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Promoting Gastrointestinal Health and Decreasing Inflammation with Whole Grains in Comparison to Fruit and Vegetables through Clinical Interventions and in vitro Tests

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Promoting Gastrointestinal Health and Decreasing Inflammation with Whole Grains in Comparison to Fruit and Vegetables through Clinical Interventions and *in vitro* Tests

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The incidence of metabolic syndrome and its side effects are increasing throughout the world. Diet plays a key role in reducing the risk of metabolic syndrome, possibly mediated by metabolic activity of the gut microbiota. Whole grains (WG) and fruits and vegetables (FV) are two food groups that are commonly promoted for their ability to reduce the risk of metabolic syndrome. However, these two food groups provide vastly different nutrients that likely impact health via different mechanisms. Therefore, the primary objective of this research was to determine the impact of consuming recommended amounts of whole grains and fruit and vegetables against a background of their typical diet on inflammatory makers and gut microbiota composition in overweight or obese individuals that have low intakes of these food groups. The WG treatment significantly reduced tumor necrosis factor and lipopolysaccharide binding protein (TNF-α and LBP), while the FV treatment reduced IL-6 and LBP. Both treatments induced individualized changes in microbiota composition, with the FV treatment causing a significant increase in α-diversity. Thus, WG and FV both have the capability of decreasing inflammation associated with metabolic syndrome, but by different mechanisms. A follow-up in vitro study was performed using the fecal microbiota collected from subjects in the WG and refined grain (control) treatment

groups to gain insight into changes in metabolic activity of the microbiota that may help explain the reduction in inflammatory markers identified in the feeding trial. Microbiota collected at the end of the WG intervention tended to increase the fermentation of carbohydrates from WG compared with before the intervention. Correlation analysis suggested that changes in *Corpococcus* may be related to increased short chain fatty acids production by the microbiota and subsequent reduction in inflammatory markers. Thus, this *in vitro* approach enabled possible insight into the relationship between WG intake, changes in the gut microbiota, and reduced inflammation.

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Chapter 1 Introduction

It has been found that both whole grains (WG) and fruit and vegetables (FV) can reduce markers of metabolic disease, possibly mediated through activity of the gut microbiota (Martínez et al., 2013; North et al., 2009; Vetrani et al., 2016). However, no previous studies have compared these two food groups to determine their relative effects on human health. This thesis is focused on promoting gastrointestinal health and reducing subclinical inflammation in obese individuals though intake of WG products in comparison with FV. By comparing WG with FV, observations can be made regarding which food group may have the most positive impact on health.

Chapter 2 of this thesis reviews research that has been published about WG and FV. Key foci are clinical WG and FV interventions and epidemiological studies that address the metabolic benefits of WG and FV.

Chapter 3 of this thesis presents results from a feeding trial comparing the metabolic effects of consuming the recommended levels of WG or FV in comparison with refined grains (RG) in an overweight/obese population with habitually low intakes of WG and FV. The objectives were to 1) perform a 6 week feeding trial with WG, FV and RG (control) treatments; 2) determine changes in inflammatory markers, fecal microbiota composition, and microbial metabolite concentrations after treatments; and 3) determine how changes in inflammatory markers may be related to changes in the gut microbiota composition during the interventions.

WG and FV have a wide variety of nutritional benefits. Consumption of WG have shown to decrease inflammatory markers and increase beneficial bacteria in gut bacteria

(Ampatzoglou et al., 2016; Martínez et al., 2013; North et al., 2009). Epidemiological studies correlated WG consumption to lowering metabolic type diseases (D. et al., 2003; McKeown et al., 2009). FV interventions and epidemiological studies have connected FV to lowering risk of metabolic syndrome through decreased inflammation (Holt et al., 2009; Mirmiran et al., 2012; Yeon et al., 2012). Based on WG and FV epidemiological and intervention studies, I hypothesize that the WG intervention will be as effective in improving beneficial bacteria and reducing subclinical inflammation compared to a FV intervention, both of which will be better than RG.

Chapter 4 of this thesis describes results from an *in vitro* fermentation experiment using stool samples collected from the individuals in the WG and RG treatments at baseline and at the end of the study. These stool samples were used as a source of gut bacteria for in vitro fermentation of pre-digested whole wheat flour to determine changes in carbohydrate fermentation and SCFA production by the microbiota as a result of the intervention. Previously, others have shown that individuals with higher habitual intake of plant foods (like WG and FV) harbored a microbiota that was better equipped to ferment the dietary fibers in WG and produce more butyrate compared with the microbiota from individuals with lower intake of plant foods (Brahma et al., 2017). Therefore, I hypothesize that the fecal microbiota collected from subjects after a WG intervention will be better able to ferment the dietary fibers in whole wheat and produce butyrate compared with before the intervention.

The final chapter of this thesis includes overall conclusions and prospective research ideas.

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Chapter 2

Abstract

The importance and benefits of fruits and vegetables (FV) in a healthy diet is well accepted; however, the importance and benefits of whole grains (WG) are less recognized. WG and FV clinical interventions have shown to increase beneficial gut microbiota, increase short chain fatty acid (SCFA) production, and lower anti-inflammatory markers. Epidemiological studies have also tied WG and FV to decreasing anti-inflammatory markers and lowering risks of obesity, metabolic syndrome, and associated diseases. What is less known is how whole grains, fruit and vegetables are lowering the risks for metabolic syndrome and such diseases. This review will explain and compare the key benefits of whole grain, fruit, and vegetables with evidence from clinical trials and epidemiological studies. The results will show that FV clinical trials tend to decrease anti-inflammatory markers and through epidemiological studies, WG are linked to decreased risks for obesity, metabolic syndrome, and associated diseases.

Introduction

Fruit and vegetables (FV) contain a wide variety of vitamins, minerals, and polyphenols that can influence human health (Lampe, 1999). A few of these nutrients include vitamin C, folate, dietary fiber, beta carotene and flavonoids. Different FV have unique nutritional profiles; therefore, an assortment is recommend to promote health (Slavin et al., 2012)

FV have been linked to lowering risks of metabolic syndrome by lowering inflammation. Correlations have been made to explain some interactions between fruit and vegetable components and inflammatory markers. Within a population of 265 adolescent girls and boys, C-reactive protein (CRP) inversely correlated with intake of fruit, vitamin C, and folate. Interleukin 6 (IL-6) inversely correlated with intake of legumes, vegetables, beta carotene, and vitamin C (Holt et al., 2009). CRP and IL-6 are indicators of inflammation and are markers of increased risk of metabolic diseases (Marsland, McCaffery, Muldoon, & Manuck, 2010).

Whole grain products contain the germ, endosperm, and pericarp (bran) of the seeds (Oldways Whole Grains Council, 2017). Whole grains contain dietary fiber, phytoserols, and vitamin E that have been shown to improve health through lowering blood cholesterol levels, heart disease, stroke, obesity, and type 2 diabetes (Anderson et al. 2009; Prasad 2011; Slavin et al., 2012).

There have been numerous feeding trials to investigate the benefits of WG and FV. Many of these trials focus on individual food groups (vegetables or whole grains), rather than connecting different food groups. For instance, there are not many published studies comparing the benefits of WG and FV in a feeding trial. The purpose of this

review was to investigate the benefits of WG and FV with respect to lowering metabolic syndrome through inflammatory makers and gut microbiota composition.

Benefits of Fruits and Vegetables

Fruit and Vegetables

There are over 4,000 different fruit and vegetables in the world. The 5 top fruits consumed in the U.S are bananas, apples, berries, melons, and oranges, and the top 5 vegetables are potatoes, lettuce, onions, tomatoes, and carrots ("Produce for Better Health Foundation," 2015). The reference daily intake (RDI) of FV is 5 servings (about ½ c each) (United States Department of Agricutlure, 2017).

FV have a wide variety of nutrients that can help lower risks of metabolic syndrome. For instance, vitamin C plays major roles as a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine, and neurotransmitters, helps metabolism of cholesterol to bile salts, and helps improve absorption of Fe by keeping it in its reduced state (Fe^{2+}) (Carr et al.,1999). A cross sectional study was performed on 22,671 adults, and it was found that high intakes (≥85 mg/day) of vitamin C and high physical activity can lower the risk of metabolic syndrome (Kim et al., 2016).

Flavonoids are phytochemicals found in fruits and vegetables. There are over 20 different flavonoids in fruits and vegetables (Harnly et al., 2006). Flavonoids have been associated to lower the risk of stroke, coronary heart disease, and markers of inflammation (Holt et al., 2009). Flavonoids have been shown to decrease plasma cholesterol, hyperinsulinemia, and blood glucose in diabetic rats (Mulvihill & Huff, 2010).

Folate consumption has been associated with decreased risk of certain types of cancer, heart disease, and stroke (United States Department of Health & Human Services, 2016). The RDI of folate is 400 mg/d, which assists with RNA and DNA synthesis and metabolizing amino acids. In a folic acid supplementation epidemiological study, meta-analysis was able to indicate a 10% lower risk of stroke and 4% lower risk of overall cardiovascular disease (Y. Li et al., 2016).

On average, fruit or vegetables have about 4 grams of dietary fiber per 100g depending on the FV (United States Department of Agriculture, 2017a). Depending on the type of fruit and vegetable the fiber content changes and the ratio of insoluble and soluble fiber changes as well. The main insoluble fibers in FV are lignin, cellulose, and xyloglucan, and the soluble fibers are inulin and pectin. Many epidemiological studies focusing on FV fiber have shown non-significant decreases in metabolic syndrome related diseases (N. M. McKeown et al. 2009; Rimm EB et al. 1996; Schulze et al. 2007).

Fruit and Vegetables Clinical Trials and Epidemiological Studies

There have been many clinical interventions that investigate the changes in immune responses and inflammatory markers in response to consuming FV (Table 1). In a low (1 serving per day) verses high (12 servings per day) fruit and vegetable diet clinical study, IL-6 was significantly decreased in the high fruit and vegetable diet and hs-CRP was increased in the low fruit and vegetable diet (Yeon et al. , 2012). This study also showed that there is a relationship between high fruit and vegetable diet and lowering lymphocyte DNA damage and proinflammatory cytokine production, which reduces risks of metabolic diseases. A study where subjects had an option of a wide variety of 8 servings of FV per day showed a significant decrease in hs-CRP (Watzl et al.,

2005). In a dietary intervention with 10 g of broccoli sprouts with sulforaphane (225 µmol concentration), the intervention was shown to significantly reduced IL-6 and TNF-α and significantly lower hs-CRP (Mirmiarn et al. 2012). In a dietary intervention of wild blueberry juice (25g wild blueberry powder), it was shown that inflammatory markers were not significantly decreased but levels of endogenously oxidized DNA bases were reduced (Riso et al., 2013). These studies suggest that individual fruits and vegetables could have significant effects on inflammatory markers. These results suggest that people consuming increased levels of fruits and vegetables could potentially have lower inflammatory markers, thus, lower chances for inflammatory diseases.

Large scale epidemiological studies have shown the benefits of high FV diets in reducing inflammatory markers and the risk of chronic disease (Table 3). Holt et al., (2009) was able to show inverse correlations between CRP and intake of fruit, vitamin C, and folate, as well as an inverse relationship between IL-6 with intake of legumes, vegetables, beta carotene, and vitamin C. The intake of fruit and vegetables has also been inversely linked to lowering risks of coronary artery disease and certain cancers (Riboli et al., 2003; Steffen et al. 2003). Clinical trials and epidemiological studies have shown the significant effects of FV on inflammatory diseases, cancer, and metabolic health.

Benefits of Whole Grains

Whole Grains

The most commonly consumed whole grains are brown rice, wheat, oats, corn, rye, and barley. The most consumed whole grain products in the U.S are whole wheat bread, oatmeal, popcorn, whole grain cold cereal, and whole grain pasta (Oldways Whole Grain Council, 2015.). The current recommendation of whole grains is at least three servings (1 serving = 1 ounce) per day**.** Certain whole grains are good sources of dietary

fiber, vitamin E, and phytosterols (Harvard School of Public Health, 2017). The main soluble dietary fiber in WG are, ß-glucan, and inulin, and the main insoluble dietary fiber are cellulose, arabinoxlyan, and lignin.

Arabinoxlyan (AX) is a non-starch polysaccharide that is found in many cereal grains. It is the one of the most abundant polysaccharides in the cell wall (Mendis et al., 2016). In the body, purified AX helps control blood glucose and insulin and improved postprandial metabolic responses (Garcia et al., 2007). *Bifidobacterium*, *Bacteroides*, and *Clostridium* have shown significant increases in fermenting AX in the gut (Mendis et al., 2016).

The main source of ß-glucan is in the cell walls of barley and oats. Consuming 3 g or more ß-glucan per day has been shown to lower cholesterol levels (Anderson et al., 2009). Other benefits include lowering dyslipidemia, hypertension, and obesity (Khoury et al., 2012).

Cellulose is an insoluble fiber that acts as a bulking agent that helps move food through the digestive system. This promotes regular bowel movement and can help reduce constipation. Cellulose can also be fermented in the gut by *Rumminicous* (J. et al., 2013).

One of the notable phytonutrients in whole grains is vitamin E. This antioxidant can inhibit lipid peroxidation and reactive oxygen species production. One study showed that vitamin E is linked to lowering CRP levels, which is a marker for inflammation associated with heart disease. Vitamin E can also lower cholesterol by down regulating HMG-CoA reductase in cholesterol synthesis (Parsad, 2011).

Phytosterols are plant based sterols that are a subgroup of steroids. In whole grain products phyotserols reduce serum cholesterol (Salvin, 2003). They can inhibit dietary and biliary cholesterol absorption by displacing cholesterol from micelles which increases excretion. The average western diet includes 200-300 mg/d of plant sterols. The consumption of whole grains high in phytoserols, rye bread and wheat germ, could increase phytoserol level in the body. Consumption of 2-3 grams per day of phytoserols can lower an individual's LDL by 10% (Mendis et al., 2016).

Whole Grain Clinical Trials and Epidemiological Studies

Although wheat-based food products, even those made with whole grains, have been implicated as causative agents for obesity, diabetes, and many other diseases in the popular literature (Davis 2011), scientific studies have found that wheat-based products lower the risk of type 2 diabetes and cardiovascular disease (Figure 2). Some studies have investigated the influence of a diet rich in whole grain products on the gut microbiota composition (Costabile et al., 2008; J. et al., 2013; Martínez et al., 2013). Intake of high fiber whole grain rye bread and low fiber wheat bread in Finnish adults during a 12-week intervention decreased *Bacteroides vulgatus, Bacteroides plebeius,* and *Prevotella tannerae* and increased *Clostridum cellulosi, Clostridum leptum, Anaerotruncus colihominis* (Lappi et al. 2013). Clinical trial treatments with increased consumption of whole grain and wheat bran cereals increased Lactobacilli/enterococci and *Bifidobacterium* (Costabile et al., 2008). In another 17 week cross over study with treatments of whole grain, barely, and rice, Bacteroidetes decreased and Firmicutes increased (Martínez et al., 2013).

Bacterial changes and SCFA production could be impacting anti-inflammatory markers. Ampatzoglou et al, (2016) showed a significant decrease in IL-10, CRP, and

insulin with a treatment of 80 grams whole grain per day. Through an intervention of 60 grams of whole gran barely and brown rice, IL-6 was lowered (Martínez et al., 2013).

Numerous epidemiological studies have been completed to investigate the intake of whole grains with metabolic health (Table 3). Whole grain consumption has been associated to lower body mass index (BMI), adipose tissue, and obesity (J. Davis, Alexander, & Ventura, 2009; Nicola M McKeown et al., 2009; van de Vijver, van den Bosch, van den Brandt, & Goldbohm, 2009). Ma et al. (2008) showed that total intake of dietary fiber, soluble fiber, and insoluble fiber was inversely related to CRP concentrations. Lastly, the intake of whole grains and their fibers have been associated with lower risks of cardiovascular diseases and Type 2 diabetes (D. et al., 2003; Rimm et al., 2004; M B Schulze et al., 2007). Through clinical interventions and epidemiological studies whole grains have shown to be associated with to lower metabolic syndrome through the interactions of gut bacteria fermentation and inflammatory markers.

Comparison of WG and FV Diets

Reviewing FV and WG clinical studies, FV treatments tended to show more consistent decreases in anti-inflammatory markers (Figure 2.1-2.2). Five out of the eight FV studies reviewed showed decreases in anti-inflammatory markers with FV treatment. Two out of the nine WG clinical trials reviewed showed decreases in anti-inflammatory markers with WG treatment.

The WG treatments tended to show more significant changes in gut microbiota and production of SCFA. Four studies saw significant changes in gut microbiota. These changes were seen by increases or decreases in OTUs, increased microbial diversity, and changes in individual taxa. Five studies had changes in SCFAs or branched chain fatty

acids (BCFAs). Most studies involving FV did not report changes in gut microbiota and SCFA production. Only 1 FV study reported changes in the gut microbiota, and this was an overall bacterial community shift.

Not many intervention studies have shown the relationship between increasing beneficial bacteria with the link of decreasing anti-inflammatory markers. Martinez et al., (2013) showed this relationship with a treatment of 60 grams of whole grain barely and brown rice by an increase in the ratio of Firmicutes/Bacteroidetes, and a change in abundance of the genus *Blautia* with a decrease of IL-6. This shows that certain bacteria could have an impact on lowering anti-inflammatory markers. On the contrary other studies have shown that Firmicutes/Bacteroidetes do have impacts on certain fibers and obesity could impact the certain bacteria ratios like Firmicutes/Bacteroidetes (Brandt et al., 2012; Flint et al., 2012).

Throughout the FV and WG clinical trials there are variable results. Each clinical trial had a unique treatment to their study. In the FV trials some studies were investigating certain FV while others were investigating a wide variety of FV. This could be one of the main causes of their variable results. The WG interventions also had studies focusing on a particular food option as well as using a wide variety of WG. The treatments also were giving different levels of whole grains or fiber. All these factors would be creating the variable results.

Through the reviews of epidemical studies where both whole grain, fruit, and vegetable intakes were analyzed, WG intake showed more significant relationships with metabolic syndrome and associated diseases. Five out of the five studies comparing WG and FV within the same study showed WG intake was more strongly related to reduced

risks of cancer, obesity, and cardiovascular diseases. Thus, it can be concluded that FV clinical treatments have shown greater reductions in anti-inflammatory markers, which help lower metabolic syndrome compared to WG consumption, while epidemiological studies have linked WG consumption to reduced risk of metabolic disease more so than FV consumption.

