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Effect of Grain Sorghum Polyphenols on Fecal Fermentation

A thesis submitted in partial fulfillment
of the requirements of the degree of
Master of Science in Food Science

by
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University of Arkansas
Bachelor of Science in Food, Human Nutrition, and Hospitality

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This thesis is approved for recommendation to the Graduate Council.

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Abstract

Obesity is an increasing epidemic which during 2015-2016 afflicted 39.8% of adults and 18.5% of youth in the United States. Not only can obesity affect quality of life, but it can lead to life-threatening diseases such as type 2 diabetes, heart disease, stroke, and some cancers. Animal and human research have demonstrated a pattern of gut microbiota perturbation in overweight and obesity, characterized by a reduction of beneficial bacterial species and increase in harmful species. Diet has been established as a contributing factor in gut ecology, and foods including fiber, resistant starch, and polyphenols have been found to both enhance desirable species and inhibit pathogens. Short chain fatty acids (SCFA), the products of microbial fermentation of carbohydrates, have also been implicated in body weight maintenance. Sorghum is the world's fifth leading crop and highly undervalued in the US as a source of nutrition. The diversity and abundance of polyphenols in sorghum bran has been identified, however limited information is available on the effects of sorghum polyphenols on gut microbiota. We hypothesized that polyphenolic extracts of black and sumac sorghum brans would impact production of SCFA, alter composition of human fecal microbiota in favor of beneficial species, and improve the overall microbiota profile of overweight/obese individuals. The objectives of this study were to determine the contents of polyphenolic compounds in sorghum bran and to evaluate the change of gut microbiota composition and the effect on SCFA production in response to sorghum bran polyphenols in normal weight (NW) and overweight/obese (O/O) subjects. Black and sumac sorghum brans displayed individually unique polyphenol profiles. Total SCFA production tended to be higher in the NW group, while butyrate production from FOS tended to be higher in the O/O group. Sorghum bran phenolic extracts modulated the gut microbiota and stimulated

Prevotella and the butyrate-producing bacteria *Roseburia*. They also worked synergistically with FOS to enhance *Lactobacillus* and *Bifidobacterium*. Varying responses to sorghum polyphenols were seen between bacteria in NW and O/O. Our results support gut health-enhancing actions of sorghum polyphenols, some of which may depend on body weight status.

Table of Contents

Introduction	1
Chapter 1: Literature Review	3
1. Grain Sorghum.....	3
1.1 Components of Grain Sorghum	4
1.2 Polyphenols of Grain Sorghum.....	5
1.3 Health Effects of Grain Sorghum	8
2. Human Gut Microbiota	10
2.1 General Overview	10
2.2 Roles of HGM in Health and Disease.....	13
3. Functional Roles of Human Gut Microbiota	24
3.1 Fermentation Pathways	24
3.2 Prebiotics.....	27
3.3 Polyphenols.....	28
4. Grain Sorghum and the Human Gut Microbiota.....	30
References	33
Chapter 2: Publication Manuscript.....	49
Abstract	49
1. Introduction	50
2. Materials and Methods.....	52
1.1 Sorghum Brans, standards, and reagents	52
1.2 Preparation of Polyphenols from Sorghum Bran Samples.....	52
1.3 Analysis of Polyphenol Content and Antioxidant Activity	53
1.4 Human Fecal Fermentation	55
1.5 Statistical Analysis	57
3. Results.....	58
2.1 Polyphenol Profiles and Antioxidant Properties of Sorghum Bran and Sorghum Bran Extracts	58
2.2 Short Chain Fatty Acid Production	59
2.3 General Changes in the Microbiota.....	60

2.4 Specific Changes in Microbial Populations	61
4. Discussion.....	65
5. Conclusions and Next Steps	75
Tables and Figures	76
References	89
Conclusion.....	94
Appendix A: IRB Approval Letter.....	95
Appendix B: IBC Approval Letter	96
Appendix C: IBC Personnel Form	97

Introduction

Grain sorghum is rich in polyphenols, with proanthocyanidins, phenolic acids, and flavonoids that are concentrated in the bran fraction (Awika et al., 2005). Sorghum bran polyphenols have been shown to positively affect cancer cytotoxicity and tumor inhibition, and display considerable antioxidant activity (Shih et al., 2007; Yang et al., 2009; Wu et al., 2011.)

Research in recent years has focused on how diet can be applied to promote a healthy human gut microbiota, which plays crucial roles in maintaining health. Gut dysbiosis has been observed in several health conditions including overweight and obesity (Collado et al., 2008), and foods that help correct this dysregulation may contribute to body weight maintenance strategies. Of particular interest have been carbohydrates, which are metabolized by bacteria through the process of fermentation and increase short chain fatty acid (SCFA) production and produce shifts in bacterial populations (Rossi et al., 2005). Prebiotics have recently been defined by the International Scientific Association for Probiotics and Prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). While few food compounds are considered unquestioningly as prebiotics, several foods are being investigated as potential candidates, and these include polyphenols. Polyphenols from numerous sources have been shown to undergo metabolism in the colon and consequently affect the microbiota (Tomas-Barbaran et al., 2016).

While there is abundant evidence of interactions between polyphenols and the gut microbiota, studies involving polyphenols from sorghum bran are limited. Following the redefinition of "prebiotics" we sought to investigate the prebiotic potential of these compounds. It has also been proposed that polyphenols may enhance utilization of

carbohydrates to produce SCFA (Tzounis et al., 2008), but little research has been done to test this theory. We hypothesized that sorghum bran polyphenols would impact production of SCFA, increase beneficial gut microbiota in both groups, and improve bacterial profile of overweight/obese individuals. The objectives of this study were to determine the contents of polyphenols in sorghum brans and to evaluate the change of gut microbiota composition and the effect on SCFA production with sorghum bran polyphenols in fecal samples from normal weight and overweight/obese subjects.

Chapter 1: Literature Review

1. Grain Sorghum

An often-overlooked member of the cereal grain family, sorghum bicolor (L.) Moench (commonly known as sorghum) is one of the world's most important cereal crops, topped only by wheat, maize (corn), rice, and barley (Dykes et al., 2011). Often categorized with millets, sorghum displays high tolerance to arid, dry conditions relative to other grains, making it a staple food in areas prone to drought, such as Africa and India (Awika, 2011). While its consumption is historically high in these areas, it has seen a decline since the early 20th century, being gradually replaced by maize. During drought stress, maize is more readily afflicted by fusarium molds which can result in carcinogenic compounds, and this increase utilization of maize in Africa has been associated with increased diagnoses of squamous carcinoma of the oesophagus (Awika, 2011; Isaacson, 2005). According to the USDA Foreign Agriculture service, the United States was the number one producer of sorghum in 2016/2017, followed by Nigeria and Mexico (USDA, 2018). However, acreage of planted sorghum declined from 6.7 million acres in 2016 to 5.6 million acres in 2017, and production from 364 to 480 million bushels (USDA, 2016, 2018). These statistics, however, do not accurately depict consumption, as most sorghum products produced in the US are exported to other countries. Of the sorghum produced and utilized in the United States, only 3% makes its way to the human food industry, with most being used for animal food (17%), ethanol production (21%), or as an export commodity (55%) (The Sorghum Checkoff, 2016). But as American consumers continue to seek functional foods and healthy products on grocery shelves, sorghum is becoming more desirable

for its numerous health benefits which include glucose and insulin control (Poquette et al., 2014), anti-cancer properties (Awika and Rooney, 2004), and colonic health improvement (Fedail et al., 1984).

1.1 Components of Grain Sorghum

Like most cereal grains, the sorghum kernel is composed of an inner endosperm, germ, and outer layer of bran (Hubbard and Hall, 1950). Macro- and micronutrient composition differs amongst varieties, containing approximately 9-14% protein, 2-4% fat, 65-75% starch, and 1.5-2.5% fiber (Hubbard and Hall, 1950; Neucere and Sumrell, 1980). Poquette et al. (2014) found 76% of starch to be slowly digestible starch (SDS) or resistant starch (RS), considered by many as dietary fiber and contributing many of the same positive effects of fiber (frequent and regular bowel movements) without negative consequences such as abdominal pain and flatulence. Sorghum is also gaining interest due to its lack of gluten, making it a safe and palatable alternative to wheat products for those suffering from celiac disease (Ciacca et al., 2007).

The bran fraction of sorghum contains not only the majority of its fiber, but also the vast majority of its polyphenols (Awika et al., 2005). Awika et al. (2003, 2005) found that sorghum brans of several varieties including brown, black, red, and white contained 4-6 times the dietary fiber and 3-5 times the concentration of polyphenols compared to the whole grain of each variety. Polyphenol content also differs amongst sorghum varieties. Sumac sorghums typically have higher total polyphenol content due to large amounts of condensed tannins, while black

sorghums are richer in flavonoids, especially the 3-deoxyanthocyanins (Awika et al., 2003, 2004, 2005; Dykes et al., 2005).

1.2 Polyphenols of Grain Sorghum

Polyphenols are chemical structures composed of one or more phenol rings and are classified by number of rings and attached components into four categories: Phenolic acids, flavonoids, stilbenes, and lignans. Polyphenols are widely spread in plants and relatively abundant in diets high in fruits and vegetables. In plants, polyphenols are secondary metabolites that serve as protection against ultraviolet light, pathogens, and predators. In foods, they contribute positive actions such as protection against oxidation, resilience to light and pH, flavor, and effectiveness as coloring agents (Pandey and Rizvi, 2009).

Although most have been demonstrated *in vitro* only, numerous health-promoting biological activities have been proposed for plant polyphenols (Manach et al., 2004). Polyphenols are well established antioxidants, and have been known to increase antioxidant activity *in vivo*, providing protection against free radicals and other potential oxidative threats (Manach et al., 2004). While these antioxidant activities occur during catabolism of polyphenols, their metabolites may also act upon target sites. Polyphenols also affect the metabolism of xenobiotics by competitively inhibiting various metabolic pathways (Manach et al., 2004). High intake of polyphenol-rich fruits and vegetables has been associated with decreased risk of chronic disease, and health-related actions include anti-cancer, cardioprotective, anti-diabetic, anti-aging, anti-viral, anti-bacterial, and neuro-protective (Manach et al., 2004, Kumar and Pandey, 2013).

Sorghum polyphenols can be categorized into three major groups: phenolic acids, condensed tannins (proanthocyanins), and flavonoids (Burdette et al., 2009). Previous research has found total polyphenol contents of 7.6-35.6 mg/g and 22.5-88.5 mg/g for black and brown sorghum bran, respectively (Awika et al., 2004, 2004, 2005). Polyphenols in sorghum bran have been of interest for decades, mostly for the sake of animal feed and food quality purposes rather than human nutrition.

There are two subclasses of phenolic acids, cinnamic acid and benzoic acid derivatives (Pandey and Rizvi, 2009; Manach et al., 2005). Phenolic acids have demonstrated high antioxidant activity *in vitro*, and have been found in several sorghum varieties, including white which do not contain the other phenolic compounds commonly found in colored brans (Awika and Rooney, 2004). In 1966, Guenzi and McCalla identified ferulic acid, p-coumaric acid, syringic acid, vanillic acid, and p-hydroxybenzoic acid in sorghum by paper chromatography (Guenzi and McCalla, 1966). Other phenolic acids in sorghum include gallic acid, protocatechuic acid, genistic acid, salicylic acid, caffeic acid, cinnamic acid, and sinapic acid (Dykes and Rooney, 2006).

