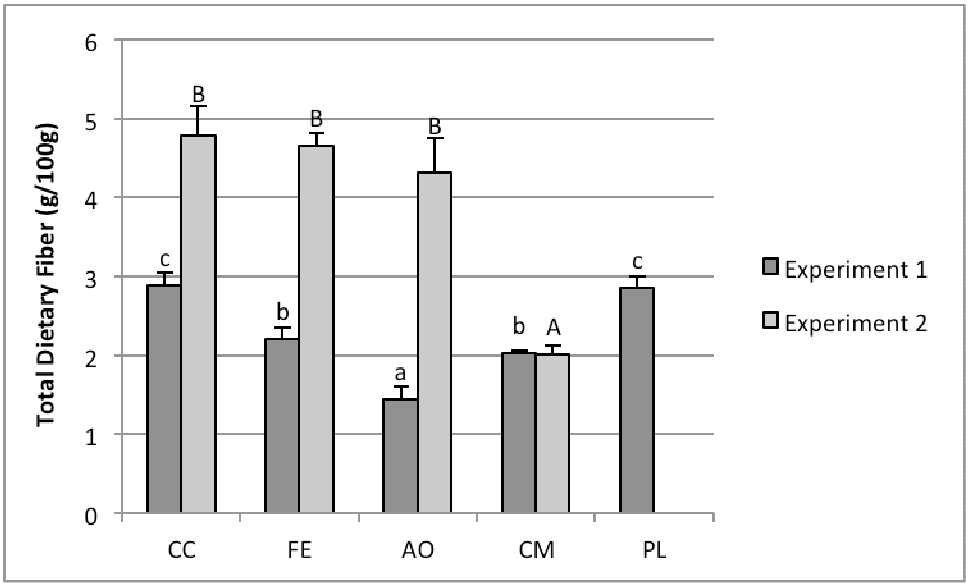
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-6. Red Giant Mustard Vitamin C Concentration (mg/100g Fresh Weight) in Harvested Biomass – Experiment 1 and Experiment 2.



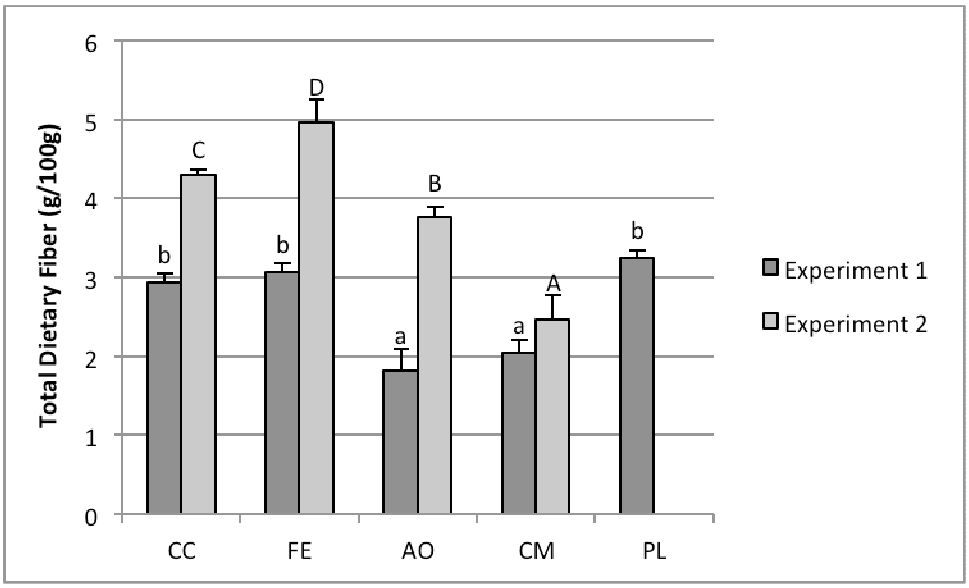
Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-7. Total Dietary Fiber in Mizuna (g/100g Fresh Weight) Determined with AOAC 991.43.



Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

Figure 6-8. Total Dietary Fiber in Red Giant Mustard (g/100g Fresh Weight) Determined with AOAC 991.43.



Data shown are mean ± standard deviation for two different replicate experiments. Significant differences among mean values are indicated with different letters above bars (p<0.05) with lower-case letters for experiment 1 and capital letters for experiment 2. Results analyzed using ANOVA Tukey HSD for significance testing.

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Appendix

Summary and Contribution to the Field

Prebiotic dietary fibers play a critical role in helping develop and maintain a healthy gut microbiome and improving digestive health. Because of their ability to ferment as a primary carbon source for beneficial taxa, prebiotics have the long-term potential to improve the gut microbiome. *In vitro* models provide mechanistic fermentation kinetics, laying the foundation for clinical feeding studies to further elucidate the health impacts that the fermentation of these compounds may have.

Key scientific contributions made as a part of these studies included the finding of partially hydrolyzed guar gum (PHGG) to increase the beneficial genera of *Parabacteriodes*, a key bacteria that has been shown in clinical studies to be inversely associated with irritable bowel syndrome (IBS) symptoms and disease. Previous to our findings, PHGG was known to be therapeutic for individuals affected with IBS, but this was for unknown reasons. In follow-up studies, our research was able to show that there can be over a 2-fold difference in SCFA production, potentially the contributing factor for why some people find PHGG consumption therapeutic, and others may not. This is hypothesized to be due to the differences in the gut microbiota between individuals, but with our small samples size, we were unable to determine any significant correlations between these differences in donors. Our experimental results supported our initial

hypothesis that PHGG was a readily fermentable fiber, provided a substantial amount of SCFA production, and provided growth to potentially therapeutic taxa.

All of the dietary fibers analyzed in this study are emerging prebiotics in that limited studies have been conducted to show their prebiotic capacity. Our studies were able to show that PHGG and wheat dextrin promote lactogenic and bifidogenic growth within 12 h of exposure, which was previously unknown. Of all the dietary fibers measured, the xylooligosaccharide fiber was the most bifidogenic fiber, and is the most effective prebiotic dietary fiber in that regard. Although these compounds promote the growth of beneficial bacteria *in vitro*, in still remains unknown if they have the same potential in clinical studies over longer durations. Future studies building on these findings should investigate the ability of these compounds to promote host health as part of a diverse, long-term diet. Studies should be formulated testing the hypotheses that xylooligosaccharides promote the growth of *Bifidobacteria* as part of a well-balanced, healthy diet *in vivo*, and that all the compounds included in these studies would still provide beneficial amounts of SCFAs when consumed daily as part of a healthy diet and lifestyle.

Investigating the effects that fertility treatments can have on nitrate accumulation and nutrients in produce grown in Minnesota is critical in determining the safety and effectiveness of these treatments. Our study was unique in that it examined the effects of fertility treatments currently being used in Minnesota by many growers. Based on these

findings, nitrate accumulation in the analyzed plants may be above recommended values, primarily due to excess nitrogen in the fertility treatments. Because of the differences in experiments, it is still unknown if certain fertility treatments can have consistent influences on the levels of minerals and vitamins in the harvested plants. Future studies should be formulated to test the hypothesis that certain organic fertility treatments can result in produce higher in nutritive value, with nitrate values in the harvested tissue in acceptable ranges for consumer health.