Table 2.1 Review of Clinical Trials with Fruit and Vegetables Interventions

Author	Subject Characteristics	Feeding Trial and Treatments options	Time periods	Not Significant Results	Significant Results
Basu et al., 2010	Twenty-seven subjects with metabolic syndrome, 2 males and 25 females; BMI: 37.5±2.15 kg/m2; age: 47.0±3.0y	4 cups freeze-dried strawberry beverage (50g freeze-dried strawberries ~ 3 cups fresh strawberries in 2 cups water	Eight weeks in a randomized controlled	glucose, triglycerides, HDL-cholesterol, blood pressure, and waist circumference, dietary intake differences	↓ total and LDL-cholesterol, vascular cell adhesion molecule-1 (VCAM-1),
Li et al. 2009	12 women and 5 men healthy, nonsmoking men and women, 20– 40 y old, and recruited on the basis of GSTM1, GSTT1, and	4 diets 1) low-phytochemical basal diet devoid of fruits and vegetables and whole-grain, high-fiber foods [major food items consumed included bagels, pasta, white rice, ready-to-eat cereal, dairy 2) a "single-cruciferous" diet [i.e. basal diet + 7 g	Randomized, crossover, controlled feeding study, 2 14d intake	Total energy, Carbohydrate, Fat, Protein,	Overall bacterial community composition differed between the 2 consumption periods Bacterial community was individual specific in vegetable diet ↑ Urinary TIC excretion, fiber consumption tRFLP fragments that were significant

	CYP1A2 genotypes.	cruciferous vegetables/(kg body weight×d) 3) “double-cruciferous diet” [i.e. basal diet + 14 g cruciferous vegetables/(kg body weight×d) 4) a mixed diet [i.e. basal diet + 7 g cruciferous vegetables/(kg body weight×d) and 4 g apiaceous vegetables/(kg body weight×d)			correlated with double vegetable Firmicutes, Bacteroidetes, and Actinobacteria
Mirmiran et al. 2012	81 type 2 diabetic patients,	Broccoli sprouts powder (BSP) with high sulforaphane concentration Treatments 1- 10g/d 2- 5g/d 3- placebo	Random 4 week study	Fasting blood glucose	↓ C-reactive protein in all BSP groups ↓ non-significant decrease in IL-6 ad TNF-α between treatment groups but significant in group A
Ngondi et al. 2009	102 healthy, overweight and/or obese volunteers	150 mg of IGOB131 (wild African Mango extract) twice a day	Randomized, double-blind, placebo controlled design for 10 weeks	All results were significant	↓ C-reactive protein , LDL, total cholesterol, weight, waist, leptin, Glucose, adiponectin
Pierce et al. 2007	3088 women previously treated for early stage breast cancer who were 18 to 70	Telephone counseling program, cooking classes, and newsletters targeted to get participates to consume 5 vegetable servings, 16 oz vegetable juice, 3 fruit	Multi-institutional randomized controlled trial of dietary change	baseline demographics, characteristics of the original tumor, baseline dietary pattern, breast cancer treatment	↑servings of vegetables, +65%; fruit, +25%; fiber, +30%, and energy intake from fat, -13%. No other significant changes

	years old at diagnosis	servings and 30 g of fiber a day			
Riso et al. 2013	20 Male, healthy subjects with at least one risk factor for cardio vascular disease (CVD) based on American Heart Association guidelines, needed to consume2< >5 portions of FV/day)	WB-25 g of wild blue berry powder mixed in 250 ml of water PL -250 mL water, 7.5 g fructose, 7 g glucose, 0.5 g citric acid and 0.03 g blueberry flavor	6 week cross-over design, 6 week wash out period	Vitamin C, folate, Vitamin B12, blood pressure, plasma total nitric oxide and soluble vascular cell adhesion molecule-1, weight, BMI, glucose, TG, LDL, HDL, IL-6, CRP, TNF-α	No significant results besides reducing the levels of endogenously oxidized DNA bases
Watzl et al. 2005	Sixty-four healthy men, nonsmoker and didn't use dietary supplements or medication	1 of 3 treatment groups 1) 2 servings/d 2) 5 servings/d 3) 8 servings/d Vegetable intake included carrots, green beans, peas, broccoli, zucchini, tomatoes, kohlrabi, Brussels sprouts, red cabbage, cauliflower, spinach, corn, and salsifies. Salad intake included lettuce, tomatoes, carrots, corn, radishes, cucumbers, fennel, and cabbage.	Randomized longitudinal trial of two 4-wk treatments	Plasma Vitamin C, Vitamin E, weight, BMI, energy intake, protein, fat, carbohydrates, cholesterol, LDL, HDL, TAG, blood glucose, IL-2, interferon, IL-13, IL-12, TNF-α, NK cell cytotoxicity (%) NKp46+, CD3-/CD56+, CD3+, lymphocyte proliferation	↓ C-reactive protein in the treatment with 8 servings/d of FV a day compared with 2 servings/d

		Fruit intake consisted of apples, pears, kiwis, bananas, peaches, nectarines, cherries, strawberries, and red currants.			
Yeon et al., 2012	22 overweight women, non-smoker and didn't use dietary supplements or medication	Low FV diet 1 serving of fruits and 1 serving of vegetables High FV diet- 6 servings of fruits and 6 serving of vegetables The composition of vegetable and fruit in high-VF diet consisted of 2 servings of dark green vegetable; 2 servings of dark orange vegetable; 2 servings of dark red product; 2 servings of other vegetables; 2 servings of vitamin C-rich fruits/ juices (e.g., orange, strawberries)	2 week dietary treatment with a 2 week wash out		Significant change in decreasing IL-6 and IL-1 between treatment groups

Table 2.2 Review of Clinical Trials with Whole Grains Interventions

Author	Subject Characteristics	Feeding Trial	Treatments options	Time periods	Not Significant Results	Significant Results
Ampatzoglou et al. 2016	12 men, 21 women; ages 40–65 y, BMI: 20–35 kg/m2, habitual WG consumption <24 g/d	WG High diet- >80 g/day Low diet-16 g/day	Bread, rice, pasta, snacks, breakfast cereals	6 week cross over study	IL-1, IL-6, IL-8, TNFα, CD8+, ghrelin, GIP, GLP-1, glucagon, leptin,	↑ WG Consumption ↑ Fiber intake ↓ IL-10, CRP, insulin, CD4+ T cells, C-peptide, PAI-1
Brownlee et al. 2010	316 18-65 years, BMI 25, consume <30 g WG/;d	Control (no dietary change), intervention 1 (60 g WG/d for 16 weeks) and intervention 2 (60 g WG/d for 8 weeks followed by 120 g WG/d for 8 weeks)	An option of whole wheat bread, shredded wheat, cheerios, porridge oats, brown basmati rice, whole wheat pasta, weetabix porridge, oat bar, chips	16 week randomized, controlled dietary intervention	Total cholesterol, HDL, LDL, TAG, glucose, insulin, NEFA, QUICKI, R-QUICKI, sialic acid, CRP, IL-6, fibrinogen, PAI-1, ICAM-1, VCAM-1, E-selectin, systolic BP, diastolic BP, weight, waist, body fat percentage	No changes in biomarkers of CVC health ↑ WG Consumption Good compliance
Cooper et al., 2017	25 female and 21 male healthy adults; between the ages of 19 to 46 years with BMI 20 to 28 kg/m2, “low whole grain consumers” < 1 serving of whole	Based on estimated energy output ex:2000 kcal per day WG - 13.7 g fiber/d RG- 4.2 g fiber/d	Bread, rice, pasta, snacks, breakfast cereals, tortilla, baking mixes	6 week study randomized, controlled dietary intervention	BMI, HDL, triglycerides, gastrointestinal symptoms, microbiota relative abundance in a particular taxa,	No significant changes in gut bacteria ↓ LDL, non HDL cholesterol, fasting blood glucose ↑ bowel movement

	grains/whole grain products per day,					
Costabile et al. 2008	32 healthy adults	48 g breakfast cereal Whole grain Wheat bran	cereal	Double-blinded randomized crossover study, 6 week	Feacal SCFA,, fasting blood glucose, insulin, total cholesterol, TAG, HDL, stool habits	↑ *Bifidobacteria* and in whole grain cereal ↑ *Lactobacilli/enterococci* with whole grain and whole bran cereal ↑ WG Consumption ↑Feurlic acid both treatments
Lappi et al., 2013	25 males, 26 females; 40–65 y, a BMI of 26– 39 kg/m2, and at least 3 other features of metabolic syndrome:	RG- Rye breads high in fiber WWB – refined wheat breads low in fiber	Breads	randomized, parallel, 2-arm 12-wk intervention whether	Intestinal microbiota composition did not significantly differ between the groups, certain nutrients,	↑ WG Consumption ↓ plasma Androgen receptor
Martinez et al., 2013	17 females and 11 males healthy adults; age 25.9±5.5 years	WGB- 60 g of whole grain barely BR- 60 g of brown rice WGB+BR- 30 g brown rice, 30 g of whole grain barely	Whole grain barely , Brown rice	randomized cross-over trial with four-week treatments	Cholesterol, HDL, non-HDL, hs-CRP, LBP, fasting blood glucose and insulin, microbial diversity Chao1,	↑ microbial diversity Shannon's and Simpson's indices ↑ Firmicutes, Incertae Sedis XIV,*Blautia, Roseburia, Bifidobacterium, Dialister* ↓ Bacteroridetes, Bacteriodaceae, Porphyromonadaceae, *Odoribacter* ↓OUT *Odoribacter splanchnicus* ↑Eubacterium *rectale*, Roseburia *faecis*, Roseburia *intestinalis*, Blautia *wexlerae*, Blautia *spp*, Eubacterium *rectale* ↓ in IL-6 in BR, BR+WGB treatment groups

McOrist et al. 2011	16 males, 30 females; age 55.9 ± 2.0 y (range 31–66); body weight 79.5 6 3.6 kg (range 57–109). Illnesses and participants on antibiotics were excluded	The NSP diet ~25 g of NSP/d- as 50 g BranPlus, 50 g cooked carrots, 50 g couscous, and 2 g unprocessed bran The RS diet supplement ~22 g of RS/d - 25 g of NSP/d as an extruded, high-amylose barley breakfast cereal, 50 g of a commercial canned legume preparation, 50 g Greenwheat Freekeh and 20 g Hi-maize	Carrots, couscous, bran, barely breakfast, legume hi maize, freekeh	randomized cross-over study, 4 week	Age, weight, height, BMI, diet, propionate, ammonia, fecal, pH and moisture	↑ acetate, butyrate, and total SCFA in RS diet compared to NSP diet and entry Fecal butyrate and ammonia excretions were positively associated ↑ fecal weight
Sheflin et al. 2015	7 adults, no history of food allergies or major dietary restrictions, not currently taking cholesterol-lowering medications or non-steroidal anti-inflammatory drugs (NSAIDs)	SRB-7 meals and 6 snack options with 30 g/d of SRB Non SRB-7 meals and 6 snack options with similar nutrient content but no SRB	Meals or snacks made with heat stabilized rice bran (SRB)	four-week, pilot, randomized-controlled, single-blinded dietary SRB intervention study	Phyla level bacteria, SCFA, acetate, propionate, , valeric, caproic and heptanoic acids were	Increases in eight operational taxonomic units (OTUs), including three from *Bifidobacterium* and *Ruminococcus* genera ↑Isovalerate, isobutyrate, secondary bile acids in SRB ↓Butyrate Various stool metabolites that had significant correlations

Vanegas et al. 2017	49 men and 32 postmenopausal women [age range: 40–65 y, BMI from 20 to 35, a report of the consumption of a low-fiber diet (men: ,<7 g/1000 kcal; women: ,8 g/1000 kcal) for >2wk before enrollment	Meals high in WG (16 g/1000 kcal) or RG (8 g/1000 kcal)	Western Style Meals- high protein and high fat	2 week run in then 6 week diet	Stool characteristics, propionate, butyrate, α and ß-diversity, relative abundance at phyla level, IgA, DTH, IFN, IL-17, TNF-α, IL-6, TGF-ß, white blood cells, lymphocytes, monocytes, eosinophils, ba-sophils, or neutrophils	WG compared to the RG diet ↑plasma total alkyresorcinols, stool frequency, Changes in acetate and total SCFA, percentage of terminal effector memory T cells and LPS-stimulated ex vivo production of tumor necrosis factor-a, Positive correlation between *Lachnospira* with acetate and butyrate
Vetrani et al. 2016	23 men and 31 women overweight/obese with metabolic syndrome	Whole grain cereal Refined grain cereal	Cereal products	12 week dietary intervention	glucose, BMI, HOMA, TAG, cholesterol, HDL, hs-CRP, IL-1 ra, IL-6, and TNF-α, and SCFAs	↓ postprandial insulin ↑in fasting plasma propionate concentrations

Table 2.3 Review of Epidemiological Studies Associated with FV and WG intake and Metabolic Health

Study	What Investigating	Parameters	Results relating to WG, RG, and FV	Improved health from WG or FV
Bradlee et al. 2010	Food group intakes associated with central obesity anthropometry among children and adolescents (covariance modelling with PROC GLM)	3761 children and 1803 adolescents with single 24h dietary recalls and anthropometric measures of central body fat	Adolescents' central body fat measure were inversely associated with grain intake. Central obesity adolescents consumed significantly less total grains and FV	WG
Cassidy et al. 2010	Diet, lifestyle factors and telomere length (change in z score)	2284 female participants	Dietary fiber intake was positively associated with telomere length specifically cereal fiber FV was not significant	WG
D. et al. 2003	Examine fiber consumption from fruit, vegetable, and cereal sources (including whole grains and bran) is associated with incident CVD in elderly persons	3588 men and women aged 65 years or older and free of known CVD at baseline	Cereal fiber consumption was inversely associated with incident CVD. Fruit and vegetable fiber were not associated with lowering CVD.	WG
Davis, Alexander et al., 2009	Changes in dietary intake with changes in adiposity and risk factors for type 2 diabetes (Partial correlations)	Overweight Latino youth (n = 85; aged 11–17 y)	Increases in total dietary fiber and insoluble fiber were associated with decreases in visceral adipose tissue FV was not tested	WG

Holt et al. 2009	Intakes of fruit and vegetables, antioxidants, folate, and total flavonoid with associated with markers of inflammation and oxidative stress (Spearman Correlations Coefficients)	285 adolescent boys and girls, 13-17 yrs	Urinary F2-isoprostane was inversely correlated with intakes of total fruit and vegetables, vitamin C, beta carotene, and flavonoids. CRP was significantly inversely associated with intakes of fruit, vitamin C, and folate. IL-6 was inversely associated with intakes of legumes, vegetables, beta carotene, and vitamin C. TNF-α was inversely associated with beta carotene and lutein. WG were not tested	FV
Ma et al. 2008	Examine longitudinal associations between dietary fiber intake and CRP (simple linear regression analysis)	524 subjects, 20 and 70 y	Inverse association between intake of total dietary fiber (separately for soluble and insoluble fiber) and CRP concentrations in both cross sectional and longitudinal analyses FV not tested	WG
McKeown et al. 2009	Examine the association between whole and refined grain intake and measures of body fat distribution and the relations between cereal, fruit and vegetable fiber, and body fat distribution (Multivariate-adjusted means (95% CI))	434 free-living adults (177 men and 257 women) aged between 60 and 80 y	Cereal fiber was inversely related to BMI, body fat, and trunk fat mass while fruit and vegetable fiber were not significant related.	WG
Riboli et al., 2003	Examine evidence from case-control and prospective studies	Case-control or cohort studies evaluating the	Fruit and vegetables reduce your risk of esophagus, lung, stomach, and colorectum cancer.	FV

	on fruit and vegetable intake and cancer risk with	relationship between total vegetable and/or total fruit consumption and risk of cancer (esophagus, larynx, stomach, colon and rectum, breast, lung, and bladder); with incidence or mortality as the endpoint	WG was not tested	
Rimm EB et al. 1996	Examine the relationship between dietary fiber and risk of coronary heart disease. (Relative Risk (95% CI))	43 757 US male health professionals 40 to 75 years of age and free from diagnosed cardiovascular disease and diabetes completed	A 10-g increase in total dietary fiber was associated to decreasing your relative risk for myocardial infarction. When comparing fruit, vegetable and cereal fiber, cereal fiber was more associated but not significantly.	WG
Schulze 2007	Examined associations between fiber and magnesium intake and risk of type 2 diabetes (Relative Risk (95% CI))	9702 men and 15 365 women aged 35 to 65 years who were observed for incident diabetes	Relative risk of Type 2 diabetes was significantly lowered with cereal fiber. Fruit lowered RR but not significantly. Vegetable fiber increased the RR for Type 2 diabetes but not significantly.	WG

Steffen et al. 2003	Examine relations of whole-grain, refined-grain, and fruit and vegetable intakes with the risk of total mortality and the incidence of coronary artery disease (CAD) and ischemic stroke (Multivariate-adjusted hazard rate ratios (and 95% CIs))	11 940 participants (4083 white men, 4754 white women, 118 8African American men, and 1915 African American women)	FV intake were inversely associated with coronary artery disease among the African American population but not white population. Whole grains were inversely associated with total mortality and incident of coronary artery disease.	FV and WG
van de Vijver et al. 2009	Whole grain and cereal fiber intake with BMI and risk of being over weight (Regression coefficients)	2078 men, 2159 women, 55-66 years old, no cancer	Inverse association between WG consumption BMI and risk of overweight and obesity FV was not tested	WG

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Chapter 3: Promoting Gastrointestinal Health and Reducing Subclinical Inflammation in Overweight and Obese Individuals through Intake of Whole Grain Products in Comparison With Fruits and Vegetables

Abstract

A randomized parallel arm feeding trial was completed on forty-nine overweight or obese subjects with typically low intake of fruit and vegetables (FV, <2 servings/d) and whole grains (WG, <1 serving/d). Individuals were randomized into three groups: WG (3 servings/d; n=17), FV (5 servings/d; n=18), and refined grains (RG; 3 servings/d; n=14). Treatment foods were incorporated into the subjects' overall diets. Stool and blood samples were collected at the beginning of the study and after 6 weeks. In the blood samples, inflammatory markers [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), lipopolysaccharide binding protein (LBP), and high sensitivity C-reactive protein (hs-CRP)] were measured. Stool sample analysis included short/branched chain fatty acids (S/BCFA) and microbiota composition. There was a significant decrease in LBP on both the WG (-0.2 μg/mL, p=0.02) and FV (-0.2 μg/mL, p=0.005) diets, with no change on the RG diet (0.1 μg/mL, p=0.08). The FV diet induced a significant change in IL-6 (-1.5 pg/mL, p=0.006), but no significant change in the other treatments (RG, -0.009 pg/mL, p=0.99; WG, -0.29, p=0.68). The WG diet resulted in a significant decrease in TNF-α during the intervention (-3.7 pg/mL; p<0.001), whereas no significant effects were found on the other diets (RG, -0.6 pg/mL, p=0.6; FV, -1.4 pg/mL, p=0.2). The feeding treatments induced highly individualized changes in microbiota composition such that treatment group differences were not identified, except for a significant increase in α-diversity in the FV group. These data support the positive impact that WG and FV intake can have on metabolic health in overweight or obese individuals with normally low

intake of WG and FV, although it appears that these effects are mediated via different mechanisms

Introduction

Poor diet is the leading risk factor for premature death and disability in the United States (US Burden of Disease Collaborators, 2013). Poor diets lead to metabolic syndrome and its associated diseases such as heart disease and diabetes, which rank first and seventh among common causes of death, respectively (Xu et al., 2012). The health care cost of treating these chronic diseases is in excess of $600 billion annually (CDC, 2012; CDC, 2014; Mozaffarian et al., 2015). Consequently, the government has directed policy towards promoting a healthier society, especially to promote healthier eating.