Flavonoids in sorghum are mainly 3-deoxyanthocyanins, namely apigeninidin, luteolinidin, and their derivatives, and other flavonoids in sorghum include naringenin,

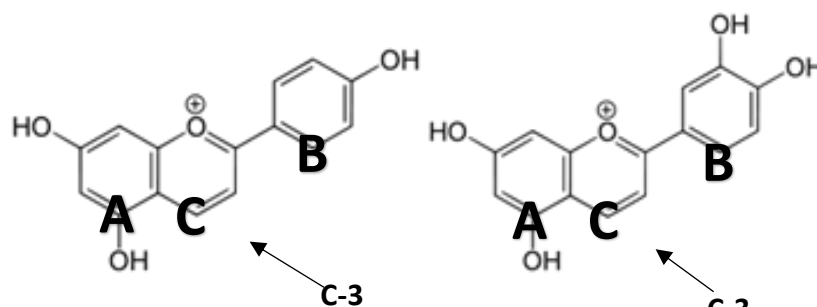


Figure 1. 3-Deoxyanthocyanins apigeninidin (left) and luteolinidin (right)

apigenin, and eriodictyol. As opposed to other anthocyanins, 3-deoxyanthocyanins lack a hydroxyl group at the C-3 position of ring 3 (**Figure 1**). This characteristic makes 3-deoxyanthocyanins especially stable in the presence of light and pH changes, and potential antioxidants and cancer cell cytotoxic agents (Sousa et al., 2016; Shih et al., 2007; Yang et al., 2009). Sorghum polyphenol research has focused extensively on 3-deoxyanthocyanins, because it is the most significant dietary source of these compounds identified thus far (Awika and Rooney, 2004). Apigeninidin, luteolinidin, and their glucosides were identified in sorghum using HPLC analysis, with 36-50% of anthocyanins being apigeninidin and luteolinidin. These results were confirmed by Dykes et al. (2009), who identified luteolinidin, apigeninidin, 5-methoxyluteolinidin, and 7-methoxyapigeninidin as the four major 3-deoxyanthocyanins in sorghum (Dykes et al., 2009). Quantification also revealed that black sorghums contained over two times the 3-deoxyanthocyanins of red and brown sorghums and concentrations in black and brown sorghum bran ranged from 1.7-6.1 mg/g and 0.5-2.8 mg/g, respectively (Awika and Rooney, 2004; Wu and Prior, 2005).

Condensed tannins, or proanthocyanins, are polymeric flavanols, the most common in sorghum being catechin, epicatechin gallate, procyanidin B₁, and polyflavan-3-ol (Awika and Rooney, 2004) (**Figure 2**). Degree of polymerization has been found to positively correlate with

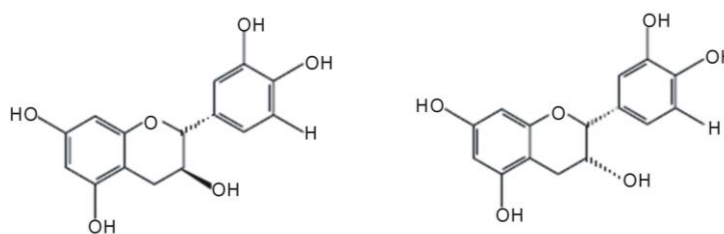


Figure 2. Common monomers of proanthocyanidins, catechin (left) and epicatechin (right)

antioxidant potential, and high molecular weight tannins are much more potent antioxidants than monomeric polyphenols (Hagerman et al., 1988). Additionally, animal studies have found condensed tannins to be significant antitumor agents in multiple types of cancer, including lung cancer and cervical cancer (Wu et al., 2011). Price and Butler used colorimetry and spectrophotometric methods to determine presence of condensed tannins in sorghum (Price and Butler, 1977). They were also identified in sorghum by HPLC and colorimetric methods (Dykes et al., 2005; Gu et al., 2007). Brown sorghum brans contained 28.2- 50.1 mg/g of condensed tannins, however none were detected in black brans (Awika et al., 2005).

1.3 Health Effects of Grain Sorghum

1.3.1 Cancer

Whole grain consumption has been associated with prevention of gastrointestinal cancers, and epidemiological studies have suggested sorghum to be more effective than other grains (Chen et al., 1993; Isaacson, 2005), possibly due its relative abundance and variety of polyphenols (Yang et al., 2012). Anti-cancer activities of sorghum polyphenols have been demonstrated *in vivo* and *in vitro*. Sorghum 3-Deoxyanthocyanins exhibited anti-proliferative actions against colon cancer cells *in vivo* (Yang et al., 2009) while procyanidin-rich sorghum extracts inhibited lung tumor formation and growth in mice (Wu et al., 2011). Sorghum phenolic extract also reduced proliferation and induced apoptosis of prostate cancer cells *in vitro* and subsequently reduced the severity of tumor metastasis in cancer-induced mice (Ryu et al., 2017). Though studies utilizing crude sorghum extract are limited, one *in vivo* investigation with 40 rats found that diets containing 6% black or brown sorghum bran suppressed colon carcinogenesis compared to a cellulose control diet, as evidenced by development of fewer

aberrant crypts. No effect was found for white sorghum bran, which supports the hypothesis that anti-cancer effects of sorghum are due to the color-conferring phenolic compounds (Turner et al., 2006). Demonstrated mechanisms of sorghum anti-cancer activities include induction of detoxification/antioxidant enzymes by 3-deoxyanthocyanins (Yang et al., 2009; Wu et al., 2011), estrogen receptor activation by apigenin and naringenin (Yang et al., 2012; Yang et al., 2015), and chemo-sensitization (Dia et al., 2016).

1.3.2 Diabetes

Incidence of diabetes is continuously rising, and it is the 7th leading cause of death in the USA (American Diabetes Association 2016). When managing Type I or Type II diabetes, maintaining low glucose and insulin response is crucial. Due to its high levels of slowly digestible starch (SDS) and resistant starch (RS), sorghum shows promise as an alternative to traditional therapies for controlling these factors. It has been found to exert hypoglycemic effects in diabetic rats by altering hepatic gluconeogenesis (Kim and Park, 2012), and has also lowered both glucose and insulin response in healthy men (Poquette et al., 2014). Inclusion of whole sumac sorghum flour in a rat diet (n=40) not only improved glucose and insulin homeostasis, but also protected pancreatic islet function in high-fat diet induced obesity (Moraes et al., 2017). Sorghum has also demonstrated lowering of protein glycation, a non-enzymatic reaction that is believed to contribute to development of diabetes (Farrar et al., 2008).

1.3.3 Colonic Health

As attention continues to shift to the gut and its implications for health in multiple bodily systems, colonic health and the human gut microbiota have become important targets of

research focusing on nutrition and functional foods. Consumption of whole grains retaining the bran was linked to a decrease in colorectal cancer in a cohort study of 60,000 women (Larsson et al., 2005). Sorghum bran has also been found to support healthy colon function. When compared to wheat bran, sorghum bran displayed comparable health benefits such as frequent defecation and softening of stools, while negative side effects such as abdominal pain and excessive flatulence was caused only by wheat bran (Fedail et al., 1984). Consumption of extruded whole-grain sorghum enhanced colonic health in rats by decreasing pH and inducing antioxidant enzymes in the caecum (Llopart et al., 2017). Polyphenols from various foods, including sorghum, have also been found to positively affect colonic health through modulation of the gut microbiota (Cueva et al., 2012; Ritchie et al., 2015).

2. Human Gut Microbiota

2.1 General Overview

Microbiota has been defined as the “entire population of microorganism that colonizes in a particular location,” (Jandhyala et al., 2015). The human flora has been studied since the mid to late 1900s (Drasar et al., 1976), but the intricate roles of the human gut microbiota (HGM) in health has gained new appreciation in the recent years. Most of the gastrointestinal tract is inhabited by bacteria, and the distal gut, or the colon, is the most heavily populated. Early on, occupation of fecal material in the colon by microbiota was reported as 10^{12} bacteria or more per gram (Moore and Holdman, 1974). Since then, estimates have ranged from 1.5×10^{11} to 5×10^{11} bacteria/gram (Sender et al., 2016). Early studies characterized 100+ bacterial species (Moore and Holdman, 1974), but a more recent compilation of large culture databases

and scientific literature reported 2172 species isolated in humans (Hugon et al., 2015). Bacteria within the human gut are most abundant of phyla Firmicutes and Bacteroides, but also include Actinobacteria, Verrucomicrobia, and Proteobacteria (Arumugam et al., 2011). Individual gut microbiotas are often classified by enterotypes, which are determined by dominant genus, and Arumugam et al. (2011) determined from a study of 39 individuals that HGM enterotypes can be categorized into three groups: type 1 enriched in *Bacteroides*, type 2 enriched in *Prevotella*, and type 3 enriched in *Ruminococcus*.

2.1.1 Age

A stable adult microbiota can be characterized by three enterotypes, and development of this state is a dynamic process beginning at birth. Mode of delivery plays a crucial role in establishing initial microbiota; a study of ten newborns reported that infants born vaginally displayed a microbiota dominated by the mother's vaginal bacteria e.g. *Lactobacillus* and *Prevotella*, where cesarean section infant displayed microbiota resembling maternal skin bacteria (Dominguez-Bello et al., 2010). These differential effects may diminish with age, however, as a recent study of 78 children ages 9-16 found no correlation between mode of delivery and gut microbial composition (Riva et al., 2017). By two years of age, the HGM resembles a stable adult population (Gibson et al., 1995), however, studies have demonstrated significant differences between child and adult microbiota, the most significant being higher abundance of *Bifidobacterium* in children than adults (Agans et al., 2011). Lower diversity is also observed in children, supporting continued development throughout adolescence (Ringel-Kulka et al., 2013). A stable state is reached during the 3rd decade of life (Jandhayala et al., 2015),

though the natural aging process is paralleled by decreased species diversity and a shift in population proportions (Claesson et al., 2011).

2.1.2 Diet

Diet is another factor that strongly affects HGM composition. As mentioned above, the initial HGM is established upon delivery and determined by route of passage. An infant's HGM taxonomy is further affected by diet, however. In 1983, a study of 13 infants found that those fed breastmilk developed a HGM consisting predominantly of *Bifidobacterium*, which have been attributed beneficial effects including short chain fatty acid (SCFA) production, detoxification of toxins, immunomodulation, and vitamin production (Yoshioka et al., 1983, Gibson et al., 1994). Formula-fed infants, on the other hand, harbored only a fraction of *Bifidobacterium* compared to the breast-fed infants, and had greater levels of *Bacteroides* as well as the potentially harmful *Enterobacteria* and *Enterococci* (Yoshioka et al., 1983). These results were corroborated in a 1987 study, which also observed an increase in *Clostridia*, another potentially harmful bacteria, in bottle-fed infants (Mevissen-Verhage et al., 1987).

While Arumagam et al. (2011) did not conclude a correlation with diet when establishing the three enterotypes, another study of 98 individual HGM found a strong association between high levels of *Bacteroides* (Type 1) and a long-term diet high in protein and animal fat, and high levels of *Prevotella* (Type 2) and long-term diet rich in carbohydrates (Wu et al., 2011). These results agreed with previous findings of not only increased *Prevotella* in children with a high fiber diet, but also an increased Bacteroidetes:Firmicutes ratio and increased short chain fatty

acid (SCFA) production compared with children with a western Diet (De Filippo et al., 2010).

These results promote the idea that numerous plant foods contribute substrates for desirable bacteria, and support development of a healthy gut.

Other dietary factors that affect the HGM include plant polyphenols and resistant starch (RS). Polyphenols have been found to alter HGM *in vitro* as well as in animal studies (Kemperman et al., 2013; Queipo-Ortuno et al., 2012), and RS modulates both bacterial population and SCFA production (Topping and Clifton, 2001; Martinez et al., 2010). Polyphenol and RS as HGM effectors will be discussed more in depth in another section.

2.2 Roles of HGM in Health and Disease

The HGM is considered by many to be an organ itself, with specific functions and roles in the body (Jandhyala et al., 2015). Colonic bacteria metabolize nutrients that bypass the small intestine unaltered and/or unabsorbed, such as polyphenolic compounds, fiber, and resistant starch (Dall'Asta et al., 2012; Topping and Clifton, 2001). The HGM can metabolize proteins that reach the colon and is also involved in lipid metabolism (Jandhyala et al., 2015). Vitamin synthesis is an additional intriguing role of bacteria in the gut. HGM have been found to promote biosynthetic pathways of multiple vitamins, with enterotype 1 (dominated by *Bacteroides*) associated with biotin, riboflavin, pantothenate, and ascorbate synthesis, and enterotype 2 (dominated by *Prevotella*) with thiamine and folate synthesis (Jandhyala et al., 2015). Possibly the most obvious function of the HGM is its pro/anti-microbial effect. It is vital for a healthy gut to maintain a profuse population of beneficial bacteria while preventing or inhibiting growth of pathogenic bacteria. Beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* inhibit harmful bacteria through multiple mechanisms, including competition

for nutrients and production of metabolites (e.g. SCFAs) that lower pH to promote an environment toxic to pathogens (Gibson et al., 1994). The HGM also combats pathogens (bacteria and others) through interactions with the immune system, such as development of gut associated lymphoid tissues, involvement in proliferation and homeostasis of T-cells, and control of inflammation (Hooper et al., 2012). One example of immune modulation by the HGM is its stimulation of plasma cells to produce immunoglobulin A (IgA). IgA is a crucial element in preventing bacterial translocation across the intestinal epithelium. Through these and other immune mechanisms, intestinal bacteria contribute to the fidelity of the gut mucus barrier, which bars pathogens from interacting with colonocytes and entering circulation (Sommer and Backhed, 2013). For example, *Lactobacillus* and *Bifidobacterium* spp. reduced inflammation and reversed permeability in interleukin-10 (IL-10) gene deficient mice (Madsen et al., 2001). In the following sections we will discuss associations between dysbiosis, or dysregulation of the normal microbiota, and some health conditions.

2.2.1 Obesity

Obesity is an increasing epidemic which during 2015-2016 afflicted 39.8% of adults and 18.5% of youth in the United States, and the rates in Arkansas closely follow this trend (CDC 2016). Not only can obesity affect quality of life, but it can lead to life-threatening diseases such as type 2 diabetes, heart disease, stroke, and some cancers. The high prevalence of obesity is due mainly to lifestyle factors such as decreased physical activity and unhealthy diet, but other physiological factors that may play a role in development of overweight/obesity, such as the HGM, must not be overlooked.

Several, but not all *in vivo* studies of HGM in obesity support a link between obesity and the ratio of Bacteroidetes to Firmicutes. Ley et al. (2005) found a 50% reduction of Bacteroidetes paralleled by an increase in Firmicutes in obese mice compared to lean mice. A following human study found that the Bacteroidetes:Firmicutes ratio in 12 obese individuals was positively correlated with weight loss during a year on a restricted calorie diet (Ley et al., 2006). In conflict with the previous reports, a study of 98 human subjects found the opposite correlation, namely a higher proportion of Bacteroidetes in obese and overweight individuals (Schweirtz et al., 2009). Others identified no change in the Bacteroidetes:Firmicutes ratio in overweight/obese individuals (Duncan et al., 2008; Arumugam et al., 2011). In support of early findings, a 2017 pediatric study of 96 children found a significant reduction of this ratio in obesity compared to normal weight (Riva et al., 2017). With the conflicting evidence presented, further study is needed to corroborate this association.

Correlations with obesity have been found at the genus and species level, especially *Staphylococcus aureus*, *Bifidobacterium* and *Akkermansia muciniphilia*. Increased *S. aureus* and decreased *Bifidobacterium* has been consistently observed in overweight/obesity, in animal as well as human studies (Collado et al., 2008; Kalliomaki et al., 2008, Santacruz et al., 2010). *A. muciniphilia* is a mucin-degrading member of Verrucomicrobia that colonizes in the mucus layer of the gut (Derrien, et al., 2008). This mucus layer serves as the gut barrier and plays important roles in minimizing gut permeability, which is increased in diabetes and obesity (Derrien et al., 2008; Everard et al., 2013). Derrien et al. (2008) found *A. muciniphilia* to represent 1-3% of bacterial cells in 96 human fecal samples, and this species has been negatively correlated with weight gain and obesity through *in vivo* animal studies (Everard et al., 2013; Schneeberger et

al., 2015) and human studies (Dao et al., 2016; Karlsson et al., 2012). Also, several human clinical trials indicate anti-obesogenic actions for some strains of *Lactobacillus* (Crovesy et al., 2017). These findings implicate diets that increase *Bifidobacterium*, *Lactobacillus* and *A. muciniphilia* as possible interventions in overweight/obese individuals.

Though findings regarding overweight/obesity and SCFAs have been mixed, several studies have observed protective effects of SCFAs against progression of obesity. Dietary supplementation of acetate was seen to suppress high fat diet (HFD)-induced weight gain by 72% in mice (Lu et al., 2016). In a study with six overweight/obese men distal colonic acetate injections led to improved metabolic and inflammatory markers, including fat oxidation and glucose and insulin levels (van der Beek et al., 2016). Recently, supplementation with acetate or propionate abolished HFD-induced weight gain and improved glucose and insulin homeostasis in mice (Weitkunat et al., 2017). Multiple murine studies have found dietary propionate and butyrate to block or reduce HFD-induced weight gain, as well as ameliorate oral-glucose tolerance and insulin levels (Henagan et al., 2015; Lin et al., 2012; Lu et al 2016). One human trial found that propionate intake significantly reduced intrahepatocellular and body weight gain over 24 weeks in 60 overweight adults (Chambers et al., 2015).

Contrarily, increased production of acetate by the gut microbiota was found to promote obesity and metabolic syndrome in rats via stimulation of appetite hormones (Perry et al., 2016). In a human study with 94 adults, total fecal SCFA concentrations were significantly higher in overweight/obesity compared to their normal weight counterparts (Fernandes et al., 2014). Also, the 2017 pediatric study found that butyrate, propionate, and acetate were all

significantly elevated in the obese group, though their findings do not necessarily implicate a causative role of SCFA in obesity (Riva et al., 2017).

Possible mechanisms for amelioration of body weight gain by SCFA include modulation of circulating appetite hormones and effects on energy metabolism. It has been demonstrated that dietary, as well as acute injections of acetate result in altered expression of neuropeptides in favor of appetite suppression, and decreased circulating free fatty acids in mice (Frost et al., 2014; Ge et al., 2008). Lin et al. (2012) found that satiety-promoting hormones Glucagon-like peptide 1 (GLP-1) and Glucose-dependent insulintropic polypeptide (GIP) were both suppressed by butyrate, while propionate exerted effects on GIP alone. Psichas et al. (2015) also observed increases in polypeptide YY (PYY) and GLP-1 in response to propionate in rats. While subsequent *in vivo* human trials found these hormones to be stimulated also by a single supplemental dose, long term (24 week) trial resulted in no effects on PYY and GLP-1 despite reduce body weight gain, so it is unclear if hormone modulation by SCFA contribute to long-term weight maintenance (Chambers et al., 2015).

Recent work demonstrates several ways through which SCFA exert control of energy metabolism and homeostasis in the body. Den Besten et al. (2015) demonstrated that a reduction in HFD-induced obesity in mice following SCFA supplementation was mediated through a PPAR γ -dependent transition from lipogenesis to fat oxidation via adenosine monophosphate-activated protein kinase (AMPK). This supports previous reports of acetate activating AMPK, which is a key factor in cellular energy homeostasis (Sakakibara et al., 2006). Furthermore, rectal administration of physiological concentrations of butyrate, propionate, and acetate in 12 overweight/obese men resulted in significantly increased fasting fat oxidation,

increased resting energy expenditure (REE), and increased levels of circulating PYY (Canfora et al., 2017). Additionally, SCFAs have been linked to “browning” of deleterious white adipose tissue (WAT) and reduction of whole-body adiposity and mice (Sahuri-Arisoylu et al., 2016). Weitkunat et al. (2017) also found that prevention of HFD-induced weight gain was accompanied by significant increases in body temperature, which is indicative of adipose tissue “browning”. These studies lay a groundwork for long-term human studies to examine by which mechanism enhanced SCFA production can help alleviate obesity in humans.

Not only is there clear evidence of correlations between the HGM and body weight, but multiple murine studies have suggested a causative role of microbiota, on the basis that excessive weight and fat gain can be transmissible through fecal transplant. In 2004, Backhed et al. (2007) inoculated gut microbiota (GM) to germ free (GF) mice, which resulted in a 60% increase in body fat despite reduced food intake. These findings highlight the ability of the gut microbiota to extract energy from food that is otherwise unutilized by the digestive tract and excreted in the feces. They later found that despite similar chow consumption, there were significant differences in body weight gain between conventional and GF mice being fed a western diet, with conventional mice gaining on average more than twice that of GF mice. This same study observed no significant differences between GF mice fed the high-fat western diet and GF mice fed a low-fat and polysaccharide-rich diet, indicating the presence of GM has greater effect than diet on body weight (Backhed et al., 2007). This concept was further explored in 2008, when GF mice were inoculated with GM from either lean mice or mice with diet-induced obesity. Though no significant differences in food intake were observed between groups, significantly greater increase in body fat was seen in mice treated with microbiota from

diet-induced obese donors (Turnbaugh et al., 2008). Though dysbiosis is likely caused by diet and other environmental factors, the above study establishes that the dysbiosis observed in overweight/obesity can further augment undesirable weight gain.

Proposed mechanisms by which the human gut microbiota may regulate body weight include its interactions with bile acid metabolism and the farnesoid X receptor (FXR) signaling pathway, and reduction of circulating lipoprotein fasting-induced adipose factor (FIAF). Gut microbes have the ability to transform bile acids through multiple enzymatic reactions including deconjugation, oxidation-reduction, and hydroxylation, which in turn activate the nuclear receptor FXR (Midtvedt 1974; Wang et al., 1999). FXR signaling pathways play an intricate role in both bile and lipid homeostasis, and genetic disruption of the receptor in mice has resulted in improved blood glucose and insulin sensitivity as well as reduced adiposity and weight gain in both genetic obesity and exposure to HFD (Sinal et al., 2000; Prawitt et al., 2011). In a study with germ free mice, Sayin et al. (2013) discovered that microbiota regulate bile acid homeostasis by metabolizing bile acids to forms that serve as ligands for FXR, alleviating its suppression. In 2017, Parseus et al. explored the effect of FXR on HFD-induced weight gain in GF and conventional mice. In agreement with previous findings, conventional mice gained significantly more weight than GF mice, however this difference was abolished in FXR deficient mice, implicating FXR as a requisite in GM-induced obesity (Parseus et al., 2017). This is in agreement with an earlier study, which showed that reduction of *Lactobacillus* led to increased levels of tauro- β -muricholic acid (known FXR antagonist), resulting in inhibited FXR signaling (Li et al., 2013). Future therapies to combat obesity may attempt to support a gut microbial population favoring FXR suppression rather than activation.