There have been numerous human feeding trials showing that consuming whole grain (WG) or fruits and vegetables (FV) can have significant impacts on markers of metabolic syndrome (Martínez et al., 2013; Cooper et al, 2017; Shefin et al, 2015). Unfortunately, FV and WG intakes are typically far below recommended intake levles (US Department of Agriculture, USDA 2013). In a typical 2000 kcal diet, the current recommendations from the USDA are to consume 5 servings of FV and 3 servings of WG per day. However, in a 2015 report 76% of the US population did not meet the recommended intake of fruit and 87% did not meet the recommended vegetable intake (Latetia V. Moore et al., 2015). Over the past 5 years FV consumption has decreased by 7% due to declines in vegetables as a side dish and consumption of juice at breakfast (Produce for Better Health Foundation, 2015). Likewise, a 2009-2010 survey revealed that only 2.9% of children/adolescents and 7.7% of adults in the US consumed at least 3 whole grain servings/d (Reicks et al., 2014). The typical American consumes only 1 serving of WG per day (Rehm et al., 2016).

There are several inflammatory mediators that can be assayed in blood as markers of metabolic syndrome, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), lipopolysaccharide binding protein (LBP), and high sensitivity C-reactive protein (hs-CRP). TNF-α is a pro-inflammatory cytokine secreted by macrophages that helps regulate cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation (Banday et al., 2017). IL-6 is a pro-inflammatory cytokine that is secreted by macrophages during infection after trauma to stimulate immune responses (Schafer et al., 2007). LBP is a protein that is an acute-phase immunologic response to lipopolysaccharide (Banday et al., 2017). The levels of LBP in blood are a measure of endotoxemia. hs-CRP is secreted by the liver in response to inflammatory cytokines, and if most often associated with heart disease risk (Gao et al., 2004).

The metabolic benefits of WG and FV are thought to be mediated, at least in part, through their interactions with the gut microbiota (Salonen, 2014; Costabile et al., 2008; Li et al., 2009). One way that the gut microbiota may mediate the anti-inflammatory effects of dietary fibers is through short chain fatty acid (SCFA) production (Brahe et al 2013), the major metabolic end products of dietary fiber fermentation. These acids are known to have trophic effects locally on epithelial cell functions as well as distally via distribution in blood. For instance, SCFA help with maintenance of gut barrier function by increasing mucin production, inhibiting growth of enteropthogens, and increasing nutrient absorption. Distally, SCFA are signaling molecules for carbohydrate and lipid metabolism. SCFA are associated with a decreased risk for cancer and obesity (Ríos-Covián et al., 2016).

Typically, human feeding trials confine subjects to a single test food to determine its benefits on health (Basu et al., 2010; Martínez et al., 2013; Mirmiran et al., 2012; Sheflin et al., 2015). However, these interventions are not very practical as part of a long-term dietary regimen to improve health. In contrast, Cooper et al., (2017) provided subjects with a variety of options for treatment foods. Another option is to completely change someone's diet to see if certain diets have impacts on metabolic syndrome. The Mediterranean diet (plant foods like fruit, vegetables, whole grains, legumes and nuts and replacing vegetable oils with olive oil and canola oil) has been studied to see decreases in lowering effects of metabolic syndrome (Michalsen et al., 2006; Romaguera et al., 2009). These studies focus on whole diet changes with counseling on how to cook with these types of foods. Smaller changes or whole diet changes could be more sustainable for subjects to continue eating healthy after the study. This purpose of this study was to determine the impact in participants consuming recommended amounts of whole grains and fruit and vegetables against a background of their typical diets on inflammatory makers and gut microbiota composition in overweight or obese individuals that have low intakes of these food groups.

Materials and Methods

Intervention Study

110 individuals responded to flyers advertising this trial at grocery stores and University buildings and through social media advertisements. Individuals were screened during the initial contact period to determine if they qualified for the study and were interested in participating. Inclusion criteria were: body mass index (BMI) >25 kg/m^2, no diagnosed gastrointestinal diseases, no antibiotic use for 3 months, <1 h/week of structured exercise, and low intake of FV and WG. BMI was calculated by measuring

weight and height (in light clothing without shoes). To verify a low intake of FV and WG, an online validated food frequency questionnaire was used (DHQII, National Cancer Institute, 2017). The diet survey analyzed participants' yearly diet and included questions about serving size. Responses were converted to daily intake of FV or WG using Diet*Calc software (Bethesda, MD, USA). Sixty one individuals were excluded due to low BMI, high intake of FV or WG, working out regularly, recent antibiotics use, health reasons, or scheduling conflicts. Fifty two participants met the inclusion criteria and were enrolled in the study. Three participants dropped out by the end of the study. The feeding trial was a randomized parallel arm design. The Institutional Review Board of the University of Nebraska approved all study protocols described herein (IRB Approval Number: 20141214525FB). Written informed consent was obtained from all subjects before being enrolled in the study. The clinical study was posted on clinicaltrials.gov.

Enrolled subjects were randomized into three groups: WG (3 servings/d; n=17), FV (5 servings/d; n=18), and refined grains (RG) (3 servings/d; n=14). Participants were instructed to incorporate these foods into their normal diet. The RG group served as the control. These subjects were supplied 3 servings/d of RG products to maintain interest and contact with them during the study; however, all subjects already consumed at least 3 servings/d of RG per day as part of their normal diet. This treatment therefore represented minimal to no intervention. For the WG and FV treatment groups, 3 servings/d and 5 servings/s were selected because these are the reference daily intakes (RDI) recommended in the US (United States Department of Agriculture, 2017)

One week prior to beginning the study and each week during the study participants visited the clinical facility. During these visits they filled out a "menu" for the following week. The menu consisted of a list of foods within each treatment group that the subjects could choose from (Table 1). These foods were commonly available at the local grocery store. They marked on the menu how many servings of each of the foods they wanted for the following week. They could choose any combination of any of the foods on the list, but were required to order at least 21 (WG or RG) or 35 (FV) servings for the week. They were allowed to order up to 44 servings of FV or 30 servings of WG per week if they wished.

At all of the weekly visits during the trial period, subjects turned in several daily diaries that they were required to keep. One diary was a daily diet diary, which included intake of all test foods. The log consisted of intake of FV, WG, and RG and how many servings participants consumed. A secondary document was given to participants to discuss what constituted a serving of grain and FV. The servings from the log were transferred to an online database that calculated weekly amounts of FV, grains, WG, RG and other nutrients (United States Department of Agriculture, 2017). This diary was used to assess compliance to the dietary regimens. Additionally, subjects were asked to verbally indicate compliance when they visited the facility each week.

Participants also filled out a weekly GI symptom questionnaire (Appendix A.3). Questions consisted of number of days per week where symptoms were experienced together with the severity. Symptoms included stomach pains, heart burn, acid reflux, hunger pains, nausea, rumbling stomach, bloating, burping, flatus, constipation, diarrhea,

loose stools, hard stools, urgent bowels, and feeling of incomplete bowel emptying. The severity was rated on a 0-4 scale (0=no discomfort, slight, mild, moderate, and 4=severe).

Biological Sample Collection and Analysis

Stool and blood samples were collected at the beginning of the study and after 6 weeks. The blood samples were drawn using standard venipuncture techniques by experienced phlebotomists from the University of Nebraska Medical Center. Blood was collected into two vacutainer tubes (367815, Vactutainer Serum Tubes, and 367986, Vacutainer Serum Separation Tubes, BD, Franklin Lakes, NJ USA). About 5 mL was drawn into each tube. After being collected, samples were left at room temperature for 15-30 min to allow the blood to clot. The samples were then centrifuged (2,000 x g for 10 min). Supernatant (plasma) was aliquoted into smaller test tubes for storage. Tubes were stored at -80˚C until analysis.

TNF-α and LBP were assayed from the plasma recovered from the Vactutainer Serum Tubes; IL-6 and hs-CRP were assayed from the plasma recovered from the SST Serum Separation Tubes. All analyses were carried out using commercial ELISA kits according to the manufacturer's instructions (hs-CRP: HU8817; TSZ, Waltham, MA USA; TNF-α: KHC3011, Invitrogen, Frederick, MD USA; IL-6: HS600B, R&D Systems Minneapolis, MN USA; LBP: 0628D2100, Sigma Aldrich, St. Louis, MO USA).

Stool samples were collected using a commode collection kit (02-544-208, Thermo Fischer Scientific, Waltham, MA USA). An insulated cooler with ice packs was provided to keep samples cool until they could be delivered to the research center for immediate storage at -20˚ C. The samples were obtained and frozen within 2 h of defecation

DNA was extracted using the method described in Martínez et al. (2009). Stool samples were thawed and 1 g of sample was diluted ten-fold in ice cold phosphate buffered saline (PBS). Fecal homogenates were transferred into a 2 mL sterile bead-beating tube containing 300 mg of 0.1-mm zirconium beads, and bacterial cells were collected by centrifugation at room temperature at 8,000 x g for 5 minutes. The bacterial pellet was washed three times in ice cold PBS. The pellet was resuspended in 0.75 mL of lysis buffer (200 mM NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) containing 20 mg/mL of lysozyme (611930010, ACROS Organics) and incubated at 37 °C for 30 min. Eighty five µL of 10% SBS solution (S0287, Teknova) and 30 µL of proteinase K (19133,Qiagen) (15mg/mL) was added and then incubated for 15 min at 60 °C. The pellet was by adding 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1) and bead beated (MiniBeadbeater-8, BioSpec Products, USA) on high for 2 min. In a refrigerated (4 °C) centrifuge the tube was spun at 10,000 x g for 5 min. The top layer was removed into 1.5 mL tube and 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube. Then the tube was spun at 10,000 x g for 5 min. The top layer was removed and put into a new 1.5 mL tube. This step was repeated 3 times followed and each time top layer was remove into a new 1.5 mL tube. 0.6 mL ethanol was added and then placed in -80°C. It was then centrifuged at 15,000 rpm for 20 min, whereupon the supernatant was discarded. The pellet was washed with 0.5 mL of 70% ethanol and was centrifuged at 15,000 rpm for 20 min. The liquid was discarded carefully, and the DNA pellet was dried for 30 min at room temperature. The DNA was resuspended in 0.1 mL of tris buffer (10mM, pH8). The DNA pellet was then frozen at -80°C for further experiments.

Sequencing was performed by University of Minnesota Genomics Center following the procedure by (Gohl et al., 2016). The V4-V5 region of the 16S rRNA gene was amplified by PCR using primers Meta_V3_F_Nextera: (5'-CCTACGGGAGGCAGCAG-3')
Meta_V4_806_R: (5'-GGACTACHVGGGTWTCTAAT-3'). PCR reactions are performed using KAPA HiFidelity Hot Start Polymerase. After the first round of amplification, PCR 1 products are diluted 1:100 and 5 ul of 1:100 and then ran through a second PCR. Pooled, size-selected sample was denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20% PhiX, and heat denatured at 96C for 2 minutes immediately prior to loading. A MiSeq 600 cycle v3 kit was used to sequence the sample.

Initial Filtering of Low Quality Reads. The base sequence quality information was confirmed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). A stringent quality filter process was applied in which the Illumina sequence reads with (1) base quality scores below the minimum quality score (30 per base) across the whole read or (2) read length less than 30 nucleotides were removed using Trim Galore (ver. 0.4.0) (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). Note that the quality scores of 30 (Q30) correspond to 0.1% expected base pair call error rates. Also, any unknown ('N') or ambiguous nucleotide from the Illumina paired-end reads was trimmed.

Quantitative Analysis of the Microbiome Composition. Filtered sequences were analyzed through the software package QIIME pipeline (ver. 1.9) (Caporaso et al. 2010); using scripts implemented within QIIME, paired-end reads were merged and were clustered into operational taxonomic units (OTUs) at a sequence similarity level of 97% by UCLUST (default parameter). The numerical values of the sequence reads that map to

OTUs were used to evaluate differences in taxonomic abundance and alpha diversities. The statistical differences across the samples were determined using DESeq2 (ver. 1.14) (Love et al., 2014) in the R Bioconductor package (http://www.bioconductor.org) (ver. 3.1.2). OTUs to have less than 10 reads mapped were removed.

Nucleotide sequence accession numbers. All Illumina raw sequences from this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number pending.

The short chain fatty acid analysis was performed on the fecal samples and fermented samples based on the procedure described in Pollet et al. (2012) with modifications. In a 2 ml plastic centrifuge tube that is immersed in ice mix together 0.4 ml of fermented media, 0.1 ml of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 ml of 9 M sulfuric acid, and 0.16 g of sodium chloride and vortex. In another 2 ml plastic centrifuge tube that is immersed in ice mix together 0.4 ml water, 0.1 ml of standard mix in 1 M potassium hydroxide, 0.2 ml of 9 M sulfuric acid, and 0.16 g of sodium chloride and vortex. Just before injecting onto the GC, add 0.5 ml of diethyl ether and shake for 30 s to 1 min. Allow layers to separate and transfer diethyl ether layer to a fresh screw cap microfuge tube with rubber septum. Inject 3-4 µl of diethyl ether phase using SCFA program on GC.

Statistical Analysis

All data was analyzed using ANOVA (PROC GLIMMIX) where treatment group was the main factor and BMI (at baseline), gender, and baseline measurement were covariates using SAS software (version 9.4, SAS Institute, Cary, NC, USA). Differences among treatments were assessed using Tukey Test; an adjusted $P < 0.05$ was considered significant. Changes in measured variables from baseline to the end of the study within

each group were also assessed after correcting for BMI (at baseline), gender, and baseline values. The fermentation outcomes (carbohydrates and SCFA) were also analyzed using a binomial sign test, $P < 0.05$. Correlations between changes in gut bacteria and changes in blood parameters and formation outcomes and between changes in diet history and changes in gut bacteria were analyzed using Pearson correlation (PROC CORR) in SAS software, $P < 0.05$.

Results and Discussion

Baseline Data

In the randomized parallel arm feeding study 54 participated and 3 participants dropped out. There were no significant differences between groups at baseline except in fecal SCFA (Table 3.2).

Treatment Compliance and Tolerance

Based on diet diaries, participants in the FV and WG groups consumed an average of 5.7 and 3.4 servings/d of treatment foods, respectively (Table 3.3). This showed that participants were compliant with the treatment protocol. The diaries also showed that the interventions were treatment specific; e.g., subjects in the FV group were consuming low WG foods. Compared to other feeding trials our treatments were very modest in that subjects consumed the minimum recommendation of that food group. Our study focused on a sustainable change of the recommended amount of WG or FV with a variety options in the treatment group. Many participants indicated informally that they planned to continue trying to consume the recommended amount of FV or WG after the study period.

Weekly gastrointestinal symptoms were recorded to track changes throughout the study. There were no significant changes in GI symptoms throughout the study (Table 3.4).

Plasma Inflammatory Markers

The WG diet resulted in a significant decrease in TNF-α during the intervention, whereas no significant changes in TNF-α were found on the other treatment groups (Figure 3.2). Additionally, there was a significant decrease in LBP in the WG and FV diet with no change on the RG diet. There were no significant changes in the RG and WG treatments for IL-6, but there was significant decrease in FV diet. There were no significant changes in hs-CRP.

The WG and FV diets had significant positive impacts on inflammatory markers. In particular, the change in LBP suggested a positive impact on gut barrier function. Lipopolysaccharide is a component of the cell walls of gram-negative bacteria; increased levels of LBP in the blood is suggestive of endotoxemia (Neves et al., 2013) .

The WG diet decreased TNF-α, which was also reported by Qi et al. (2006). In a study similar to ours, dietary fiber treatment decreased hs-CRP which differs from our results (North et al., 2009; Qi et al., 2006). In another trial, a treatment of WG rice or barley lowered the IL-6 in the population (Martínez et al., 2013). The FV diet was the only treatment that lowered IL-6. A study by Yeon et al., (2012) a FV intervention with a variety of food options, similar to this study but focusing on high consumption vs small consumption of FV, were able to significantly reduce IL-6 and IL-1.

Both treatment groups decreased inflammatory markers, but in different ways. FV decreased IL-6 and TNF-α, and WG decreased LBP and TNF-α. These different changes

suggest that consuming both FV and WG could have a synergistic effect to help lower inflammation.

With the variety of food options it is not possible to pinpoint which component of the foods was responsible for the significant changes in inflammatory markers. Both treatment groups were good sources of dietary fiber, although the composition of the dietary fibers within each group are very different. The main dietary fibers in WG are ß-glucan, arabinoxlyan, inulin and cellulose, while FV mainly contain pectin, inulin, or xyloglucan. FV contain more soluble fiber, and WG contain more insoluble fiber (Dhingra et al, 2012). WG foods can also contribute vitamin E, and phytoserols that have been linked to lowering parts of metabolic syndrome (Anderson et al., 2009; J. et al., 2013; Mendiset al., 2016; Salvin, 2003), while FV include folate, flavonoids, vitamin C, and β-carotene, which have been inversely correlated with hs-CRP and IL-6 (Holt et al., 2009).

Microbiota

There were no significant changes in fecal short chain fatty acid (SCFA) production during the study period (Figure 3.3). One study showed a significant increase in SCFAs when using a more dramatic treatment of high resistant starch (~25 g of NSP/d as 50 g BranPlus, 50 g cooked carrots, 50 g couscous and 2 g unprocessed bran) (McOrist et al., 2011). A 3 week treatment of WG cereal showed no significant difference between whole grain treatment and the control in SCFA production (Costabile et al., 2008), which compares with our data. SCFA are rapidly absorbed from the gut, which could be why many studies show no significant changes in SCFA production (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987).

There was a surprising significant increase in BCFAs in the FV treatment (Figure 3.3). This suggests a shift toward protein fermentation by the microbiota. However, because the subjects in the FV group experienced improvements in metabolic markers and protein fermentation is associated with negative health outcomes, this is unlikely (Russell et al., 2011). Perhaps the FV treatment induced changes in the absorption of BCFA from microbial fermentation.

The Firmicutes were generally higher and Bacteroidetes lower than expected (Cooper et al., 2017; Vanegas et al., 2017; Sheflin et al., 2015) (Figure 3.5). Similar studies have shown 20-40% abundance of Bacteroidetes, while this study had 7-11%. The differences could be due to amplification of different regions of the 16S gene or differences in DNA extraction protocol. Regardless, there were no significant differences between treatment groups or from baseline to the end of treatment. Opposing results from Martínez et al (2013) reported signficiant phylum-level differences in Firmicutes and Bacteroidetes after feeding 60 g/d of WG, which was generally more dramatic of an intervention than the present study. An intervention of 14g of cruciferous vegetable was able to shift overall bacterial community after a 14 day intake period, but no signficant changes in Firmicutes or Bacteroidetes (Li et al., 2009). The Firmicutes/Bacteroidetes may not be the best bacteria to investigate since recent reanalyzed data have been reporting new results that showing different relationships between Firmicutes/Bacteroidetes and obese populations (Shloss et al., 2016).

There were no significant changes in individual OTUs during the treatment. This has been previously reported in other WG interventions with a similar experimental design (Cooper et al., 2017). However, FV interventions and some WG interventions

have shown changes in individual OTUs belonging to *Roseburia* and *Blautia* (Martínez et al., 2013; Sheflin et al., 2015). When OTUs were binned according to their relative fold change during the intervention, the WG and FV treatment groups showed a shift toward a decrease in some OTUs of <0.5-fold while the RG treatment group showed a shift toward an increase of <0.5 fold (Figure 3.5).

The FV intervention resulted in a significant increase in α-diversity, while the other treatment groups did not show any differences (Figure 3.5). The wide variety of and new types of dietary fiber introduced to subjects' diets in the FV treatment could have contributed to the increase in bacterial α-diversity in the FV group. In contrast, the WG diet was mainly composed of wheat-based products which contain similar dietary fibers to the RG products that subjects consumed as part of their habitual diet prior to the study.

There were no significant changes in ß-diversity over the course of the study (Figure 3.5). Similar studies (with a wide variety diet) have also shown no significant differences between treatment groups (Cooper et al., 2017). During a 14 day cruciferous vegetables intervention there were significant changes in gut bacterial community which differs from our results (F. Li et al., 2009). Their stricter choice of vegetables could be aiding these changes.

There were no significant changes in the microbiota at the genus level by treatment group, but there were individualized responses to the treatments (Figure 3.6). Other WG and FV interventions have shown similar results of no significant changes at genus level but individualized changes (Cooper et al., 2017.; J. et al., 2013). On the contrary Martinez et al., (2013) showed genus level significant changes in *Blautia, Bifidobacterium,* and *Dialister* and Costabile et al., (2008) also saw significant changes in

Bifidobacterium from a WG intervention. WG diet consisted of mainly wheat based products. Wheat fibers can have different impacts on the gut bacteria compared to other whole grain fibers (De Paepe et al., 2017; Duncan et al., 2016).

Conclusions

A FV or WG intervention significantly reduced inflammation, but each diet induced changes in different markers. The FV treatment decreased IL-6 and LBP and the WG treatment decreased TNF-α and LBP. Both treatments had individualized effects on the gut microbiota, with a significant increase in α-diversity in the FV treatment. The data supports the positive impact that WG and FV intake can have on metabolic health in overweight or obese individuals with normally low intake of WG and FV, but in different ways.

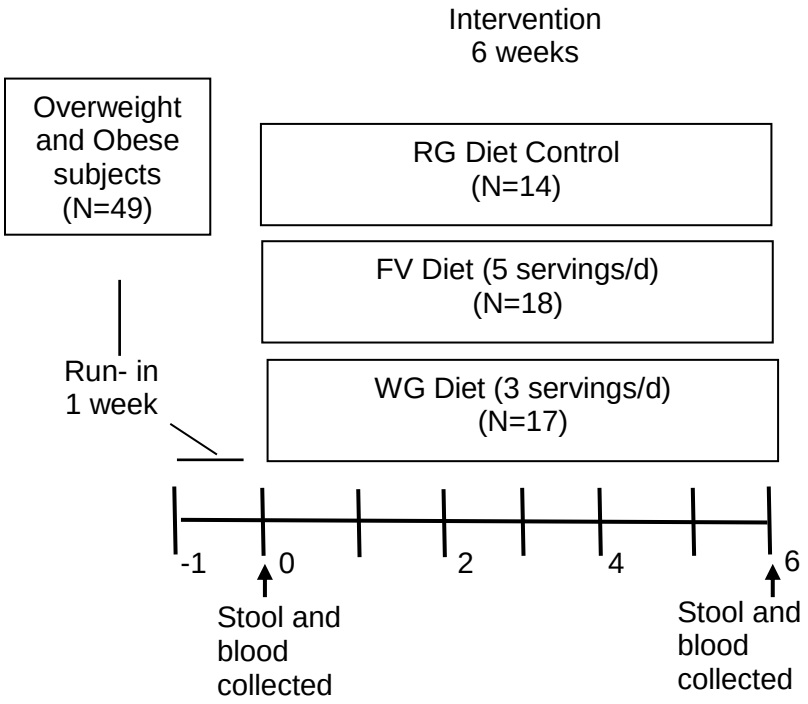


Figure 3.1 Timeline of Experimental Design

Table 3.1: Food Options During the Treatments

Treatment group	What was served	Servings	Dietary fiber (g/serving)
Fruit and Vegetables			
Banana	1 medium	2	3.9
Apple	1 medium	2	4.4
Clementine	2	2	2.6
Strawberries	½ cup	1	2.9
Blueberries	½ cup	1	1.8
Grapes	½ cup	1	0.7
Pear	1 medium	2	5.5
Pepper	1 large	2	3.4
Celery	1 stalk	1	1.2
Broccoli	½ cup	1	1.2
Cauliflower	½ cup	1	2.1
Lettuce	1 cup	1	0.9
Spinach	1 cup	1	1.4
Peas	½ cup	1	2.3
Tomato	½ cup	1	0.9
Carrot	½ cup	1	2.6
Green Beans	½ cup	1	1.4
Average dietary fiber for 5 servings			11.5
Whole grain			
Whole Wheat Bread	1 slice	0.8	2
Whole Wheat Tortilla 14 in	1 tortilla	1.9	5
Whole Wheat Tortilla 7 in	1 tortilla	1	2
Oatmeal	1 package	1.7	3
English Muffin	1 muffin	1.6	3
Whole Wheat Flakes Cereal	¾ cup	1.4	3
Ready to Eat Brown Rice	1 package	3	2
Granola	½ cup	1.1	7
Whole Grain Spaghetti	½ cup	3.5	6
Toasted Oats Cereal	¾ cup	1.1	2
Woven Wheat Crackers	5 crackers	1.5	3
Shredded Wheat Cereal	11 biscuits	3	8
Whole Grain Mac and Cheese	Single serving container	0.5	3
Oatmeal Square	1 bar	0.9	2
Popcorn	1 cup	1.25	6
Sun Chips	7-11 chips	1.3	3
Average dietary fiber for 3 servings			11.3
Refined grain			
White Bread	1 slice	1	0
Flour Tortilla 7 in	1 tortilla	1	1

Flour tortilla 14 in	1 tortilla	1	1
Wheat Farina	1 package	1	1
Plain Bagel large	1 bagel	1	1.3
Plain Bagel small	2 bagel	1	0.6
Corn Flakes	¾ cup	1	1
Ready to eat White Rice	1 package	1	1
Crispy Rice Cereal	¾ cup	1	0
Spaghetti	½ cup	1	2
Rice Flakes Cereal	¾ cup	1	0
Buttery Crackers	5 crackers	1	0
Crispy Rice Treat	1 bar	1	0
Pita Chips	7-11 chips	1	1
Mac and Cheese	Single serving container	1	1
Average dietary fiber for 3 servings			2.2

The serving size for fruit and vegetables is ½ cup. For convenience participants were given either 1 cup or ½ cup. For instance a whole banana (1 cup) was served due to the inconvenience of getting a ½ banana and it going bad. FV fiber content was retrieve from the USDA composition database (United States Department of Agriculture, 2017). The serving size for whole grains is 1 ounce or 16 grams of whole grain. All servings and grams of whole grains were calculated by the whole grains food stamp. Some products more grams of whole grains were given due to convenience like a whole tortilla instead of a half. Refined grains were serving size was matched whole grain servings. Fiber serving size was retrieved from nutritional labels of the particular foods.

Table 3.2. Baseline Data

Baseline data	Groups			
	Whole Grains	Fruit and vegetables	Refined Grains	P-Value
BMI	33.4 ± 6.5	30.5 ± 5.9	30.1 ± 5.2	0.342
Participants	17	18	14	
Female	11	12	7	0.153
Male	6	6	7	0.153
Inflammatory Markers				
IL-6 (pg/ml)	4.4 ± 1.9	4.3 ± 2.6	2.9 ± 1.5	0.5962
TNF (pg/ml)	26.7 ± 4.17	24.2 ± 5.2	23.8 ± 5.9	0.1105
HS-CRP (mg/ml)	0.8 ± 0.6	0.7 ± 0.4	0.6 ± 0.4	0.8853
LBP (mg/L)	1.9 ± 0.4	1.8 ± 0.4	1.8 ± 0.3	0.3775
Stool Short Chain Fatty Acids				
Acetate	50.4 ± 8.3	45.4 ± 7.6	75.7 ± 8.7	**0.0412**
Propionate	11.2 ± 2.1	10.5 ± 1.5	23.8 ± 6.1	0.1379
Butyrate	7.6 ± 1.5	8.4 ± 1.3	18.7 ± 3.4	**0.0235**
SCFA	69.3 ± 11.7	64.7 ± 9.8	117.92 ± 17.7	**0.0418**
BCFA	1.8 ± 0.3	1.6 ± 0.2	2.4 ± 0.4	0.2419

The serving size for fruit and vegetables is ½ cup. It is recommended to get 5 servings a day of FV. The serving size for whole grains is 1 ounce or 16 grams of whole grain. It is recommended to get 3 servings of WG per day. Refined grains were serving size was matched whole grain servings. This data was analyzed through supertracker (SuperTracker Home, 2017).

Table 3.3: Average Weekly Intake of Whole Grains, Refine Grains, Fruit, and Vegetables.

Treatment Groups	Average Daily Intake WG (servings)	Average Daily Intake of RG (servings)	Average Daily Intake of Fruit (servings)	Average Daily Intake of Vegetables (servings)	Average Daily Intake of FV (servings)
FV	0.9 ± 0.3^{b}	2.4 ± 0.4^{b}	3.1 ± 0.1^{a}	2.3 ± 0.2^{a}	5.7 ± 0.3^{a}
WG	3.4 ± 0.2^{a}	2.7 ± 0.7^{b}	0.8 ± 0.1^{b}	0.6 ± 0.1^{b}	1.4 ± 0.2^{b}
RG	0.7 ± 0.1^{b}	7.1 ± 0.7^{a}	1.4 ± 0.1^{b}	0.6 ± 0.2^{b}	2.0 ± 0.2^{b}

The serving size for fruit and vegetables is ½ cup. It is recommended to get 5 servings a day of FV. The serving size for whole grains is 1 ounce or 16 grams of whole grain. It is recommended to get 3 servings of WG per day. Refined grains were serving size was matched whole grain servings. This data was analyzed through supertracker (SuperTracker Home, 2017).

Table 3.4 GI Symptoms Reported at 0, 3, and 6 Weeks of FV, WG, and RG Treatment Groups.

Subjects	Stomach pains	Heart Burn	Acid Reflux	Hunger Pains	Nausea	Rumbling Stomach	Bloating
			Week 0				
FV	0.33 ± 0.14	0.17 ± 0.12	0.44 ± 0.22	0.77 ± 0.18	0.39 ± 0.20	0.56 ± 0.17	0.72 ± 0.21
WG	0.24 ± 0.14	0.29 ± 0.19	0.24 ± 0.14	0.59 ± 0.19	0.32 ± 0.15	0.38 ± 0.18	0.44 ± 0.16
RG	0.15 ± 0.15	0.15 ± 0.15	0.58 ± 0.34	0.54 ± 0.22	0 ± 0	0.46 ± 0.13	0.62 ± 0.24
			Week 3				
FV	0.5 ± 0.18	0.56 ± 0.28	0.17 ± 0.12	0.83 ± 0.19	0.67 ± 0.50	1.06 ± 0.51	0.61 ± 0.22
WG	0.41 ± 0.17	0.35 ± 0.21	0.35 ± 0.21	0.71 ± 0.19	0.24 ± 0.18	0.74 ± 0.21	0.74 ± 0.23
RG	0.46 ± 0.24	0.31 ± 0.21	0.38 ± 0.27	0.46 ± 0.14	0.15 ± 0.15	0.62 ± 0.24	0.62 ± 0.29
			Week 6				
FV	0.33 ± 0.20	0.22 ± 0.13	0.31 ± 0.15	0.61 ± 0.20	0.64 ± 0.29	0.58 ± 0.22	0.64 ± 0.25
WG	0.47 ± 0.19	0.29 ± 0.16	0.06 ± 0.21	0.62 ± 0.23	0.24 ± 0.13	0.62 ± 0.18	0.47 ± 0.19
RG	0.62 ± 0.23	0.50 ± 0.24	0.31 ± 0.06	0.59 ± 0.27	0.15 ± 0.15	0.54 ± 0.24	0.69 ± 0.24

The significant difference was measured between week 0 and week 6. Severity scale was 0-4, 0 being no discomfort and 4 being severe. There were significant differences in number of days between bloating and diarrhea. There was no significant difference between severities. Participates in each group FV=18, WG-17, and RG=14.

Table 3.4 GI Symptoms reported at 0, 3, and 6 Weeks of FV, WG, and RG Treatment Groups. (Continued)

Subjects	Burping	Flatus	Constipation	Diarrhea	Loose Stools	Hard Stools	Urgent Bowels	Not Empty
				Week 0				
FV	0.28 ± 0.18	1.17 ± 0.25	0.44 ± 0.25	0.06 ± 0.06	0.44 ± 0.17	0.78 ± 0.30	0.44 ± 0.17	0.39 ± 0.17
WG	0.47 ± 0.17	0.68 ± 0.26	0.29 ± 0.21	0.18 ± 0.10	0.53 ± 0.19	0.44 ± 0.22	0.76 ± 0.28	0.47± 0.19
RG	0.15 ± 0.15	0.38 ± 0.18	0.5 ± 0.25	0.54 ± 0.31	0.33 ± 0.26	0.62 ± 0.29	0.31 ± 0.31	0.46 ± 0.18
				Week 3				
FV	0.17 ± 0.09	1.28 ± 0.25	0.28 ± 0.19	0.61 ± 0.23	0.56 ± 0.23	0.22 ± 0.17	1.06 ± 0.27	0.44 ± 0.18
WG	0.38 ± 0.18	0.80 ± 0.24	0.24 ± 0.14	0.29 ± 0.21	1.00 ± 0.28	0.32 ± 0.15	0.71 ± 0.24	0.53 ± 0.23
RG	0.31 ± 0.21	0.62 ± 0.18	0.15 ± 0.15	0.15 ± 0.15	0.38 ± 0.22	0.77 ± 0.28	0.54 ± 0.27	0.62 ± 0.24
				Week 6				
FV	0.56 ± 0.40	0.89 ± 0.23	0.36 ± 0.17	0.36 ± 0.19	0.58 ± 0.21	0.52 ± 0.24	0.64 ± 0.19	0.38 ± 0.18
WG	0.56 ± 0.20	1.03 ± 0.27	0.41 ± 0.15	0.35 ± 0.19	0.47 ± 0.19	0.55 ± 0.23	0.71 ± 0.17	0.79 ± 0.24
RG	0.15 ± 0.15	1.23 ± 0.75	0.23 ± 0.17	0.15 ± 0.15	0.23 ± 0.17	0.46 ± 0.22	0.23 ± 0.28	0.70 ± 0.31

The significant difference was measured between week 0 and week 6. Severity scale was 0-4, 0 being no discomfort and 4 being severe. There were significant differences in number of days between bloating and diarrhea. There was no significant difference between severities. Participates in each group FV=18, WG-17, and RG=14.

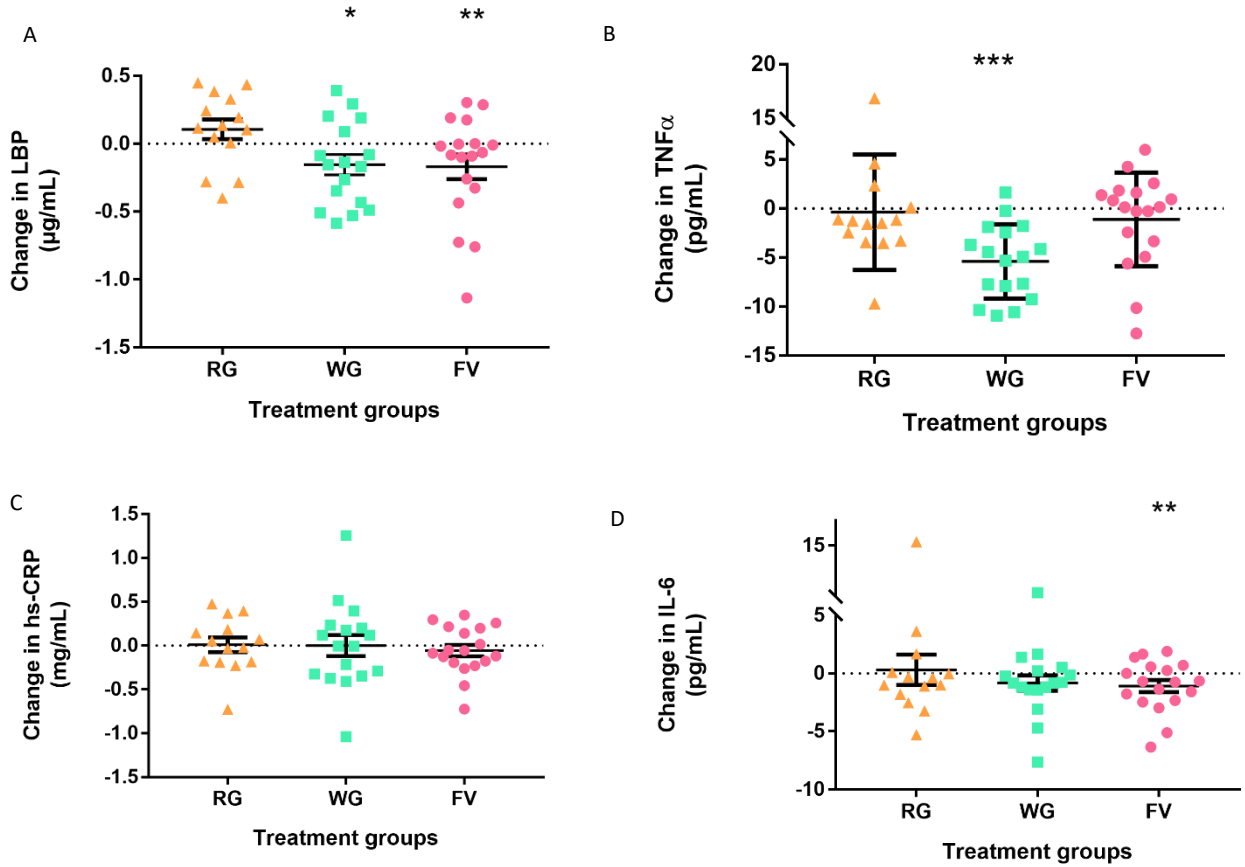


Figure 3.2: Changes in Inflammatory Markers Due to Diet
A- LBP B- TNFα C- hs-CRP D- IL-6 * < 0.05, ** < 0.01, *** < 0.