Fasting-induced adipose factor (FIAF) is protein expressed in fat and other tissues that downregulates fat storage and upregulates fat mobilization from adipocytes. Overexpression of FIAF resulted in a 50% decrease in body fat, however the translocation of fat out of adipocytes caused marked dyslipidemia, underscoring the importance of proper control of FIAF and lipid homeostasis (Mandard et al., 2006). One explanation for these effects is the ability of FIAF to inhibit both lipoprotein lipase (LPL), of which the primary function is to increase uptake of fatty acids and triglycerides into adipocytes, and peroxisomal proliferator activated receptor coactivator 1 α (Pgc-1 α), which increases expression of genes coding for fatty-acid oxidation (Yoshida et al., 2002; Backhed et al., 2004, 2007). Backhed et al. (2004) demonstrated the ability of the gut microbiota to suppress FIAF in intestinal epithelial cells and consequently increase LPL activity following inoculation of GF mice with caecal contents on conventional mice as well as isolated *Bacteroides thetaiotaomicron*. They expounded upon these findings in a later study, which showed a role of FIAF-regulated Pgc-1 α in obesity that developed upon inoculation of germ free mice with a conventional microbiota (Backhed et al., 2007).

2.2.2 Gastrointestinal conditions

It is estimated by the American Cancer Society that in 2018 there will be 50,630 deaths from colorectal cancer (CRC), which ranks 3rd in cancer deaths in the USA (Alteri et al., 2014). Risk of CRC is 50% higher in African Americans than Caucasian citizens, though native Africans rarely contract the disease (O'Keefe et al., 2009). This disparity has been explained by the consumption of a Western diet in African Americans, and their increased risk for CRC has been associated with decreased microbial production of SCFAs and in the gut (O'Keefe et al., 2009).

Butyrate has been implicated in anti-proliferative actions. Ou et al. (2013) found not only higher levels of butyrate-producing bacteria in native Africans, but also lower production of carcinogenic bile acids compared to African Americans. A number of *in vitro* studies have attributed the anti-cancer effects of butyrate to its ability to induce cell cycle arrest and apoptosis through gene regulation and upregulation of enzymes that detoxify carcinogens (Hamer et al., 2008). A study in 2011 demonstrated not only reduced proliferation of colon cancer cells by butyrate, but also a reduction of select microRNAs (miRNA) that are increased in human colon cancer (Hu et al., 2011).

Pathogenic microbial species also contribute to development of CRC. Excessive meat intake, as is seen in the Western diet, may result in increased proportions of proteolytic bacteria such as *Bacteroides* and *Clostridium histolyticum*, which in the colon metabolize proteins to toxic, cancer promoting agents (Hughes et al., 2000). Yu et al. (2017) positively associated cancer reoccurrence with *Fusobacterium nucleatum* in a cohort of 92 CRC patients. They subsequently linked *F. nucleatum* with development of chemoresistance through activation of an autophagy pathway via downregulation of specific miRNAs and alteration of innate immune signaling. It is clear that species residing in the gut play intricate roles in development of CRC, but further research is needed to elucidate the complexity of these roles and identify potential therapeutic strategies.

Irritable bowel syndrome (IBS) has been defined as “a functional bowel disorder in which abdominal pain or discomfort is associated with defecation or a change in bowel habit, and with features of disordered defecation” and has an incidence of 10-20% worldwide, affecting mostly women (Longstreth et al., 2006). IBS does not lead to severe illness or

mortality, however symptoms including abdominal pain, severe flatulence, constipation, and diarrhea can lead to a decreased quality of life comparable to more serious illnesses. Though etiology of the disease remains unclear, factors such as psychological stress and diet seem to stimulate symptoms and increase severity. Dysregulation and instability of gut bacteria has been observed in IBS and is likely to play an important role in the course of the disease (Kassinen et al., 2007). Reports regarding specific bacterial populations have been conflicting, however. Kassinen et al. (2007) found *Lactobacillus* to be nearly extinct in IBS patients, while in another study *Lactobacillus* were increased in IBS subjects compared to subjects without IBS (Tana et al., 2010). Multiple studies have demonstrated that probiotics containing *Lactobacillus* can alleviate symptoms and improve HGM stability and quality of life in IBS (Kajander et al., 2008; Ducrotte et al., 2012; Lorenzo-Zuniga et al., 2014). Rajilic-Stojanovic et al. (2001) reported a 2-fold decrease in the Bacteroidetes:Firmicutes ratio and a significant decrease in *Bifidobacterium* and *Faecalibacterium* paralleled by an increase in *Dorea*, *Ruminococcus*, and *Clostridium* in 62 subjects with IBS compared to healthy subjects. Accordingly, prebiotic intervention with GOS (galacto-oligosaccharide) resulted in increased *Bifidobacterium* and improved symptom scores, suggesting *Bifidobacterium* as a possible target of IBS therapy (Silk et al., 2009). Due to the heterogeneity of results, further research is needed to determine how bacterial composition can be normalized in IBS.

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC) and Crohn's disease (CD) and is characterized by unregulated, chronic inflammation in the gut and activation of the mucosal immune system (Hanauer 2006). Dysbiosis of gut microbiota also seems to be a feature in both IBD and UC. Human studies have indicated lower diversity, higher

populations of pathogenic bacteria, and decreased *Bacteroides* spp. in UC (Noor et al., 2010; Lepage et al., 2011). Studies investigating fecal transplant as a therapy for UC have been promising; for example, a case study reports complete amelioration of symptoms and need for medication in six UC patients (Borody et al., 2003). Kump et al. (2013) demonstrated improved symptoms and microbial population in 6 UC patients, yet no remission. However, a later study of 70 UC patients (placebo n=34, fecal transplant n=36) resulted in remission for 24% of patients undergoing treatment as opposed to only 2% of placebo group (Moayeddi et al., 2015). Fecal transplant therapy shows potential in treatment of UC and may in the future be optimized through a more individualized approach. Similarly, patients suffering from CD have microbiota characterized by reduced species diversity (particularly with the phylum Firmicutes and *Bacteroides* spp.) as well as an increase in the possibly harmful bacteria *Enterococcus* sp., *Clostridium difficile*, *Escherichia coli*, *Shigella flexneri*, and *Listeria* (Manichanh et al., 2006; Kang et al., 2010). These results were demonstrated in patients with active CD (Kang 2010) as well as those in remission (Manichanh et al., 2006), indicating that dysbiosis is not simply a result of inflammation and other symptoms of CD. Success of probiotic and prebiotic intervention has been limited, though some beneficial effects have been seen with *Saccharomyces boulardii* (Ghouri et al., 2014). Fecal transplant resulted in a 2-week remission for seven CD patients and a 6-12-week remission with no further treatments for five patients (Suskind et al., 2015). It also resulted in increased microbial diversity in 11 out of 19 patients (Vaughn et al., 2016). Though these studies hold positive implications for fecal transplant in CD, larger clinical trials are needed to confirm it as an effective therapeutic measure.

3. Functional Roles of Human Gut Microbiota

3.1 Fermentation Pathways

While most nutrients consumed are metabolized and absorbed in the stomach and small intestine, certain groups remain unaltered and provide fuel for the microbiota residing in the colon (Cummings and Macfarlayne, 1991). Most colon bacterial species are saccharolytic and glean energy from carbohydrate (CHO) in the diet including fiber and resistant starch (RS), as well as host-secreted glycoproteins and glycoconjugates (Rossi et al., 2005). Upon reaching the colon, fiber and starch are hydrolyzed to mono- and oligosaccharides by bacteria and subsequently fermented, releasing SCFAs and intermediate metabolites (e.g. lactic acid) that can be further metabolized to SCFAs (Rossi et al., 2005; Chassard et al., 2008). The importance of the HGM in energy harvest has been demonstrated *in vivo* in rodents. Germ free mice not only excreted more calories in their feces, but also incurred reduced adiposity and consumed more food than bacteria-colonized mice (Tremaroli and Backhed, 2012). Human studies have also demonstrated how dietary CHO influences the gut microbiota. In one study, a high RS diet stimulated proliferation of *Ruminococcus bromii*, *Eubacterium rectale*, and *Roseburia* spp, while a low CHO diet resulted in decreases in the same species, suggesting RS as a preferred substrate for these bacteria (Walker et al., 2011).

Short chain fatty acids are the major products of bacterial fermentation of carbohydrates in the colon. A series of reactions involving glycolytic pathways, pentose phosphate pathways, and others, convert mono- and oligosaccharides to intermediate metabolites and gasses, which are then metabolized to SCFAs, primarily propionate, butyrate, and acetate (Macfarlayne and

Macfarlayne, 2003). In fecal samples of 15 healthy human subjects, butyrate was produced in significant amounts by fermentation of starch and xylan, propionate in small amounts from all substrates (starch, xylan, casein, mucin, and cellulose), and acetate in significant amounts from all substrates (Chassard et al., 2008). SCFAs not only serve as energy sources, but they also lower pH which antagonizes unwanted bacteria; regulate cell volume, differentiation, proliferation, and gene expression; and regulate inflammation through activation of immune receptors and genetic inhibition of pro-inflammatory factors (Cook and Sellin 1998; Vinolo et al., 2011). Acetate, present at the highest concentrations in the colon, is produced by most enteric bacteria, while a limited number of butyrate and propionate producing genera have been identified thus far. Major butyrate producing bacteria found in human feces are within Firmicutes and include *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus* (Duncan et al., 2004; Louis and Flint, 2009). Butyrate can be produced directly from food sources, or from other metabolites of fermentation. As reported by Duncan et al. (2004), *Eubacterium hallii* and *Anaerostipes caccae* produce butyrate from the abundant secondary metabolite lactate. Acetate is also utilized by certain butyryl CoA: acetate CoA transferase producing bacteria for butyrate formation (Louis and Flint, 2016). These acetate-butyrate converting bacteria include *Faecalibacterium prausnitzii*, *Roseburia* spp., *Eubacterium* spp., *Coprococcus catus*, and *Anaerostipes hadrus* (Duncan et al., 2004; Louis and Flint, 2016). Propionate production has been observed in multiple species within Firmicutes, including *Veillonellaceae*, *Megasphaera*, *Coprococcus*, *Salmonella*, *Lachnospiraceae*, *Ruminococcus*, and *Roseburia*. Intestinal propionate concentration has also been positively correlated with *Prevotella* and *Bacteroides* within Bacteroidetes (Rios-Covian et al., 2016; Salonen et al., 2014). Butyrate stands out as beneficial

to human health as it is the major energy source of colonocytes, with proposed anti-colon cancer effects, anti-inflammatory activity, and mechanisms in colonic barrier function (Hamer et al., 2008). A likely mechanism of butyrate's inhibiting role in inflammation and colon carcinogenesis is its ability to cause increases in antioxidants and decreases in inflammatory mediators, which down-regulates cancer- and inflammation-promoting oxidative stress (Hamer et al., 2009). *In vitro*, butyrate increased production of glutathione-S-transferase, an anti-oxidant phase 2 enzyme (Ebert et al., 2003). *In vivo*, dietary fiber stimulated increased production of butyrate in rats, which inhibited production of tumor necrosis factor- α (TNF- α) and nitric oxide (Rodriguez-Cabezas et al., 2002). Hamer et al. (2009) found that butyrate increased production of glutathione and inhibited uric acid. These and many other studies strongly implicate butyrate's role in combatting oxidative stress in the colon. Many of butyrate's beneficial effects are due to its inhibition of histone deacetylase (HDAC), proteins involved in epigenetic mechanisms of gene control (Leonel and Alvarez-Leite, 2012). Acetate and propionate have been linked with positive outcomes as well. Propionate resulted in death of *E. coli* and *Salmonella in vitro*, most likely because of its low pH (Cherrington et al., 1991). Acetate produced by *Bifidobacterium* in mice provided protection against lethal infection with *E. coli* (Fukuda et al., 2011). A recent study elucidated mechanisms by which butyrate and propionate regulate osteoclast metabolism to protect against injurious bone loss (Lucas et al., 2018). Increased SCFA's and especially enrichment of butyrate producing species is a desirable outcome of fermentation studies and dietary interventions.

3.2 Prebiotics

Desire to manipulate bacterial populations, increasing beneficial while quenching harmful, led to the concept of prebiotics. The original criteria set forth to qualify a food as a prebiotic were: (a) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (b) fermentation by intestinal microflora; (c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing (Gibson et al., 2004). Prebiotics were previously defined as “selectively fermented ingredient(s) that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” (Gibson et al., 2010), but the Mayo Clinic later updated this definition to “nondigestible substance that acts as food for the gut microbiota...[and] stimulate growth or activity of certain healthy bacteria that live in the body.” In August of 2017, the International Scientific Association for Probiotics and Prebiotic published a consensus statement in which they proposed that prebiotics be universally defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). The three well-established prebiotics are the carbohydrate based fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose (Gibson et al., 2004), though the newest definition may allow for inclusion of non-carbohydrate substrates such as polyphenols and polyunsaturated fatty acids (Gibson et al., 2004, 2010; Slavin 2013; Gibson et al., 2017). The main bacterial targets of prebiotics are *Lactobacillus* and *Bifidobacterium* and the benefits conveyed include enhanced immune function, gut barrier function, and SCFA production, and reduced pathogenic bacterial populations (Slavin, 2013). Prebiotic action can be evaluated *in vivo* through consumption and subsequent collection of fecal samples, and *in*

vitro through fecal sample fermentations utilizing fecal preparations, prebiotic substrates, and anaerobic nutrient media. An abundance of research has demonstrated the ability of FOS, GOS, and lactulose to stimulate *Bifidobacterium* and less consistently, *Lactobacillus* (Gibson et al., 2010). Rycroft et al. (2001) performed *in vitro* human fecal fermentations of several potential prebiotics and observed significant increases in *Lactobacillus* and SCFAs and decreases in *Clostridium* for GOS, FOS, and lactulose. Fecal fermentation of FOS and inulin later resulted in enriched *Bifidobacterium* and increased production of SCFAs (acetate and propionate from FOS, butyrate from inulin) (Rossi et al., 2008). FOS are chains of 2-10 fructose derived through hydrolysis of inulin. FOS was more rapidly fermented than inulin during *in vitro* fermentation, demonstrating the relevance of structural characteristics in bacterial utilization (Stewart et al., 2008). A recent study with GOS resulted in significant increases in *Bifidobacterium* and acetate production (Rodriguez-Colinasa et al., 2013).

3.3 Polyphenols

Much like complex carbohydrates, very few polyphenols are absorbed in the small intestine, in fact 90-95% of those consumed reach the colon unaltered where they are metabolized by the residing bacteria. Metabolism of these compound involves multiple steps including cleavage of attached sugars, opening of the C ring and fragmentation of the A ring (Williamson et al., 2017). *In vitro* incubation of nine anthocyanins with pig caecal inoculum resulted in hydrolysis of all compounds to their aglycone forms, which were further degraded to phenolic acids (Keppler et al., 2005). In 2013, rutin, quercetin, chlorogenic acid, and caffeic acid were also broken down by human fecal microbiota during *in vitro* fermentation to phenolic

acids, mainly hydrocaffeic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, and phenylpropionic acid, and these microbial conversions were accompanied by increased proliferation of *Bifidobacterium* (Parkar et al., 2013). This is consistent with previous reported metabolism of chlorogenic acid by *Bifidobacterium* as well as *Lactobacillus* and *E. coli* (Couteau et al., 2001). Also, in 2011 grape seed polyphenols (mainly proanthocyanidins) were metabolized by *Lactobacillus plantarum*, but not *Lactobacillus casei*, demonstrating the metabolic diversity of bacterial species in regards to polyphenol degradation (Tabasco et al., 2011).

Although polyphenols do not undergo fermentation to SCFAs by the gut microbiota, they can have a profound effect on bacterial population via numerous other mechanisms. These include but are not limited to interference with bacterial cell membranes, inhibition of DNA and RNA synthesis, and their metabolism by certain species (Kemperman et al., 2010). Several *in vivo* and *in vitro* studies demonstrate the ability of polyphenols to enhance proliferation of beneficial bacteria, such as *Bifidobacterium*, *Lactobacillus*, and *Eubacterium*, while undermining the growth of others, particularly *Staphylococcus* and *Clostridium* spp (Cardona et al., 2013). For example, red wine polyphenols rich in proanthocyanidins administered to rats increased *Bacteroides*, *Lactobacillus*, and *Bifidobacterium*, while controls had mainly *Bacteroides*, *Clostridium* and *Propionibacterium* (Dolara et al., 2005). Multiple murine studies observed modulation of *Akkermansia* by phenolic extracts from different plant sources. Cranberry extract fed to mice over a 9 week period brought about a decrease in Bacteroidetes and increase in Firmicutes, as well as a striking increase in *Akkermansia* (Anhe et al., 2014). It is possible that the increase in *Akkermansia* was related to the phenolic acid

content of the extract, as a study in 2016 found increases in this genus in mice with feeding of caffeic acid (Zhang et al., 2016). *Akkermansia* was similarly increased in mice after supplementation with compounds present in grapes, red pitaya fruit, and rhubarb (Roopchand et al., 2015; Song et al., 2016; Neyrinck et al., 2016). Modulation of gut bacterial groups by polyphenols has also been observed in *in vitro* human studies. Human *in vitro* fecal fermentation of grape seed flavan-3-ols resulted in decreased *Clostridium histolyticum* and increased *Lactobacillus/Enterococcus* (n=3) (Cueva et al., 2013). Grape polyphenols stimulated a significantly higher increase in *Bifidobacterium* than a FOS during *in vitro* fermentation with human fecal samples (n=3) (Zhou et al., 2016). Previous work has also purported a synergistic effect between polyphenols and FOS; *Coprococcus* and *Roseburia* are both known to convert acetate to butyrate, and were increased by feruloylated arabinoxylan oligosaccharides (FAXO) and red rice bran polyphenolics when applied together but not individually during and *in vitro* fecal fermentation (Pham et al., 2017). Tzounis et al. (2008) suggests that stimulation of saccharolytic bacteria by flavanols may lead to greater fermentation capacity and increased SCFA production, rendering prebiotics more effective. Further research should investigate the ability of polyphenols to enhance the effects of well-established prebiotics, such as GOS, FOS, and lactulose.

4. Grain Sorghum and the Human Gut Microbiota

Sorghum has been an invaluable source of nourishment for centuries, however its numerous benefits are only recently gaining attention. The two main components that have drawn interest in sorghum as a possible nutraceutical are resistant starch and polyphenols. Not

only do these components contribute to weight loss, insulin and glucose control, and cancer prevention and reduction, but they also may improve colonic health through modulation of the HGM. Resistant starch is metabolized in the gut through the process of fermentation, as mentioned earlier. Rats fed resistant starch from raw potato had significantly higher numbers of *Lactobacillus*, *Streptococcus*, and *Enterobacteria*, as well as greater production of all SCFAs at 5 months than rats fed corn starch (Kleesen et al., 1997). Resistant starch engineered from corn has also been found to increase *Bifidobacteria* sp., *Ruminococcus bromii*, and *Eubacterium rectale* in humans (Martinez et al., 2010). *In vitro* fermentation utilizing human infant fecal samples (n=6) tested the ability of a whole grain sorghum cereal product to alter the infant gut microbiota; significant increases were seen in *Bacteroidaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Veillonellaceae*, whilst *Enterobacteriaceae* was decreased (Gamage et al., 2017). Sorghum bran is also highly concentrated with polyphenols, which are unique in their variety and composition. As discussed above, several studies have provided evidence that the HGM is significantly affected by polyphenol intake, though studies with sorghum polyphenols are limited. Gu et al. (2007) demonstrated that sorghum bran procyanidins underwent microbial metabolism *in vivo* in rats. A recent study investigated the actions of sorghum brans in rats with induced colitis (Ritchie et al., 2015). Sorghum bran increased species richness and diversity and effected multiple bacterial groups, with variations correlating with polyphenol content of sorghum brans. Rats fed sorghum brans exhibited higher ratios of Bacteroidetes:Firmicutes than did those fed cellulose, and also maintained higher proportions of the order *Bacillales*, though *Lactobacillus* was higher in the cellulose group (Ritchie et al., 2015). As sorghum gains more attention in

nutritional sciences, *in vitro* fecal fermentations and *in vivo* studies will further illuminate its capability to improve human health through impact on gut microbial populations and metabolite production.

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Chapter 2: Publication Manuscript

Abstract

Grain sorghum brans contain polyphenols that may promote gastrointestinal health by stimulating beneficial bacteria and inhibiting pathogenic bacteria. The products of microbial fermentation, short chain fatty acids (SCFA), have been associated with body weight maintenance and other positive outcomes. The objectives are to characterize the polyphenol compounds in sorghum bran, and to evaluate the change of gut microbiota composition and the effect on SCFA production with sorghum bran polyphenols in fecal samples from normal weight (NW) and overweight/obese (O/O) subjects. Polyphenol profiles of black (BGSB) and sumac (SGSB) sorghum brans and extracts (BSE and SSE) were determined using colorimetric and HPLC analysis. Fresh fecal samples from NW and O/O individuals were incubated with anaerobic media and one of six treatments: no treatment (NC), fructooligosaccharides (FOS), black sorghum bran extract (BSE), sumac sorghum bran extract (SSE), FOS+BSE, or FOS+SSE. Aliquots were collected over 24 hours and analyzed for SCFA at 0, 6, 12, 18, and 24 hours, and bacterial populations at 0 and 24 hours. SSB was significantly higher in total polyphenols than BSE ($P<0.01$), each displaying a unique polyphenol profile. Total SCFA production tended to be higher in the NW group, while butyrate production from FOS tended to be higher in the O/O group. Synergistic effects were seen between sorghum polyphenols and FOS to enhance *Bifidobacterium* and *Lactobacillus*, and sorghum polyphenols independently stimulated *Roseburia* and *Prevotella* and inhibited *Dorea* ($P<0.05$). Our results indicate that sorghum polyphenols have differential effects on gut health and may positively impact gut ecology, with responses varying depending on weight class.

1. Introduction

In the past decades, the human colon has come to light as a hub for microbial activities that impact many aspects of human health. The gut microbiota is composed of hundreds to thousands of species with biological activities including nutrient metabolism, vitamin synthesis, immune modulation, pro- and anti-microbial activities, and modulation of gut permeability (Jandhyala et al., 2015). Dysbiosis, or microbial balance, has been implicated in a number of chronic diseases, one of which is obesity ($\text{BMI} \geq 30$). More than one third of adults in the United States suffer from obesity, and not only does this condition threaten quality of life, but it increases the risk of other chronic diseases such as cancer, type 2 diabetes, heart disease, and stroke (CDC 2017). Considerable evidence points to marked differences in the gut microbial communities in overweight/obese compared to normal weight (Riva et al., 2017; Collado et al., 2008; Kalliomaki et al., 2008, Santacruz et al., 2010). These findings implicate the gut microbiota as a potential target of nutritional therapies to prevent/reduce weight gain and obesity.

While most food consumed is digested and absorbed in the stomach and small intestine, complex carbohydrates such as resistant starch and fiber reach the colon where they are utilized by the gut microbiota (Rios-Covian et al., 2016). These compounds are fermented by certain species to produce short chain fatty acids (SCFAs) which have numerous health implications including body weight maintenance (Canfora et al., 2015; Chambers et al., 2015; Rossi et al., 2005; Riva et al., 2017). Prebiotics were previously defined as “selectively fermented ingredient(s) that result in specific changes in the composition and/or activity of the

gastrointestinal microbiota, thus conferring benefit(s) upon host health” and the compounds that fit these criteria were limited to mainly galactooligosaccharides (GOS) and fructooligosaccharides (FOS) (Gibson et al., 2010). In 2017 however, prebiotics were redefined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). This new definition may allow for inclusion of non-carbohydrate foods that do not undergo fermentation but are utilized by beneficial species in the gut, thus enhancing their expansion and positive health benefits. Such compounds include polyphenols.