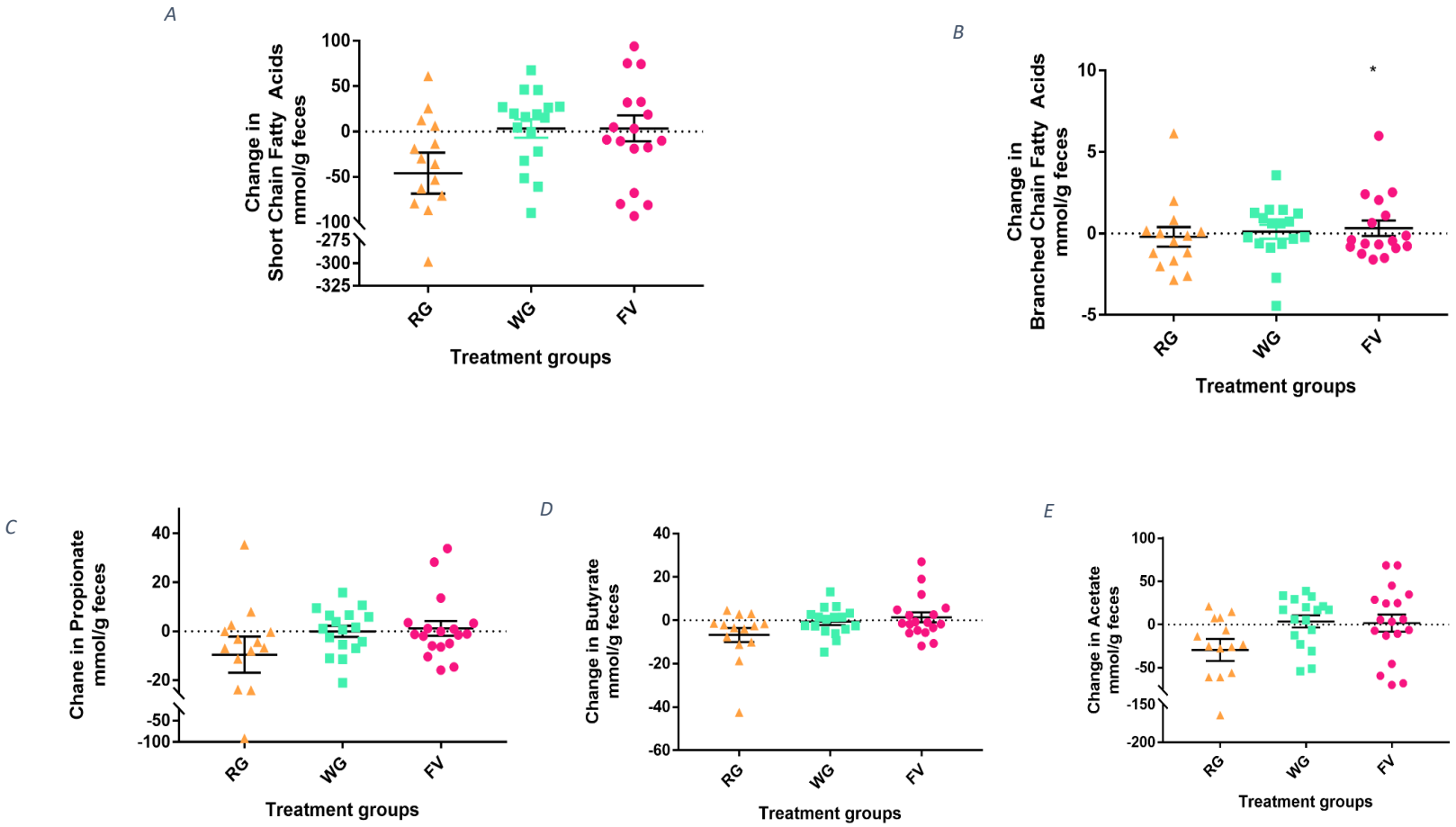


Figure 2.3: Changes SCFA and BCFA in Fecal Samples

A- SCFA B- BCFA C- Propionate D- Butyrate E- Acetate

* < 0.05, ** < 0.01, *** < 0.001

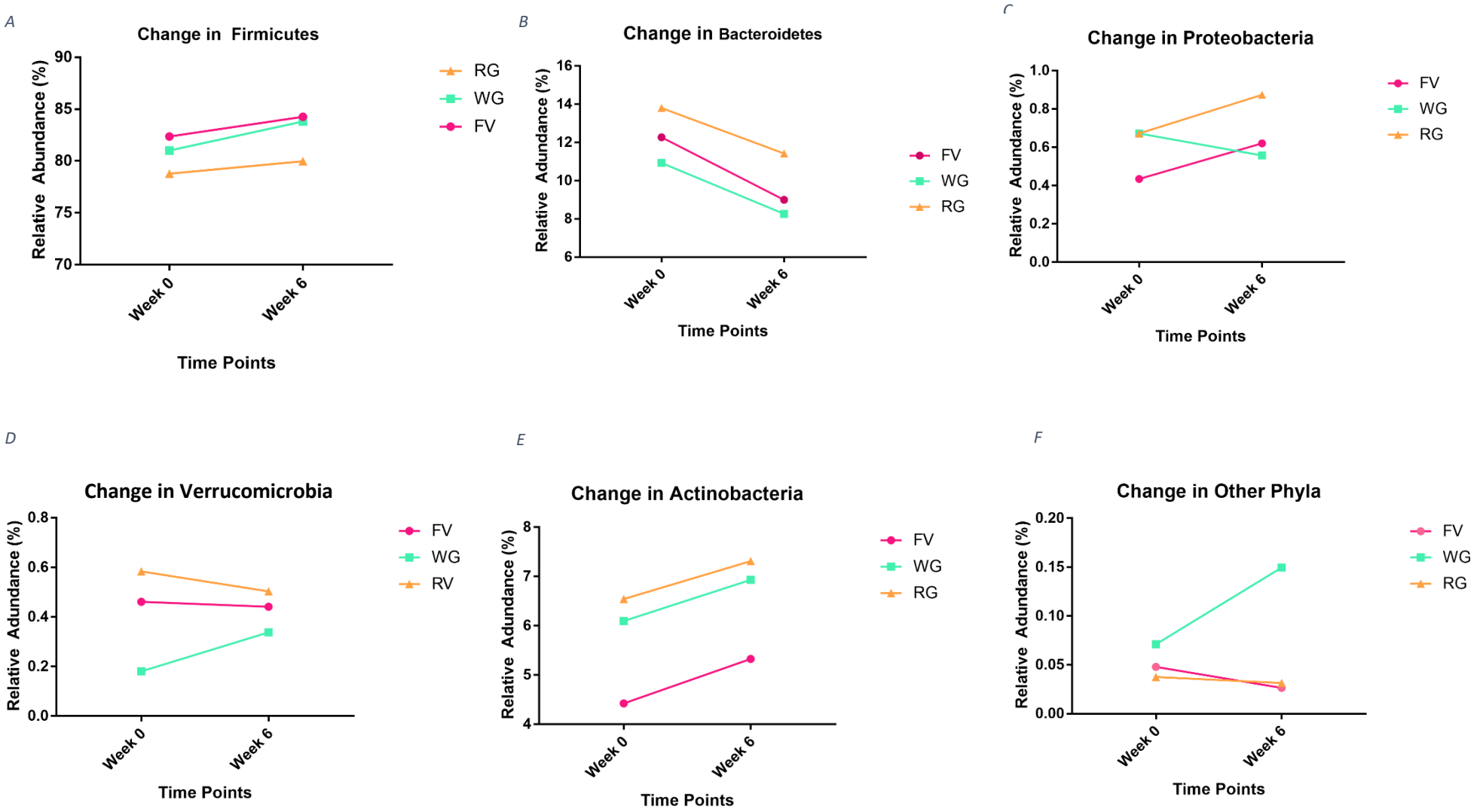
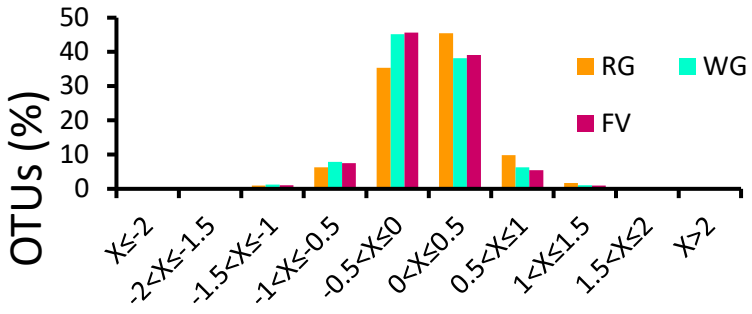


Figure 3.4: Changes in Phylum Level Bacteria (Note: No change were significant)

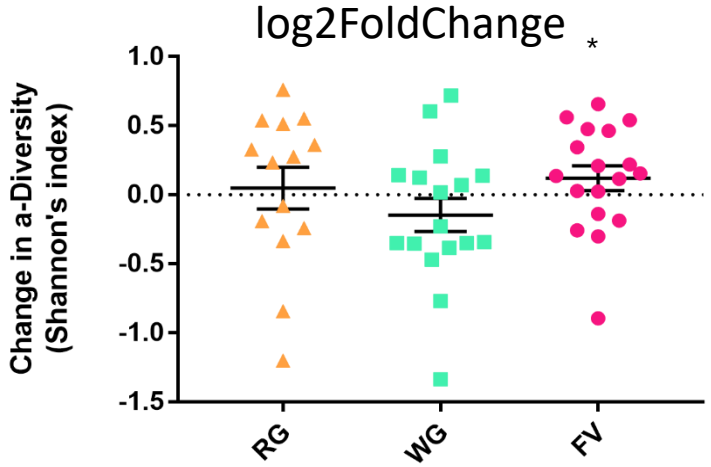
Change in Firmicutes B- Change in Bacteroidetes C-Change in Proteobacteria D- Change in Verrucomicrobia

E- Change in Actinobacteria F- Change in other Phyla

A



B



C

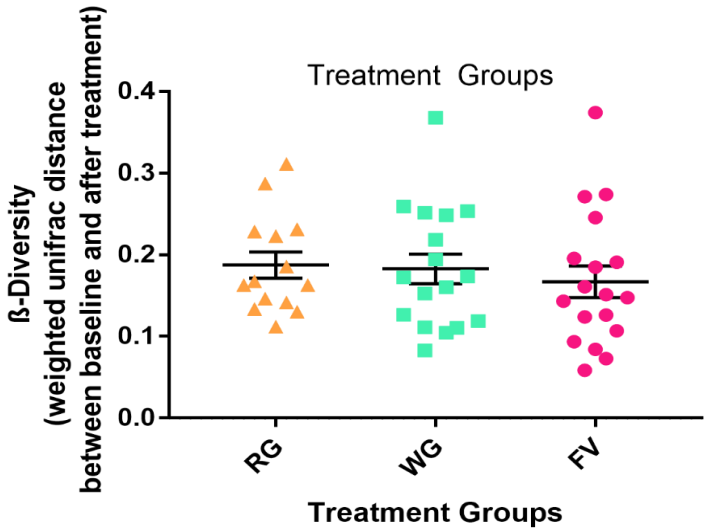


Figure 3.5 Changes in Gut Microbiota
A-Log2Fold Change of OTUs B- Change in α-Diversity (Shannon's Index) C- Weighted Unifrac Distance between Baseline and After Treatment of ß--Diversity

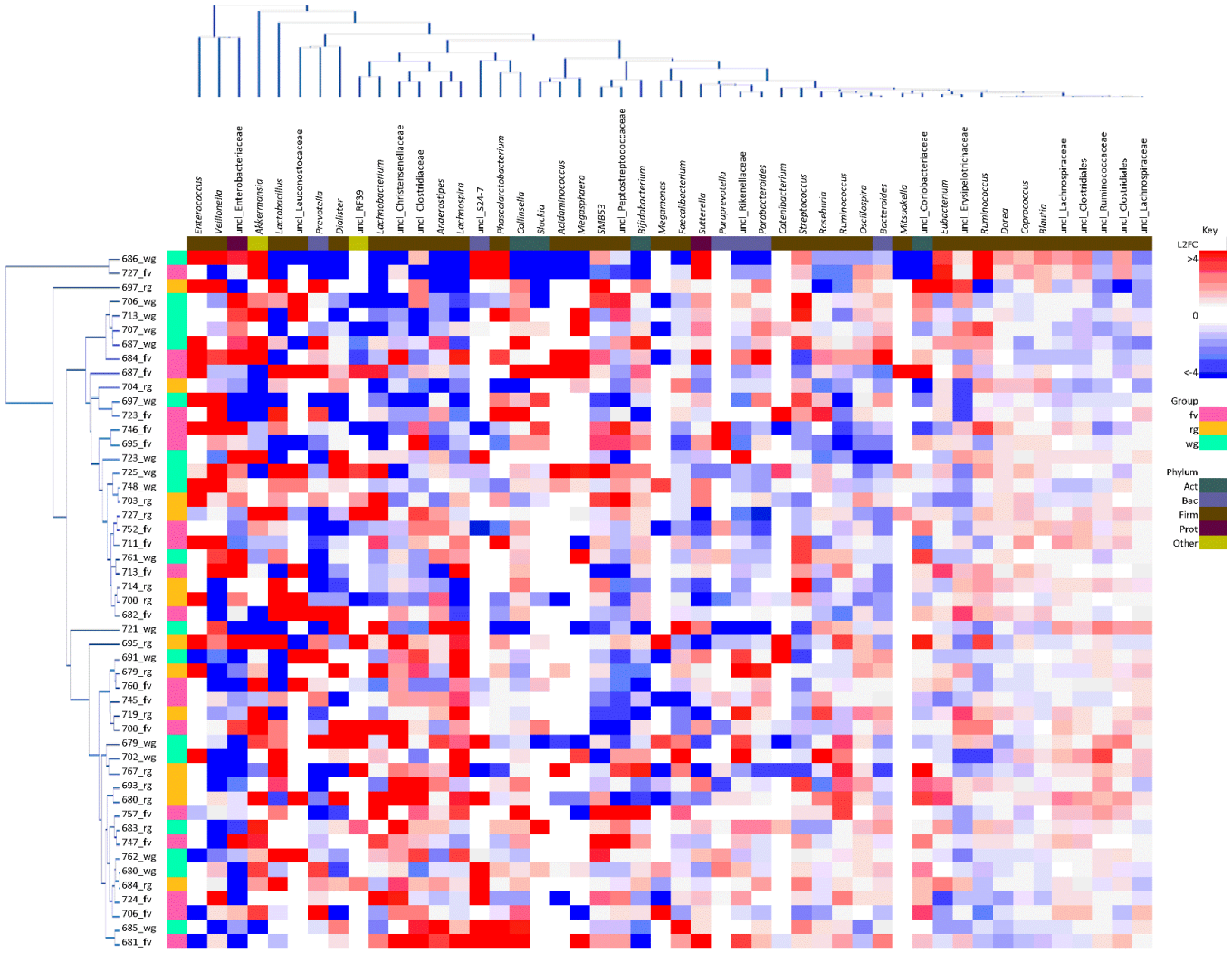


Figure 3.6: Heat Map of Log2Fold Change in dominant genera (at least 1 person with an abundance >1%) during the intervention for individual Participants.

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Chapter 4: *In Vitro* Fermentation of Pre-digested Whole Wheat Flour with Stool Samples from an *In Vivo* Human Feeding Trial with Whole Grains and Refined Grains

Abstract

Previously, a human feeding trial was conducted on obese and overweight individual with low intake of whole grains (WG), fruit and vegetables (FV) to investigate the effects of increasing WG and FV on inflammation markers in the blood and gut microbiota. The WG treatment found significant decreases in tumor necrosis factor alpha (TNF-α) and Lipopolysaccharide binding protein (LBP). These decreases could be signs of improved gut barrier function. The purpose of this study was to use an in vitro fermentation system to assess the functionality of the fecal microbiota to determine if changes in microbiota composition and function were associated with changes in inflammatory markers during the feeding trial. Pre-digested whole wheat flour was fermented in a batch *in vitro* fermentation system using stool samples collected before and after a dietary intervention of whole grains (WG) or refined grains (RG). There were no significant differences between WG and RG treatment groups for percent fermented carbohydrate or short chain fatty acid (SCFA) production. Fermentation of non-digestible carbohydrates in pre-digested whole wheat flour tended to be higher when using the fecal microbiota collected after the intervention compared with before ($p=0.07$); however, there was a high degree of variability among subjects. No change in fermentation of carbohydrates was evident in the fecal microbiota collected from subjects on the RG treatment. Correlation analysis among the subjects on the WG intervention showed that

the change in the relative abundance of *Coprococcus* was associated with increased SCFA production and decreased TNF-α and LBP during the intervention. This suggests that the beneficial effects of the WG intervention on markers of inflammation may have been due to increased capacity of the gut microbiota to ferment dietary fibers and produce beneficial SCFAs.