Polyphenols are biologically active compounds in plants with numerous roles including pathogen protection, antimicrobial, and antioxidant activities (Pandey and Rizvi, 2009). When consumed by humans, the majority reach the colon where they are broken down by the gut microbiota to smaller, absorbable compounds (Manach et al., 2004). Grain sorghum, commonly called sorghum, is the world’s 5th leading cereal crop and a rich source of polyphenols in its bran fraction (Awika et al., 2005). The polyphenol composition of sorghum bran varies with its color, with brown and sumac varieties enrich in proanthocyanidins (condensed tannins) and black in 3-deoxyanthocyanins (Awika et al., 2004). Though previous research has identified positive modulatory effects of polyphenols from grapes and other foods on the human gut microbiota (Cueva et al., 2013; Anhe et al., 2015; Roopchand et al., 2015; Song et al., 2016; Zhou et al., 2016; Neyrinck et al., 2016), to our knowledge no research has been conducted with sorghum polyphenol extracts. The objectives of this research were to characterize the major polyphenol components of two sorghum brans, and to evaluate the change of gut microbiota composition and the effect on SCFA production in response to sorghum bran polyphenols in fecal samples from normal weight (NW) and overweight/obese (O/O) subjects.

2. Materials and Methods

1.1 Sorghum Brans, standards, and reagents

Black and Sumac sorghum bran (BSB and SSB) were purchased from Nu Life Market (Scott City, KS) and FOS from Megazyme International Ireland Ltd. (Wicklow, Ireland). Folin-Ciocalteu's reagent, 4-dimethylaminocinnamaldehyde (DMAC), 2,2- diphenyl-1-picrylhydrazyl (DPPH), and butyric acid, propionic acid, and acetic acid from were obtained from Sigma-Aldrich (St. Louis, MO). Luteolinidin and apigeninidin were from Chromadex (Irvine, CA).

1.2 Preparation of Polyphenols from Sorghum Bran Samples

Polyphenol extractions were performed according to Awika et al. (2004) with modifications. Defatted sorghum bran samples (1 g) and extracting solvent (1% HCl in 70% methanol) were mixed at a ratio of 1:20. Mixtures were stirred at room temperature (RT) for 2 hours, centrifuged at 6000 rpm for 10 minutes, and the supernatants were collected. The extraction was repeated twice, and all three supernatants pooled for the final extract, BGSB (black grain sorghum bran) and SGSB (sumac grain sorghum bran). Solvent was evaporated using rotary evaporator at 30°C, extracted polyphenols were brought to 20 mL in deionized (DI) water and stored at -4 °C.

Dried polyphenol extracts were prepared to be used as substrates in the *in vitro* fecal fermentation experiment. Polyphenols were extracted by combining defatted dry bran (5 g) and extracting solvent (1:60) and stirred at RT for 2 hours. Vacuum filtration with 42 Whatman filter paper (GE Healthcare, Amersham, UK) was used to separate extract from solid particles, and

solvent was removed using rotary evaporation at 30°C. The aqueous extract was then centrifuged at 6000 rpm for 10 minutes and supernatant applied to a pre-activated C-18 solid phase extraction (SPE) column (Grace Davidson Discovery Sciences, Deerfield, IL). Sugars were eluted with 100 mL water, and remaining components with 100 mL methanol. After elution of polyphenols, 50 mL of DI water was then added to methanolic extract and methanol was removed through rotary evaporation at 30°C. The concentrated aqueous extract was then frozen and subsequently lyophilized in a VirTis Benchtop SLC freeze dryer (SP Industries, Warminster, PA). Resulting powdered extracts, black sorghum bran extract (BSE) and sumac sorghum bran extract (SSE), were then pooled and stored in a desiccator at -4°C until further analysis.

1.3 Analysis of Polyphenol Content and Antioxidant Activity

Total polyphenolic content of sorghum brans and bran extracts was determined using the Folin-Ciocalteu assay according to Singleton and Rossi (1965) with minor modifications. Standards were prepared using gallic acid in concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 ppm. BGSB and SGSB were centrifuged at 6000 rpm for 10 minutes and diluted 40-fold in water. BSE and SSE were dissolved in water for a dilution of 8000-fold. In a 48-well plate, 0.1 ml sample or standard, 0.5 mL of 0.2 N Folin-Ciocalteu's reagent, and 0.4 ml of 7.5% Na₂CO₃ were combined. The plate was incubated at room temperature (RT) for 2 hours and analyzed in Synergy TT microplate reader (BioTek Instruments, Winooski, VT) at 760 nm. All samples and standards were measured in at least triplicate. Concentration was determined by plotting against a standard curve of absorbance and concentration using gallic acid.

Total proanthocyanidin content was determined using the 4-dimethylaminocinnamaldehyde (DMAC) assay (Payne et al., 2010). For preparation of 60 mL DMAC solution, 6 mL HCl was diluted to 60 mL using reagent alcohol (95% ethanol in water), cooled at 4 °C for 20 minutes, with subsequent addition of 60 mg DMAC. Standards were prepared using catechin and reagent alcohol at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 ppm. BGSB and SGSB were centrifuged at 6000 rpm for 10 minutes and diluted 40-fold in reagent alcohol. BSE and SSE were dissolved in alcohol for a dilution of 8000-fold. In a 48-well plate, 0.15 mL of sample or standard and 0.75 mL of DMAC solution were combined, all in at least triplicate. Plates were read immediately at 640 nm in microplate reader. Concentration was determined by plotting against a standard curve using catechin.

For qualitative and quantitative analysis of 3-deoxyanthocyanins (3-DXA) a Beckman Coulter (Denver, MA) System Gold HPLC system was used. The system was equipped with a 126 pump, a 168 Detector, and a 508 autosampler. Software used for data collection and integration was 32 Karat 8. A 250x4.6 mm i.d. C18 column (YMC America Inc, Allentown, PA) was used for separation of 3-DXA in sorghum and standards. The mobile phase consisted of A: 5% formic acid in water, and B: 100% methanol. Flow rate was 1 mL min⁻¹ and injection volume was 30 µL. The gradient of Cho et al. (2004) was used, with minor modifications: 0-60 min, 2-60% B, 60-61 min: 60-2% B, 61-66 min, 2% B, 66-75 mins: 2-0% B. Wavelengths monitored were 280, 340, and 480. Apigeninidin and luteolinidin were identified by comparing retention time and λ -max with standards. Quantification use standard curves constructed from peak areas of different concentrations of standards.

Antioxidant properties were determined using the free radical 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay according to Brand-Williams et al. (2004) with modifications. DPPH solution was prepared by dissolving in methanol (0.15 mM) and stored at -20°C between uses. Serial dilutions of BGSB and SGSB, as well as BSE and SSE, were prepared in methanol. For reaction, 0.95 mL DPPH solution was added to 0.05 mL extracts of varying concentrations and shaken. Plates were read in microplate reader at 30 minutes at 517 nm. Effective concentration to reduce the radical by 50% (EC₅₀) was determined for both brans and reported as µg gallic acid equivalents per gram. All samples and standards were plated in at least triplicate.

1.4 Human Fecal Fermentation

The study has been approved by the International Review Board (University of Arkansas; IRB #17-02-433). Twenty-two subjects were recruited from the North West Arkansas area, 11 normal weight (NW, BMI<25) and 11 overweight/obese (O/O, BMI ≥25) subjects. Exclusion criteria included tobacco use, digestive disease, fasting blood glucose (FBG), current medications (besides birth control), and antibiotic use within the past six months. Consent and screening forms as well as food frequency questionnaires were filled out during screening sessions. No significant differences in macro- and micro-nutrient intake were observed between weight class groups or genders. Participant characteristics are provided in **Table 1**. Selected participants were given a stool collection kit and delivered samples within an hour of defecation. Fecal samples were transferred to anaerobic chamber immediately for fermentation experiment.

Fecal fermentation was performed according to Yang et al. (2013) with minor modifications. One liter of anaerobic fermentation medium was composed of 2 g peptone (Fischer Scientific, Waltman, MA), 2 g yeast extract (Alfa Aesar, Ward Hill, MA), 0.5 g bile salts (Oxoid, Hampshire, UK), 2 g NaHCO₃, 0.1 g NaCl, 0.08 g K₂HPO₄, 0.01 g MgSO₄·7H₂O, 0.01 g CaCl₂·6H₂O, 0.5 g L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO), 50 mg bovine hemin (Sigma-Aldrich, St. Louis, MO), 2 ml Tween 80, 10 µl vitamin K (Sigma-Aldrich, St. Louis, MO), and 4 ml 0.025% (w/v) resazurin solution. To prepare fecal slurry, 2 g fecal sample was added to 20 mL phosphate-buffered saline, vortexed to homogenization, and filtered through 4 layers of cotton gauze. Within an anaerobic chamber, 14 mL of medium was inoculated with 1 ml fecal slurry and treated with either no substrate (negative control, NC), FOS (5 g/L), BSE polyphenols (1 g/L), SSE polyphenols (1 g/L), FOS (5 g/L) + BSE (1 g/L), or FOS (5 g/L) + SSE (1 g/L). Test tubes were incubated at 37°C and aliquots (2-4 mL) taken at time points (TP) 0, 6, 12, 18, and 24 hours. Aliquots were added to 0.1-0.2 mL of 2M KOH stop solution and stored at -80 °C.

Short chain fatty acid (SCFA) content was determined for all samples at all time point aliquots using a modified method of Bourquin et al. (1993). After thawing at room temperature, samples were vortexed and 450 µL combined with 50 µL of a prepared mixture. The mixture contained 50 g meta-phosphoric acid and 1.6 g CuSO₄/liter, and 314.6 µL 4-methyl valeric acid was used as an internal standard. After incubation at room temperature for 10 minutes, the mixture was centrifuged at 11,500 rpm for 5 minutes and the supernatant collected and stored at -20°C until analysis. SCFA analysis was performed using a Varian CP-3800 GC (Agilent, Santa Clara, CA) with a CP-8400 autosampler and a HP-FFAP modified polyethylene glycol (25m x 32mm) column. One µL of sample was injected with a split of 30:1 and a flow rate of 1.3

ml/min. The gradient used was 3°C/min from 65°C to 110°C and 8°C/min until 150°C. Total and individual SCFA were quantified against reference standards for butyric acid (BA), propionic acid (PA), and acetic acid (AA).

Change of microbiota profile to treatments were determined by analyzing bacterial DNA of samples from time point 0 and 24 hours. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Gaithersburg, MD), combined with AccuPrime Pfx SuperMix and primers and amplified by polymerase chain reaction (PCR) using Eppendorf Mastercycler Pro S (Eppendorf, Hamburg, Germany). After confirming amplification through agarose gel electrophoresis, DNA samples were normalized with SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, WA). The V4 region of 16S-rRNA of bacteria in samples was sequenced utilizing the Illumina MiSeq platform (Kozich et al., 2013). Fastq files generated through Illumina were demultiplexed and quality filtered, operational taxonomic units (OUT's) assigned using SILVA database, and the data processed through Mothur 1.35.1 (Schloss et al., 2009).

1.5 Statistical Analysis

Statistical analysis was performed using SPSS (IBM, Armonk, NY). One-way ANOVA was used for analysis of sorghum bran components. Friedman's test with Bonferroni correction was used to analyze the differences in SCFA incremental concentration between treatments at 6, 12, 18, and 24 hours. Non-metric multidimensional scaling was utilized to analyze similarities in bacterial communities between treatments. PAST 3.15 was used for non-metric multidimensional scaling and analysis of similarities (ANOSIM) according to the Bray-Curtis

index, and species diversity was calculated according to the Shannon index. Data will be presented as Mean \pm standard deviation (SD) or standard error of the mean (SEM), and results will be considered significant at $P < 0.05$.