Introduction

It is difficult to assess the metabolic activity of the human gut microbiota. *In vitro* fermentation can be a tool to assess the functionality of the microbiota in terms of its ability to ferment certain substrates and produce metabolites that are associated with human health (Smidt etl al., 2009). In a recent study, increased carbohydrate fermentability and butyrate production came from people consuming higher quality diets compared to low quality diets (Brahma et al., 2017). Certain bacteria will colonize better with certain fiber; for instance, inulin is a better substrate for *Ruminococcus*, *Faecalibacterium prausnitzii*, and *Prevotella copri*, while wheat bran increased Eubacterium *xylanophilum* and members of Lachnospiraceae (De Paepe et al., 2017; Duncan et al., 2016).

Quality of diet and particular fibers can alter the gut bacteria, but what is not known is how these fibers affect gut bacteria and metabolic benefits. Some previous investigators have used in vitro fermentation to help explain results found in in vivo studies. Koecher et al., (2014) and Bliss et al., (2013) tried to link the benefits between *in vivo* and *in vitro*, but both studies found that their results were not significantly related to improved dietary *in vitro* fermentation through an *in vivo* supplementation. Daniel et al. (1997) and Wisker et al. (1998) were able to show that using mixed diet (mixtures of dietary fibers in intact food) and non-starch polysaccharide (NSP) *in vitro* can give sufficient accuracy compared to *in vivo* testing. When using intact or whole food rather than individual dietary fibers *in vivo* and *in vitro* can be connected to understand the connection between gut bacteria and metabolic outputs.

Through *in vitro* fermentation outcomes, correlations can be suggested about diet and gut bacteria in response to metabolic health. A study analyzed eighteen subjects and

correlated dietary records with dietary fiber degradation and short-/branched-chain fatty acid (BCFA) and ammonia production during in vitro fecal fermentation. It was found that butyrate was correlated with fecal donor intake of some nutrients which were contributed to grain, nut, and vegetable based foods while BCFA production was correlated with intake of unsaturated fatty acids (Yang et al., 2014). These types of correlations help link the relationship between gut bacteria, diet, and human health.

In a previous study, a human feeding trial was conducted on obese and overweight individuals with low intake of WG, fruit and vegetables FV to investigate the effects of increasing WG and FV on inflammation markers in the blood and gut microbiota. Participants consumed 3 servings of whole grains a day or 3 servings of RG a day. The WG treatment resulted in significant decreases in tumor necrosis factor alpha (TNF-α) and lipopolysaccharide binding protein (LBP). Presently, an in vitro fermentation experiment was performed to assess the changes in functionality of the microbiota following a WG and RG intervention. Correlations were analyzed to examine relationships between diet, gut microbiota, and fermentation outcomes.

Materials and Methods

Subjects, Dietary Records, and Stool Sample Collection

In chapter 3, dietary records and stool samples were collected from 31 participants from the RG and WG treatment groups. This population had not taken antibiotics in the last six months, had low intake of FV (<2 servings/d) and WG (<1 serving/d), had a BMI over 25, and did not participate in structured activity. Stool samples and dietary records were collected as described in chapter 3. All protocols involving human subjects were approved by the University of Nebraska-Lincoln's Institutional

Review Board before initiation of the study (no. 20141214525FB) All subjects gave voluntary informed consent before enrollment in this project.

In Vitro Starch Digestion and Fermentation

Wheat samples (cultivar NW15677) were milled in a Butler mill to separate and collect bran and flour. Cyclone grinder was used to grind bran to 1 mm, then the flour and bran was mixed. The milled samples were then subjected to in vitro digestion according to Yang & Rose 2014.

In vitro batch fermentation was performed based on the procedure described in Yang & Rose, 2014. Two batch 12 h fermentations were completed using stool samples at baseline and stool samples after the 6 week intervention. The results from the baseline fermentation were correlated with subject measurements at baseline. The changes in fermentation outcomes between the two fermentations were correlated with changes during the intervention.

Fermentation Analysis

The short chain fatty acid analysis was performed on the fecal samples and fermented samples based on the procedure described in Pollet et al. (2012). The soluble and insoluble carbohydrate analysis was performed based on the procedure described in Arcila et al. (2015).

Statistical Analysis

All data was analyzed using ANOVA (PROC GLIMMIX) where treatment group was the main factor and BMI (at baseline), gender, and baseline measurement were covariates using SAS software (version 9.4, SAS Institute, Cary, NC, USA). Differences among treatments were assessed using Tukey Test; an adjusted $P < 0.05$ was considered significant. Changes in measured variables from baseline to the end of the study within

each group were also assessed after correcting for BMI (at baseline), gender, and baseline values. The fermentation outcomes (carbohydrates and SCFA) were also analyzed using a binomial sign test, $P < 0.05$. Correlations between changes in gut bacteria and changes in blood parameters and fermentation outcomes and between changes in gut bacteria were analyzed using Pearson correlation (PROC CORR) in SAS software, $P < 0.05$. Baseline diet history was correlated with fermentation outcomes using Spearman partial correlations with partial variables with energy intake, age, gender, and BMI.

Results and Discussion

Baseline Correlations

Correlations were performed on baseline diet and fermentation outcomes using the fecal microbiota collected at baseline to determine if subjects' diets before the study were associated with in vitro fermentation outcomes (Table 4.1). The correlations revealed a relationship between carbohydrate quality and SCFA production: diets high in dietary fiber were associated with higher SCFA production, especially butyrate, whereas diets high in sugar were associated with reduced SCFA production. "Healthy fat" (ω-3; polyunsaturated) intake was also positively related to SCFA production during the in vitro fermentation. Intake of several vitamins and minerals were also correlated with the SCFA.

These correlations were similar to Yang et al. (2014), but in a different population. Yang et al. (2014) comprised a generally younger population with lower BMI while this study included older adults with higher BMI. The median age in this study was 31 and mean BMI was 30, while Yang et al., (2014) had a median age of 24 and median BMI of 25. Interestingly, although many of the correlations were similar between the two

groups, production of SCFA, especially butyrate, was much lower in the present population compared with the previous, healthier population (Figure 4.1).

Correlations were performed between microbiota composition and fermentation outcomes using baseline fecal samples (Table 4.2). Butyrate showed the most correlations with microbiota composition, mainly with members of the Firmicutes phylum. An unclassified genus in the Ruminococaceae family, *Faecalibacterium,* and *Dialister* were highly correlated with SCFA and are known butyrate producers (Brahe et al., 2013; Scott et al., 2014). Notably, *Faecalibacterium* abundance was positively correlated with nearly every fermentation outcome, including the fermentation of the non-digestible carbohydrate fractions. Unexpectedly, *Lachnospira* was highly correlated with SCFA production, including butyrate. *Lachnospira* are not butyrate producers but may be important cross-feeding partners with butyrate producers (Cornick et al., 2015).

Two similar studies have found comparable results when looking at changes in gut microbiota in grain based dietary fiber. Brahma et al. (2017) found *Faecalibacterium* was positively correlated with butyrate and Ruminococaceae was negatively correlated with acetate and SCFA. In another study investigating the in vitro fermentation of six dietary fibers, *Faecalibacterium* was positively correlated with SCFA (Yang et al., 2013). Yang et al. (2013) also had some conflicting results to the present study: *Faecalibacterium* was negatively correlated with acetate, and *Blautia* also had opposite correlations. The differences in correlations may be due to the different types of substrates under study.

Changes in Fermentation Outcomes Using Stool Samples from Before and After the Intervention

Pre-digested whole wheat flour was fermented in a batch *in vitro* fermentation system using stool samples collected before and after dietary intervention. Initially, there were no significant changes in percent carbohydrate fermented during in vitro fermentation when comparing before and after each intervention (Figure 4.2). However, it was evident that there was a high degree of inter-individual variability and, for many fecal samples from the WG group, the percent carbohydrate fermented at week 6 was higher than at baseline. Indeed, a binomial sign test indicated that the percent carbohydrate fermented after the intervention tended to be higher compared with week 0 for individuals in the WG group ($p=0.07$). Such was not the case for fecal samples collected from individuals in the RG group ($p=0.29$). Thus, this suggests that the microbiota tended to be able to ferment more WG dietary fibers after the intervention compared to before the intervention.

The % fermentable CHO at baseline is negatively correlated with the change in fermentable CHO across time points ($r=-0.71$) (Figure 4.3). This relationship is also true for butyrate production ($r=-0.81$). These correlations are not significant for any other fermentation outcomes. This indicates that people with low ability to ferment CHO (or produce butyrate) at baseline showed an increase with the WG treatment, while those with a high capacity to ferment CHO (or product butyrate) at baseline did not improve further. This is very common. For instance, individuals with very high *Bifidobacterium* at baseline typically do not increase further when administered a prebiotic (Davis et al., 2011).

There were no significant differences in SCFA or BCFA production between fermentations performed using stool samples from week 0 and week 6, regardless of intervention (Figure 4.5). Brahma et al., (2017) found a significant difference in SCFA between a high quality diet and a low quality diet. Subjects were divided into groups based on habitual diet quality. Perhaps the lack of change in the present study is because the intervention was not long enough or was not severe enough.

Being able to link the results of an *in vivo* study with the results from an *in vitro* has been the subject of a few previous studies. Bliss et al. (2013) found that supplementation of dietary fibers of psyllium, gum arabic, and carboxymethylcellulose could not significantly increase degradability of fiber using batch *in vitro* system. Daniel et al. (1997) and Wisker et al. (1998) both used mixed intake fiber diets rather than single fibers as the substrate. When comparing *in vivo* and *in vitro* it was found fermentation outcomes were similar when assaying carbohydrate fermentation from the whole diet. However, when estimating carbohydrate fermentation from an isolated fiber there were large differences between in vivo results and in vitro fermentation. Thus, in vitro fermentation experiments tend to agree well with in vivo outcomes when using intrinsic and intact dietary fibers as opposed to isolated dietary fibers.

Correlations between changes in fermentation outcomes and inflammatory markers during the intervention

Correlations were performed on changes in bacteria with the changes in blood markers and fermentation outcomes of pre-digested wheat flour with the WG treatment. Changes in only one genus was significantly correlated with both inflammatory markers that showed significant decreases during the intervention (TNF-α and LBP) and several fermentation outcomes: *Coprococcus* (Table 4.3).

These correlations suggest a connection between changes in gut microbiota composition (specifically changes in *Coprococcus*) and function (specifically SCFA production) during the intervention and reduction in inflammatory markers during the intervention (Figure 4.6). *Corpococcus* are gram positive chemoorganotrophs known to produce butyrate and propionate (Ezaki et al., 2015). A review discussed that microbiota-generated SCFAs are associated with reduced risk of inflammation (Rial et al.,2016).

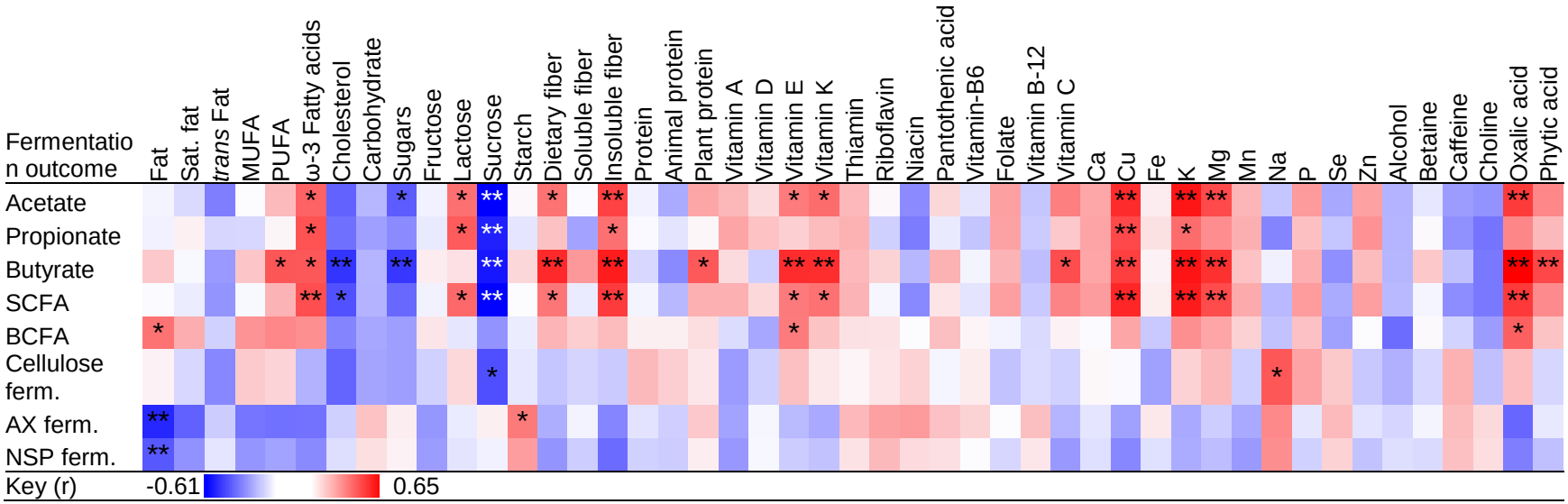
Correlations were performed between changes in inflammatory markers, fecal microbiota, and in vitro fermentation results during the RG intervention (Table 4.4). *Dorea, Bacteroides, Parabacteroides, Oscillopsira,* and *Dialister* had negative correlations with IL-6. *Corobacteriacea, Ruminococcus,* and *Lactobacillus* had positive correlations with IL-6. Bifidobacterium was negatively correlated with TNF-α, propionate, and butyrate. Because the RG intervention introduced no dietary intervention to the subjects and there were no significant changes in inflammatory markers during the study, the relevance of these correlations may be minimal.

Conclusions

Baseline correlations found that diets high in dietary fiber, ω-3 fatty acids, and polyunsaturated fatty acids and several vitamins and minerals are associated with higher SCFA production. It was also found that Ruminococaceae family, *Faecalibacterium,* and *Dialister* are highly correlated with SCFA production during fermentation. After a WG intervention, the microbiota tended to ferment more CHO after the WG intervention, but was related to baseline ability of the microbiota to ferment carbohydrates. Such was also true for butyrate production. Correlations between results from the intervention trial and the in vitro fermentation suggested a link between changes in the composition and

function of the microbiota and changes in inflammatory markers. *Coprococcus* appeared to play a central role in the production of SCFA during fermentation of carbohydrates and subsequent reduction in TNF-α and LBP. These data show that *in vitro* fermentation can help develop hypotheses explaining the relationship between diet, metabolic health, and gut bacteria.

Table 4.1 Correlations between results from in vitro fermentation of pre-digested whole wheat flour and habitual nutrient intake of the fecal donor; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; ferm., fermented (%); AX, arabinoxylan; N SP, non-starch polysaccharides; N=31; *p<0.05; **p<0.01.



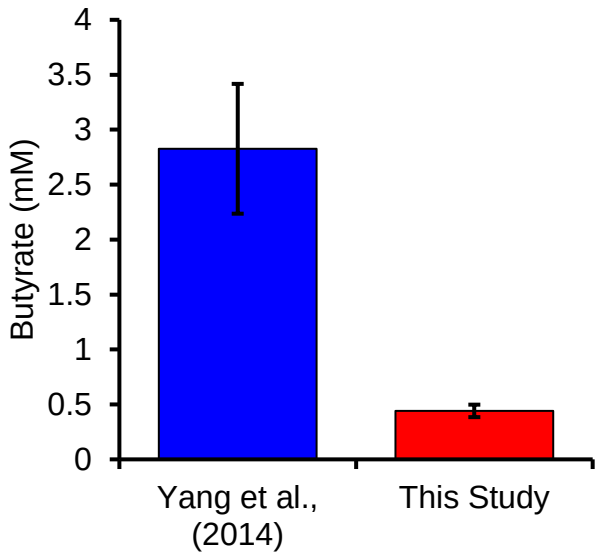
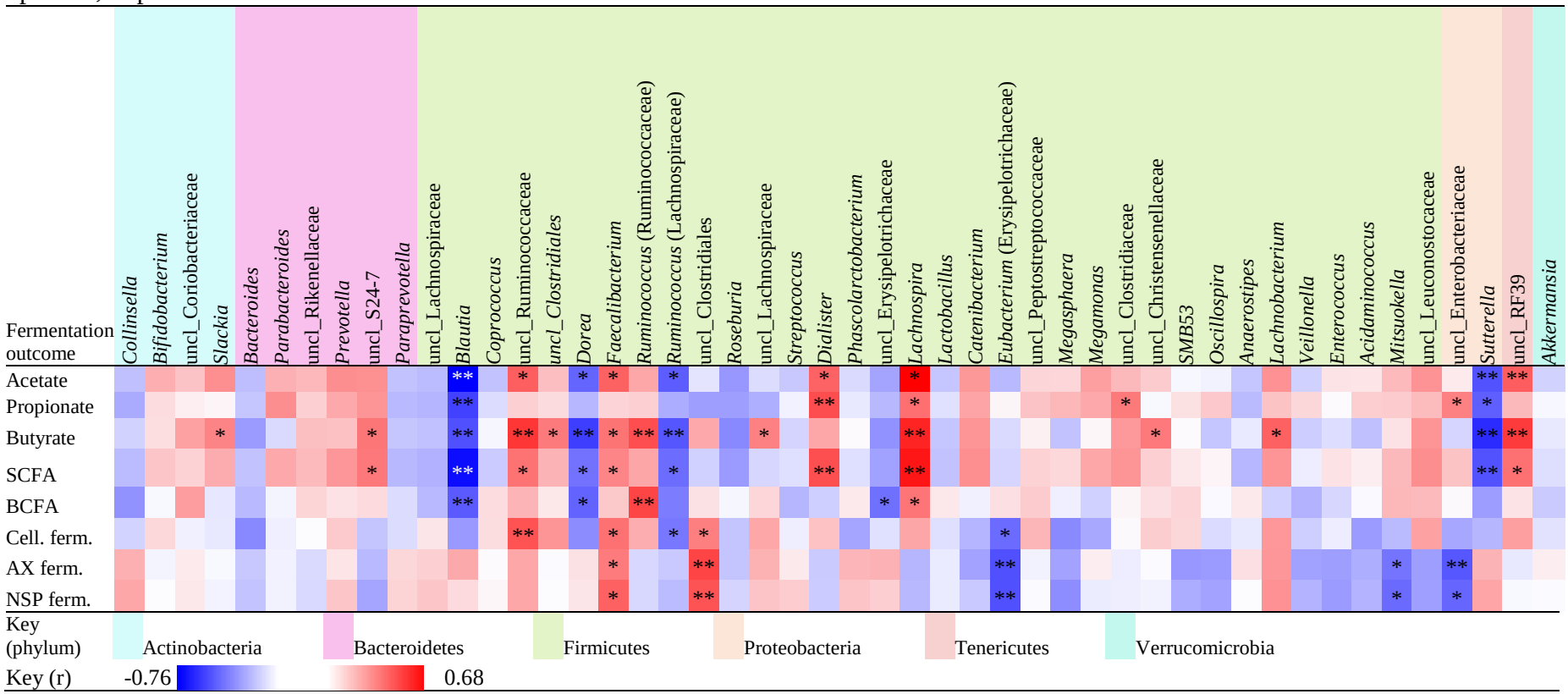


Figure 4.1 Comparative Butyrate Production Between Two Different Study Populations, Yang et al., (2014) normal weight/diet, mean BMI 25, mean age 24 ; reporting study, overweight/ poor diet, mean BMI 32, mean age 33,

Table 4.2 Correlations between results from in vitro fermentation of pre-digested whole wheat flour and fecal microbiota composition of the stool donor; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; ferm., fermented (%); cell, cellulose; AX, arabinoxylan; NSP, non-starch polysaccharides; only taxa of which at least 1 person had a relative abundance >1% are shown; N=31; *p<0.05; **p<0.01.