3. Results

2.1 Polyphenol Profiles and Antioxidant Properties of Sorghum Bran and Sorghum Bran Extracts

For polyphenol content analysis, multiple extraction procedures and solvents were tested for efficacy, and the current method chosen based on maximum values for total polyphenols. Total polyphenols and proanthocyanidins significantly higher in sumac grain sorghum bran (SGSB, 43.0 ± 2.0 mg/g and 7.8 ± 0.5 mg/g), and 3-deoxyanthocyanins in black grain sorghum bran (BGSB, 1.2 ± 0.0 mg/g) ($P < 0.01$) (**Table 2**). Polyphenol profiles of BSE (black sorghum bran extract) and SSE (sumac sorghum bran extract) closely resembled those of the bran at much higher concentrations.

Radical scavenging capability was assessed using the DPPH radical, and both black and sumac sorghum bran demonstrated antioxidant capabilities (**Table 2**). BGSB demonstrated a significantly lower EC_{50} (54.1 ± 2.9 μ g GAE/g) than those SGSB (79.9 ± 3.2), implicating a more potent antioxidant effect of black sorghum bran ($P < 0.01$). However, after extraction and lyophilization both extracts (BSE and SSE) demonstrated lower antioxidant activity, with no significant differences between the two.

2.2 Short Chain Fatty Acid Production

Short chain fatty acid analysis was performed as a measure of microbial fermentation of the various substrates applied as treatments during our experiment. No significant differences in total and individual SCFA production was observed between NW and O/O. In NW at 6 h, total SCFA concentration was significantly increased by FOS and FOS+SSE compared to the NC, BSE, and SSE (**Figure 1A**; $P<0.05$). No significant effect of FOS with or without sorghum extracts was seen in both NW and O/O (**Figure 1A and 1B**). Compared to BSE, SSE resulted in lower concentrations at time points from 12 h and 24 h in both NW and O/O.

Acetic acid (AA) is present in the colon at much higher concentrations than either butyric acid (BA) or propionic acid (PA) and makes the largest contribution to total SCFA. In NW, FOS alone increased AA compared to NC, BSE, SSE, and FOS+BSE at 6 h, and FOS+BSE displayed lower concentrations compared to FOS at 12 h (**Figure 2A**; $P<0.05$). These effects were ablated with time, and no differences between treatments were observed at time points of 18 h and 24 h. No major impacts of treatments on AA concentration were seen in O/O (**Figure 2B**). As reflected in Total SCFA, AA production was generally higher in NW than O/O, though not significantly.

Incremental propionic acid concentration was not significantly impacted by FOS with or without sorghum extracts in NW or O/O (**Figure 3**). However, sorghum polyphenols alone appeared to have an effect. In both weight classes, SSE tended to have lower PA production than BSE over 24 h. At 12 h, SSE caused lower concentrations of PA compared to NC in NW ($P<0.05$) and the trend continued at 18 and 24 h. In O/O, this same effect was seen at 12

and 18 h ($P<0.05$) with the trend continued at 24 h. A similar trend was observed in AA, which tended to be lower in response to SSE than the NC at 24 h in both weight groups.

In both NW and O/O, incremental BA concentration in FOS, FOS+SSE, and FOS+BSE treatments trended higher than NC, SSE, and BSE at all time points (**Figure 4**). In O/O, incremental BA concentrations with FOS significantly higher than NC, BSE, and SSE at 24 h (**Figure 4B**, $P<0.05$). Sorghum extracts alone did not alter BA concentrations. BA production was higher in O/O than in NW, though not reaching statistical significance.

2.3 General Changes in the Microbiota

After DNA extraction and sequencing of 264 samples, a total of 5,062,231 high quality reads were obtained for analysis of microbial populations. After 24 hours, the three most abundant genera in the controls were *Bacteroides*, *Enterobacteriaceae_unclassified*, and *Ruminococcaceae_unclassified* for NW, and *Bacteroides*, *Enterobacteriaceae_unclassified*, and *Prevotella* for O/O (**Figure 5**). Although *Prevotella* was the third most abundant genus in O/O, it was not among the top 15 genera in NW. The control fermentation resulted in a significant increase in species diversity from 1.72 (0 hours) to 2.68 (0 hours) ($P<0.05$; **Figure 6**). BSE and SSE brought about similar increases ($P<0.05$), however FOS-containing treatments saw no significant increases in diversity compared to the control at time point 0. NMDS plots revealed no differences in bacterial communities between treatments at time point 0 (**Figure 7A**), however after 24 hours clear separation was seen between the NC and FOS-Containing treatments ($P<0.05$; **Figure 7B**). Sorghum extracts alone did not significantly impact bacterial communities compared to the negative control.

2.4 Specific Changes in Microbial Populations

Due to high interindividual variation, results are being reported for the 22 subjects' samples combined as well as for individual weight class groups.

After 24 hours of *in vitro* fermentation, we observed relative increases in Firmicutes in all treatments (**Figure 8**). We found that BSE lessened this effect. BSE resulted in lower levels of Firmicutes at 24 hours compared to FOS and FOS+SSE ($P<0.05$). When analyzed by weight class, the same pattern was seen in O/O, but in the NW group there were no significant differences between treatments (**Table 3**). When treated with BSE, abundance of Firmicutes at 24 hours was higher in NW than in O/O ($P<0.05$).

As relative abundance of Firmicutes increased there was a corresponding decrease in Bacteroidetes (**Figure 8**). In all subjects BSE resulted in significantly higher abundance of Bacteroidetes at 24 hours than NC and all other treatments ($P<0.05$). Similar effects were observed in O/O ($P<0.05$), and though BSE tended to increase Bacteroidetes in NW, it did not significantly increase compared to NC (**Table 3**). The O/O group displayed higher abundance of Bacteroidetes than the NW group at 24 hours in the NC, FOS, BSE, and SSE ($P<0.05$). This effect was not seen with FOS+BSE or FOS+SSE (**Table 3**).

In the negative control, there were negligible increases of less than 0.5% in relative abundance of Actinobacteria after 24 hours (**Figure 8**). While BSE and SSE led to slightly higher abundance, FOS, FOS+SSE and FOS+BSE increased at significantly higher magnitudes of 10%, 9%, and 12%, respectively ($P<0.05$). The same pattern was observed for both weight classes,

though NW tended to display a greater response than O/O to all treatments with a significant difference seen between weight classes for BSE and FOS+SSE ($P<0.05$) (**Table 3**).

Relative abundance of Verrucomicrobia decreased by about 0.1% in NC after 24 hours (**Figure 8**). In contrast, BSE increased abundance by 0.6% though the difference between NC and BSE was statistically insignificant. A similar pattern was observed in the individual weight classes groups, and though there was a greater response to BSE in NW individuals there were no significant differences between groups (**Table 3**).

While Proteobacteria was increased by 17% at 24 hours in the NC, changes in relative abundance were 10% or lower for all treatments (**Figure 8**). FOS, FOS+BSE, and FOS+SSE were significantly different from the negative control, BSE, and SSE ($P<0.05$) after 24 hours of *in vitro* fermentation. The same patterns of response to treatments were seen in NW and O/O individuals, and O/O displayed lower abundance than NW with FOS+BSE and FOS+SSE (**Table 3**; $P<0.05$).

After 24 hours, *Bifidobacterium* relative abundance in FOS, FOS+BSE, and FOS+SSE was significantly higher than the NC, BSE, and SSE (**Figure 9**; $P<0.05$). Sorghum polyphenols alone had no effect. When analyzed by weight class, samples from NW subjects had significantly higher levels of *Bifidobacterium* after 24 hours than O/O samples when treated with FOS and FOS+SSE (**Table 4**; $P<0.05$). Additionally, the NW group displayed the same pattern as with all subjects combined. In O/O however, FOS alone did not significantly enhance proliferation, but FOS+BSE and FOS+SSE had significantly higher abundance of *Bifidobacterium* after 24 hours compared to the NC (**Table 4**).

While there was scant change (0.01%) in *Lactobacillus* in the NC after 24 hours, increases of this genus, though small, were seen in all treatments (**Figure 9**). While FOS alone did not significantly increase abundance at 24 hours compared to the NC, FOS+BSE and FOS+SSE had significantly higher levels compared to the NC ($P<0.05$). In NW group, FOS+SSE alone had a significant effect and only FOS+BSE had a significant effect in the O/O group compared to the NC (**Table 4**; $P<0.05$). Sorghum polyphenols alone did not significantly affect *Lactobacillus*. There were no significant differences between weight class groups for this genus.

Relative abundance of *Prevotella* decreased from about 20% to 5% in the NC but to a lesser extent in all treatments (**Figure 9**). At 24 hours, abundance was significantly higher than the NC for BSE, SSE, FOS+BSE and FOS+SSE ($P<0.05$) but not for FOS alone. Additionally, BSE and FOS+SSE had significant higher levels than FOS ($P<0.05$). In the NW and O/O groups, only BSE and FOS+SSE significantly increased *Prevotella* compared to NC (**Table 4**; $P<0.05$). *Prevotella* was present at significantly higher levels in O/O than NW in all treatments after 24 hours ($P<0.05$).

After 24 hours of *in vitro* fermentation, *Bacteroides* was decreased in NC and all treatments (**Figure 9**). Both BSE and SSE resulted in higher abundance at 24 hours than FOS ($P<0.05$). There were no significant differences between treatments in the NW group, and in the O/O group BSE alone resulted in higher abundance at 24 hours than FOS (**Table 4**; $P<0.05$). Though *Bacteroides* tended to be higher in NW samples, no significant differences were observed between the two groups.

Relative abundance of *Dorea* was increased after 24 hours in the NC but to a much lesser extent in the five treatments (**Figure 9**). At 24 hours, *Dorea* was present in lower abundance than the NC in all treatments except SSE ($P<0.05$). When analyzed by weight class groups only FOS had a significant impact compared to the NC (**Table 4**; $P<0.05$). O/O samples had a significantly lower abundance of *Dorea* than NW in response to BSE.

There was little change in *Faecalibacterium* over 24 hours in the NC, however abundance decreased in response to all treatments and was significantly lower in FOS, BSE, and FOS+BSE at 24 hours compared to NC, SSE, and FOS+SSE (**Figure 10**; $P<0.05$). In samples from NW subjects only FOS and FOS+BSE were significantly different from the NC, and in O/O samples only FOS+BSE was significantly lower (**Table 4**; $P<0.05$). Abundance of *Faecalibacterium* was significantly higher at 24 hours in NW than O/O in NC and all treatments except FOS.

Roseburia was stimulated by sumac sorghum polyphenols, with SSE resulting in higher abundance than NC and FOS at 24 hours (**Figure 10**; $P<0.05$). When analyzed by weight groups, NW followed the same pattern ($P<0.05$), but no significant differences were seen between treatments for OO (**Table 4**). The proliferation of *Roseburia* in response to SSE was also significantly higher in NW than O/O ($P<0.05$).

Relative abundance of *Anaerostipes* increased after 24 hours of fermentation in the NC, but to a higher magnitude in FOS, FOS+BSE, and FOS+SSE (**Figure 10**). At 24 hours, abundance was higher in FOS, FOS+BSE, and FOS+SSE compared to NC (**Figure 10**; $P<0.05$). When analyzed by weight class groups, FOS+BSE and FOS+SSE significantly increased *Anaerostipes* compared to

NC and BSE ($P < 0.05$), but FOS alone was insufficient to elicit this effect (**Table 4**). There were no differences between NW and O/O.