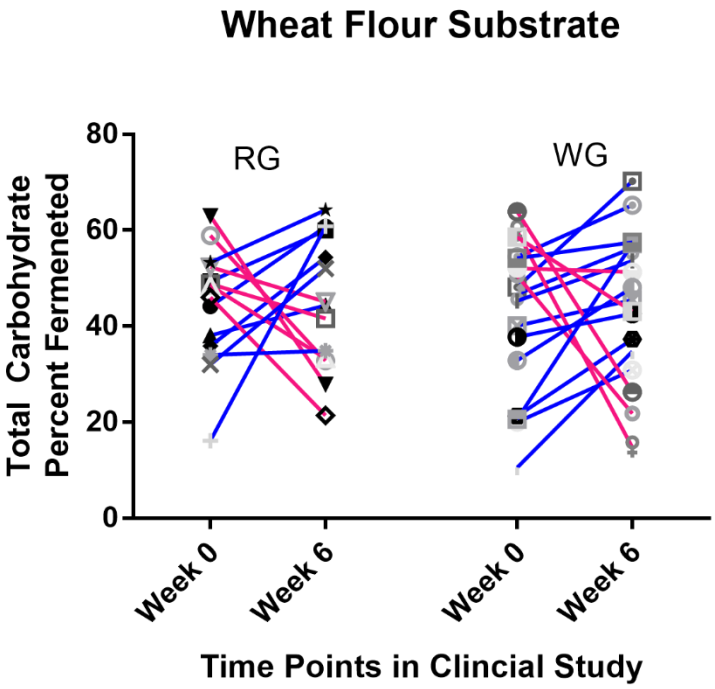
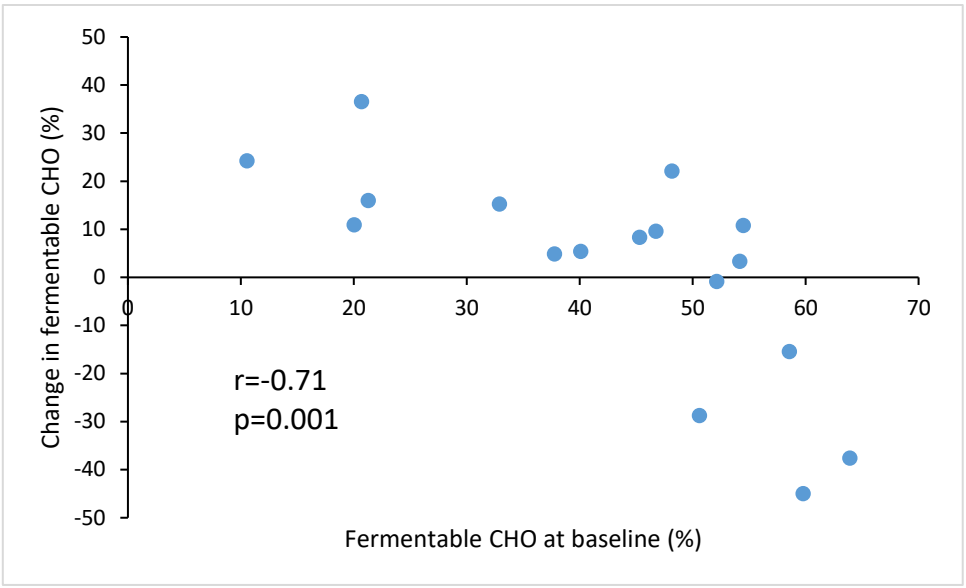


Figure 4.2 Total Carbohydrate Percent Fermentation *in vitro* Fermentation of Pre-digested Wheat Flour

A



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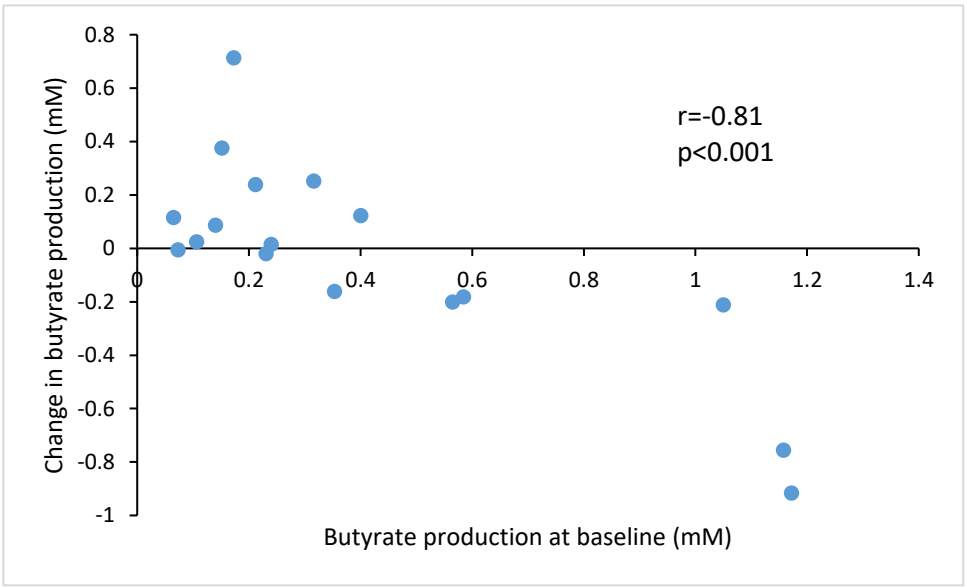


Figure 4.3 Scatter Plot of Significant Correlations of Fermentation Outcomes across Time Points A-Change in Fermentable CHO with Fermentable CHO at Baseline B-Change in Butyrate Production with Baseline Butyrate Production

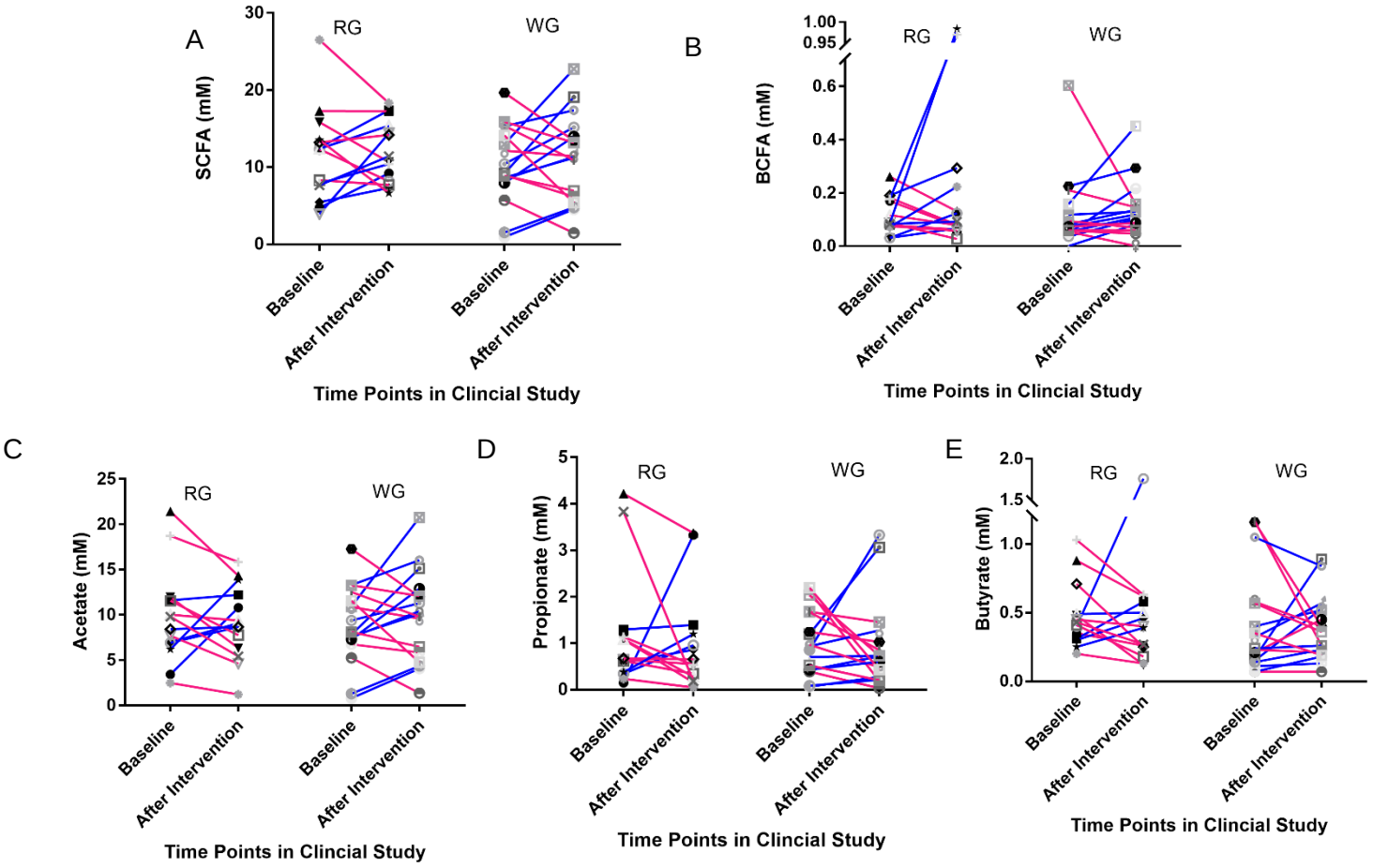
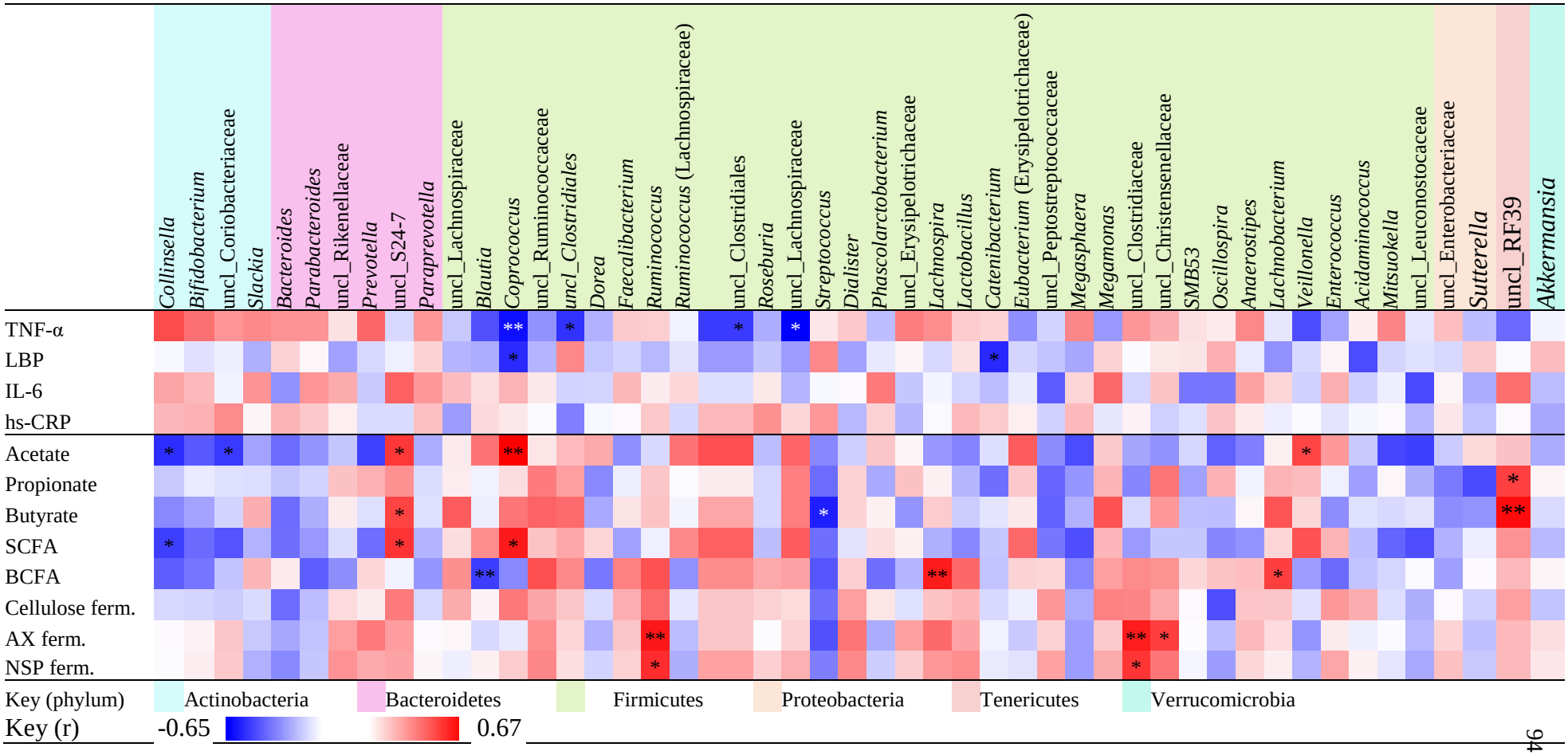


Figure 4.4 SCFA and BCFA Production during in vitro Fermentation with Pre-digested Wheat Flour (Note: Not Significant)
A- SCFA B-SCFA C-Acetate D- Propionate E- Butyrate

Table 4.3. Correlations between results from *in vitro* fermentation of pre-digested whole wheat flour with WG treatment, blood markers, and fecal microbiota composition of the stool donor; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; ferm., fermented (%); cell, cellulose; AX, arabinoxylan; NSP, non-starch polysaccharides; only taxa of which at least 1 person had a relative abundance >1% are shown; N=31; *p<0.05; **p<0.01.



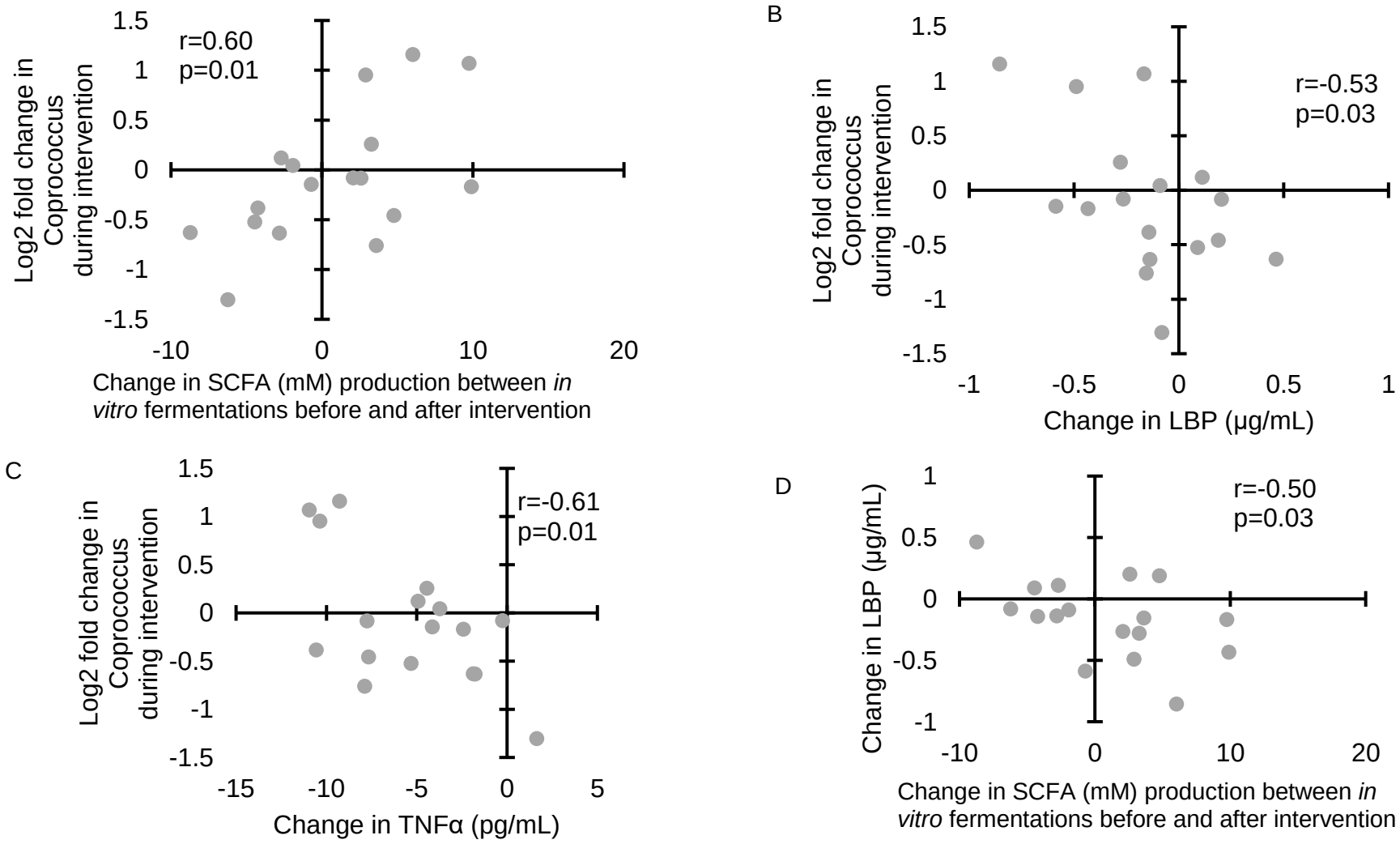
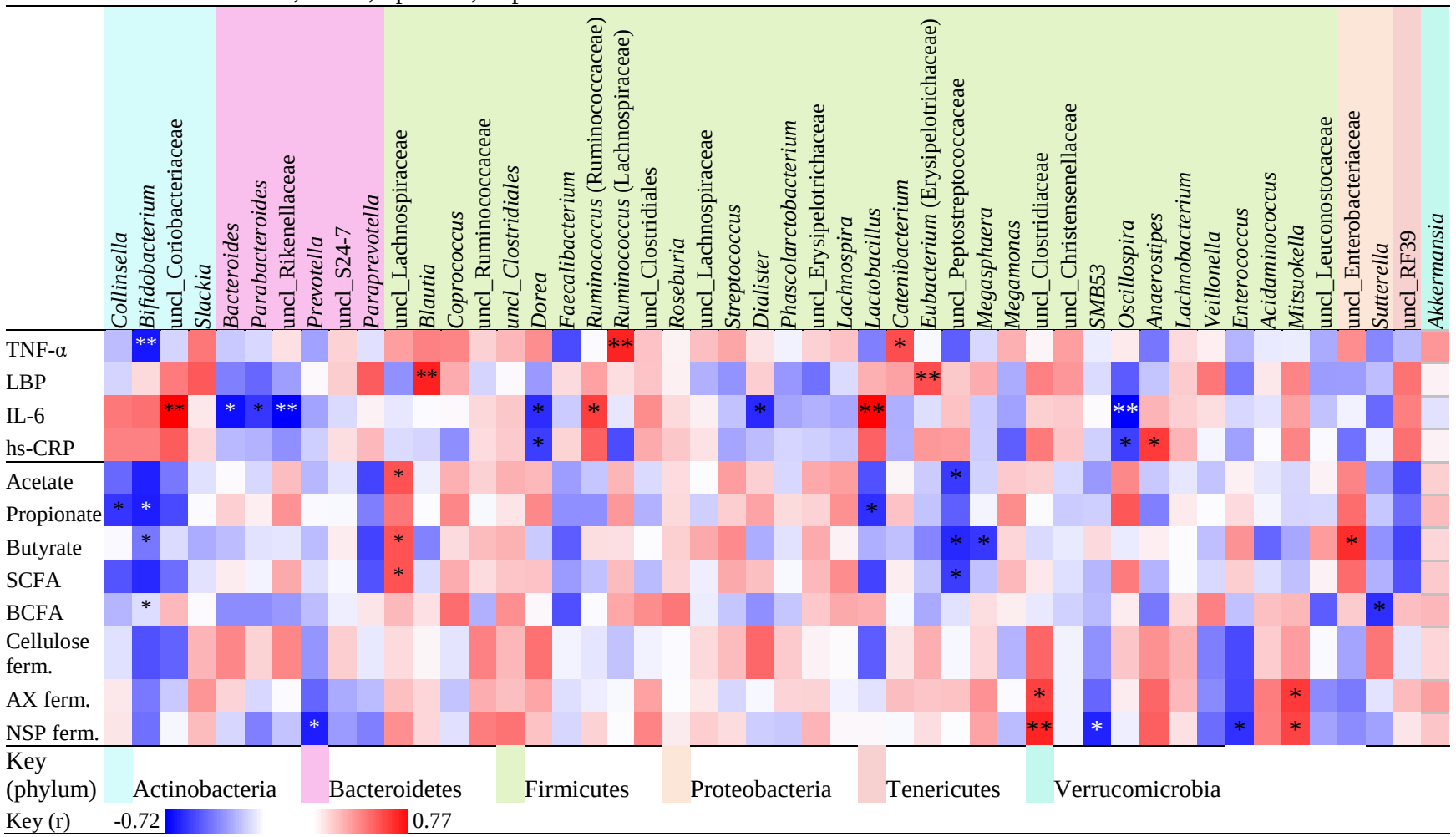


Figure 4.5 Scatter Plots of Significant Correlations A-:Scatter Plots of Significant Change in SCFA with Log2fold Change of Coprococus B-Change in TNFα with Log2fold Change of Coprococus C- Change in LBP with Log2fold Change of Coprococus D-Change in LBP with Change in SCFA

Table 4.4 Correlations between results from *in vitro* fermentation of pre-digested whole wheat flour with RG treatment, blood markers, and fecal microbiota composition of the stool donor; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; fer., fermented (%); cell, cellulose; AX, arabinoxylan; NSP, non-starch polysaccharides; only taxa of which at least 1 person had a relative abundance >1% are shown; N=31; *p<0.05; **p<0.01.



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Chapter 5: Conclusions

Conclusions

Based on whole grain (WG) and fruit and vegetable (FV) epidemiological and intervention studies, I hypothesized that the WG intervention will be as effective in improving beneficial bacteria and reducing subclinical inflammation as a FV intervention, both of which will be better than RG. To test this hypothesis I conducted a 6 week feeding trial with WG, FV, and RG. Inflammatory markers and stool samples were analyzed before and after the 6 week intervention. The data did support half of my hypothesis. The WG and FV treatments were effective in reducing subclinical inflammation compared to the RG treatment. The data did not support the hypothesis that there were equal treatment effects, but both treatments showed significant decreases in inflammatory markers. However, their effects may not be equivalent because the markers that were decreased were different depending on intervention group. Based on differing effects on inflammatory markers, I now hypothesize that WG and FV are both anti-inflammatory but by different mechanisms.

Through *in vitro* studies from the literature I hypothesized that the fecal microbiota collected from subjects after a WG intervention will be better able to ferment the dietary fibers in whole wheat and produce butyrate compared with before the intervention. The data showed a trend toward higher carbohydrate fermentation after the WG intervention, but there was a lot of individual variation. There was not a significant increase in butyrate production. Differences in SCFA production were correlated with changes in inflammatory markers during the intervention. With these data I now

hypothesize that the change in *Coprococcus* improves inflammation by fermenting whole grain dietary fiber. I also hypothesize that increased SCFA production is responsible for certain decreases in inflammatory markers.

Changes to the Experiment

The experimental design of this project was fabricated well, but there are slight changes that could have made the experiment more successful or controlled. Recruiting participants was a struggle due to finding overweight people who were not trying to get healthy. Most overweight people either work out or are trying to achieve a healthier diet. The number of participants was slightly lower than anticipated. By having a larger sample of participants more changes may have been identified.

The interventions were very modest. Participants could choose from 11-15 different options. In other WG or FV clinical treatments more strict diets and interventions were enforced (Costabile et al., 2008; J. et al., 2013; Martínez et al., 2013). With these strict interventions investigators knew exactly what they were eating and could also correlate individual nutrient components from that food to parameters in the study. With my interventions I was not able to pinpoint if the change in IL-6 was from a particular food. Conclusions could only be made that whole grain products or fruit and vegetable products are making those changes. Future clinical interventions should have specific food interventions like 1 slice of bread or 1 cup of rice a day.

During the study participants were supposed to continue with their regular diets, which were already low in whole grains and fruit and vegetables. There was no restriction on what they could and could not eat besides FV and WG. I assumed that subjects did not change their normal diet, but there are other foods in the diet (fermented

products, yogurt, seeds, and certain oils) that can be contributing to changes in gut microbiota and immune response markers. By not allowing these types of foods maybe the results would have changed a certain way.

The *in vitro* fermentation experiment did not show many significant results. The experiment had a very small sample size, but with a larger sample size potentially more significance results could be found. The fermentation time could have not been long enough. In a similar study with a fermentation time of 24 hrs more fermentation and SCFA production occurred (Brahma et al., 2017). In the *in vivo* experiment more dramatic treatments of 5 servings of WG or a single treatment of rye bread could change more of the functionality of the bacteria for the *in vitro* fermentation. With a larger sample size, dramatic intervention, or longer fermentation time potentially more changes may have been significant.

Future Research

From our results there are different future experiments that can be completed. It was shown that whole grains and fruit and vegetables decrease inflammation but in different mechanisms. There could be a synergistic effect happening with these two food products. I would propose to perform a similar intervention with RG as the control but have 1 treatment group of participants consuming their RDI of whole grains and fruit and vegetables to see how inflammation or gut bacteria changes.

Another clinical trial that could be completed is have 6 treatment groups looking at refined grains, the RDI of whole grains or fruit and vegetables, combining the RDI of whole grains, fruit and vegetables, and doubling the RDI of whole grains or fruit and

vegetables. It would be interesting to see how over consumption of WG or FV compares to control diet and the normal RDI of WG and FV.

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Appendix

Methods

Blood serum was sent to UNL Biomedical and Obesity Research Core (BORC) to be analyzed for glucose, cholesterol, magnesium, Triglycerides, and HDL using the Vitros-250 chemistry analyzer.

Results and Discussion

During testing of the plasma markers, it was shown that some of the participants had elevated glucose levels. This could be from not having a fasting blood sample. Non fasting blood sample will only effect the plasma markers and should not affect the inflammatory markers. During the analysis the participants with the evaluated glucose levels were taken out of the fasting plasma analysis.

As described in Table 1 there were evaluated glucose levels that would affect the analysis of the fasting blood samples. Figure 1 shows the change in the blood parameters. There were no significances between treatment groups. This data was very inconclusive due to the limiting participants that had a low enough glucose level to be considered a fasting blood sample. A similar study that had a 3 week treatment of whole grain breakfast cereal showed no significant differences between control and treatment diet in HDL, cholesterol, and glucose levels.

Table A.1 Baseline Data

Baseline data	Groups			
	Whole Grains	Fruit and vegetables	Refined Grains	P-Value
Fasting Plasma markers*				
Glucose (mg/dL)	88 ± 13.75	92.29 ± 2.21	92.14 ± 7.13	0.9385
Cholesterol (mg/dL)	170.33 ± 33.50	166.57 ± 29.29	141.71 ± 17.03	0.9676
Magnesium (mg/dL)	1.93 ± 0.23	1.9 ± 0.15	1.79 ± 0.11	0.9051
Triglycerides (mg/dL)	98.33 ± 36.50	100.43 ± 28.47	74.14 ± 22.87	0.6163
HDL (mg/dL)	61.67 ± 10.41	61.86 ± 17.92	52.0 ± 12.62	0.5888

Participants FV=7 WG=3 RG=7.

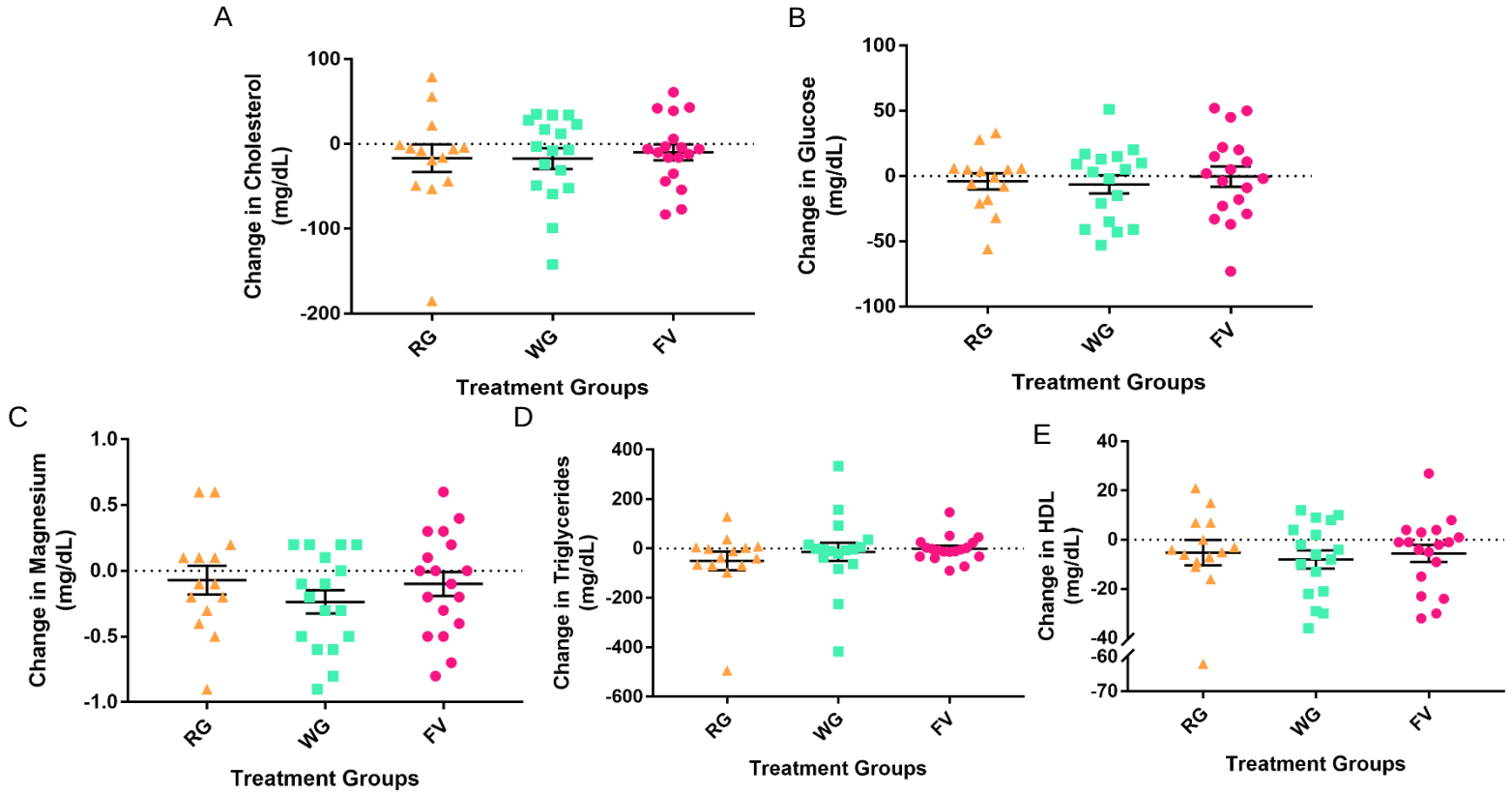

Figure A.1: Changes in Blood Markers Due to Diet
A- Cholesterol B- Glucose C- Magnesium D- Triglycerides E- HDL * < 0.05, ** < 0.01, *** < 0.001

Table A.2 FV Menu

choose 2 1/2 cups to 3 cups- put either 1, 2 or 1/2 on each day you want that item												
Food	Wednesday	Thursday	Friday	Saturday	Sunday	Monday	Tuesday	Frozen	Fresh	Can	1 cup equivalent	1/2 cup equivalent
Fruit												
Banana											large	small
Apple											large	medium
Oranges/ cutie											orange	cutie
Strawberries											1 cup	1/2 cup
Grapes											32 grapes	16 grapes
Pear											1	1/2 pear
Veggies												
Pepper											1 pepper	1/2 pepper
Celery											2 stalks	1 stalk
Broccoli											91 g or 3 spears	45 g
Cauliflower											107 g	53 g
Lettuce											2 cup	1 cup
Spinach											2 cup	1 cup
Peas											1 cup	1/2 cup
Tomato											1 large	8 cherry tomato
Baby Carrot											12	6
Green Beans											1 cup	1/2 cup
max 44 to 42												

Table A.3 WG Menu

Choose 3-4 servings per day								
Food	Wednesday	Thursday	Friday	Saturday	Sunday	Monday	Tuesday	Serving size equivalent to 1
Sara Lee Whole Wheat Bread								1 slice=1
Mission Whole Wheat Tortilla								7 in
Oatmeal								1 packet=1
English Muffin								Mini vs Big
Wheaties Cereal								3/4 cup
Ready to Eat Brown Rice								uncle Bens
Granola								48 g
whole grain Spaghetti								1/2 cup=1 serving
Cheerios Cereal								3/4 cup
Triscuit Crackers								5 chips
Mini Wheats								11 biscuits
Mac and Cheese								Individual or box
Oat Bar								1 square
Popcorn- Boom Chicka Pop								21 g
Sun Chips								7-11 chips
Max 30 servings								

Table A.4RG Menu

Choose 3-4 servings per day								
Food	Wednesday	Thursday	Friday	Saturday	Sunday	Monday	Tuesday	Serving size equivalent to 1
Sara Lee White Bread								1 slice=1
Mission Flour tortilla								14 in
Mission Flour tortilla								7 in
Cream of Wheat								1 packet=1
Plain Bagel								mini vs big
Corn Flakes								3/4 cup
Uncle Bens Ready to eat White Rice								1 package
Rice Krispies Treats								1
Spaghetti								1/2 cup=1 serving
Special K Cereal								3/4 cup
Town House Ritz Crackers								25 g
Rice Krispies								3/4 cup
Pita Chips								10-11 chips
Mac and Cheese								box vs individual
Max 30 servings per week								

Week | | Participant ID | | | |

Fill out as accurately as possible. Estimate serving sizes using the serving card provided. Do not forget condiments, snacks, and beverages.

Date	Meal	Item	Amount	Additional information

Figure A.2: Diet Diary

Please mark the boxes corresponding to your choice. Participant ID

Have you been bothered by stomach ache or pain during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by heartburn during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by acid reflux during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by hunger pains in the stomach or belly during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by nausea during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by rumbling in your stomach or belly during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Has your stomach felt bloated during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by burping during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by passing gas or flatus during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by constipation during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by diarrhea during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you ever been bothered by loose stools during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by hard stools during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Have you been bothered by an urgent need to have a bowel movement during the past week?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

When going to the toilet during the past week, have you had the feeling of not completely emptying your bowels?

Number of days	Average severity
0	No discomfort at all
1-2	Slight
3-4	Mild
5-6	Moderate
Everyday	Severe

Figure A.3 Gastrointestinal Symptom Rating Scale

RESEARCH VOLUNTEERS NEEDED

The Food Science & Technology Department at the University of Nebraska-Lincoln is currently seeking volunteers to participate in a clinical research trial to evaluate the benefits of increasing fruit, vegetable, and whole grain intake.

We are recruiting adults with low intakes of fruit, vegetables, and whole grains that are not involved in a regular exercise program.

The study involves filling out two diet history questionnaires, six visits to a study facility to pick up food and turn in questionnaires, and two visits to a clinical facility where blood and stool samples will be collected.

Subjects will receive all test foods at no cost, a personal nutritional analysis and blood chemistry profile, and modest monetary compensation.

Please contact Ms. Julianne Kopf at 402-801-2006; juliannekopf@gmail.com or Dr. Devin Rose at 402-472-2802; drose3@unl.edu for more information

University of Nebraska Lincoln

Figure A.4 Recruitment Flyer