4. Discussion

As chronic diseases such as obesity trend toward epidemic proportions, the topic of gut health, and more specifically the gut microbiota, has gained increasing attention in the realms of health and nutrition. For this reason, a recent focus of nutrition research has been identifying whole foods as well as bioactive components with the potential to positively shift dysregulated bacterial populations towards more desirable profiles. Of particular allure are plant polyphenols, as they are widely spread in nature and have been credited with the ability to positively modulate the gut microbiota. Not only grain sorghum bran is a rich source of polyphenols, but it is cost effectively and efficiently produced in the United States, making it an attractive candidate for nutraceutical applications.

Our current study found both black and sumac sorghum brans to be highly concentrated with polyphenols. Our findings of 27.5 ± 1.5 mg/g for black and 43.0 ± 2.0 mg/g for sumac are consistent with previous reports of total phenolics ranging from 7.6-35.6 mg/g and 22.5-88.5 mg/g for black and sumac sorghum bran, respectively (Awika et al., 2004, 2004, 2005). We found 3-Deoxyanthocyanins (3-DXA) to be higher in black bran (1.2 ± 0.003 mg/g) compared to sumac bran (0.14 ± 0.007 mg/g), and sumac bran to be the dominant source of proanthocyanidins (7.8 ± 0.5 mg/g) over black (0.4 ± 0.08 mg/g). These results are in consensus with multiple previous analyses of black and sumac sorghum, in which black is established as

enriched in 3-DXA, and sumac in proanthocyanidins (Awika et al., 2003, 2004, 2005; Dykes et al., 2005).

It has been well established that grain sorghum bran has significant antioxidant capabilities (Awika et al., 2003, 2004, 2005; Kamath et al., 2005; Kil et al., 2009), and our current analyses sought to compare the radical scavenging properties of the two different varieties of sorghum bran. Despite lower levels of total polyphenols, BGSB (black grain sorghum bran) had a lower EC₅₀ ($54.1 \pm 2.9 \mu\text{g/g}$) than sumac grain sorghum bran (SGSB, $79.9 \pm 3.2 \mu\text{g/g}$), implicating more efficient radical scavenging capabilities of the polyphenols present in black sorghum bran. While polyphenols were extremely concentrated in the dried sorghum extracts (BSE or SSE) compared to the bran, EC₅₀ for both black and sumac polyphenols were significantly and greatly increased, reflecting a loss of antioxidant potential through the purification and/or freeze-drying process. It was previously reported that freeze drying of water caltrop resulted in reduction of scavenging abilities paralleled by the loss of hydroxycinnamic acid, a naturally present phenolic acid (Chiang et al., 2008). It is possible that in the present study, certain phenolic compounds were altered or lost during freeze drying.

During our *in vitro* experiment, we examined the abilities of BSE and SSE to alter short chain fatty acids produced from FOS, a well-established prebiotic with known fermentability (Stewart et al., 2008). It is evident that butyric acid (BA) was the major end fermentation product in FOS-containing treatments during this experiment, as it resulted in increased concentrations in both NW and O/O over 24 h (**Figure 4**). *Lactobacillus* and *Bifidobacterium* are both known to utilize FOS to produce lactic acid (Kaplan and Hutkins 2000), which can be further metabolized to BA by genera such as *anaerostipes* (Duncan et al., 2004). In our

experiment we found increases in *Lactobacillus*, *Bifidobacterium*, and *Anaerostipes* in response to FOS-containing treatments (**Figures 9 and 10**), so it is plausible that the increases in BA were due to these cross-feeding pathways. In FOS, incremental BA concentration was higher than PA (propionic acid). These results are in accordance with the findings of Stewart et al. (2008) who reported that *in vitro* fermentation of FOS with pooled fecal samples from 3 subjects resulted in higher levels of BA (9.0-9.6 $\mu\text{mol/ml}$) than PA (1.8-2.7 $\mu\text{mol/ml}$). Interestingly, we found that FOS did not impact AA (acetic acid) compared to the NC, which is not in agreement with the report of Stewart et al. (2008) who saw production of AA from FOS. While total SCFA concentration was increased by fermentation with FOS compared to NC, it did not reach statistical significance. Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an *in vitro* fermentation system.

Our SCFA analysis suggests that SSE decreased production of PA from substrates already present in the donated fecal samples. In O/O, PA production was significantly lower in SSE compared to the NC at 12 and 18 h ($P<0.05$), and this trend continued to 24 h (**Figure 3B**). Similar results were seen in NW, but only reaching significance at 12 h. It is likely that SSE was an alternative substrate for PA-producing bacteria that would otherwise metabolize materials already present in the fecal material. Modulation of SCFA production by BSE and SSE are likely attributable to their interactions with the bacterial species that produce these substrates. Distinct differences in total SCFA production between BSE and SSE indicate differential effects of varying classes of polyphenols on microbial metabolism. Total SCFA production with SSE was significantly lower than BSE at 24 h in both weight groups (**Figure 1**; $P<0.05$). The same tendency was observed for PA and AA. This is one of the first studies to examine the impact of

sorghum bran polyphenols on SCFA production and further work is needed to corroborate these findings.

Along with SCFA we analyzed changes in bacterial populations after 24 h of fermentation. Compared with time point 0, we observed an increase in species diversity in the NC as well as with BSE and SSE, although no increase was seen with FOS-containing treatments, and at 24 h they exhibited significantly lower diversity than NC, BSE, and SSE (**Figure 6**). Diversity takes into account species richness (total number of species) as well as the evenness in distribution of those species. Our results are likely a reflection of decreasing abundance of dominant saccharolytic species (such as *Prevotella*), in turn allowing for the flourishing of other non-saccharolytic species.

Although NMDS did not reveal a significant impact of sorghum polyphenols on the overall microbial communities (**Figure 7**), the sorghum extracts modulated the gut microbiota at both phyla and genus levels, and sorghum polyphenols and FOS worked synergistically to enhance specific beneficial genera. The major bacterial phyla present in the human colon are Bacteroidetes and Firmicutes, though additional phyla such as Actinobacteria, Verrucomicrobia, and Proteobacteria are present in lower abundance. In all treatments as well as the negative control, we saw relative shifts in the two major phyla, Firmicutes increasing in abundance and Bacteroidetes decreasing (**Figure 8**). This is not surprising as many carbohydrate utilizers including the probiotic *Lactobacillus* are within Firmicutes, and the conditions of the experiment promote utilization of these substrates, including those already present in the fecal samples. This shift was less pronounced in BSE, which at 24 h had significantly higher levels of Bacteroidetes than the NC and FOS as well as lower levels of Firmicutes compared to FOS

(**Figure 8**; $P < 0.05$), It is interesting to note that FOS+BSE tended to produce less BA than FOS. It could be suggested that the compounds present in BSE are an alternative food source to FOS-utilizing bacteria, one which may not promote proliferation to the same extent and does not result in butyric acid production. When analyzed by weight class, O/O followed the same pattern as with all subjects combined, however no significant differences in Firmicutes were seen between treatments in the NW group suggesting a higher resilience of this phylum to dietary modulation in NW. Bacteroidetes followed the same pattern in the individual weight class groups as when combined. After 24 h O/O samples produced significantly higher abundance of Bacteroidetes than NW in the NC, FOS, BSE, and SSE (**Table 3**). These differences were ameliorated with FOS+BSE and FOS+BB, which did not produce a differing response in NW and O/O. NW samples tended to produce higher levels of Firmicutes, though the difference was only significant in response to BSE. These results indicate differential impacts of nutrients between weight class groups at the phylum level.

Actinobacteria composes a minute portion of human gut microbes but is of interest due to *Bifidobacterium*, another probiotic genus. BSE and SSE alone had no apparent effect, but FOS, FOS+BSE, and FOS+SSE all enhanced abundance of this phylum (**Figure 8**). No differences were seen between FOS treatments. These findings indicate that sorghum polyphenols may not interact with many bacteria within Actinobacteria. The individual weight class groups followed a similar pattern of response to treatment, and NW and O/O were only different in response to BSE and FOS+SSE.

Verrucomicrobia contains the genus *Akkermansia*, which has potential implications in obesity and body weight maintenance (Schneeberger et al., 2015). In recent years, polyphenols

from multiple sources have demonstrated the ability to enhance *Akkermansia* in mice (Anhe et al., 2015; Roopchand et al., 2015; Song et al., 2016; Neyrinck et al., 2016), however in the current study sorghum polyphenols impacted neither Verrucomicrobia (**Figure 8**) or *Akkermansia* abundance. As *Akkermansia* generally colonizes in the mucus layer over the intestinal epithelium (Derrien et al., 2008), it is likely that factors involved in phenolic modulation of this genus were not present during the present *in vitro* fecal fermentation.

Proteobacteria contains several potentially pathogenic bacteria (Shin et al., 2015), and reducing the relative abundance of this phylum is a positive outcome of nutrition strategies. Compared to the NC, lower abundance of Proteobacteria was seen with all treatments, though the difference was only significant for FOS-containing treatments (**Figure 8**). We saw the same pattern in individual weight class groups. These shifts in abundance of Proteobacteria likely reflect the increases in groups which utilize FOS (**Table 3**). Though not statistically significant, BSE and SSE caused a marked decrease in Proteobacteria compared to the NC, suggesting they may act antagonistically against some pathogenic species. These results are similar to those of Pham et al., who found that during *in vitro* fecal fermentation Proteobacteria was decreased by FOS, feruloylated arabinoxylans, rice bran polyphenols, and feruloylated arabinoxylans and rice bran polyphenols combined. Though all treatments significantly decreased Proteobacteria compared to the NC, a smaller impact was observed for polyphenols alone (Pham et al., 2017).

Though health-promoting bacterial genera in the colon are not limited to *Lactobacillus* and *Bifidobacterium*, they are the traditional targets of prebiotic supplementation (Gibson et al., 2017). With all samples combined, *Bifidobacterium* proliferation was enhanced by FOS, FOS+BSE, and FOS+SSE (**Figure 9**). When analyzed by weight group, the same pattern was seen

in NW samples. Compared to NW, O/O samples resulted in significantly lower levels of *Bifidobacterium* for FOS and FOS+SSE ($P<0.05$), and trended lower for FOS+BSE (**Table 4**). However, in O/O only FOS+BSE and FOS+SSE brought about significantly greater abundance of *Bifidobacterium* than the NC ($P<0.05$). This data suggests utilization of FOS by *Bifidobacterium* is altered in O/O microbiota, and that sorghum polyphenols may enhance fermentation of FOS by this species in O/O subjects. As decreased proportions of *Bifidobacterium* have been seen previously in overweight/obese subjects (Kalliomaki et al., 2008; Santacruz et al., 2010), nutritional therapies to increase this genus may be extremely beneficial to individuals combatting excessive body weight gain. Past *in vitro* fermentation studies have observed stimulation of *Bifidobacterium* by polyphenols such as the anthocyanin malvidin-3-glucoside (Hidalgo et al., 2012), multiple compounds known to be present in tart cherries (Mayta-Apaza et al., 2012), and grape and red wine polyphenols (Zhou et al., 2016; Dolara et al., 2005). In our experiment, however, this genus was not apparently impacted by sorghum polyphenols alone.

In all samples combined as well as in individual weight class groups, FOS and sorghum polyphenols worked synergistically to enhance *Lactobacillus*. Though FOS, BSE, and SSE alone did not significantly impact *Lactobacillus* proliferation, FOS+BSE and FOS+SSE resulted in significantly higher abundance compared to the NC (**Figure 9**). As *Lactobacillus* was present at low abundance, further studies are needed to corroborate these effects. Success of prebiotic research in stimulating growth of *Lactobacillus* has been markedly lower than with *Bifidobacterium* (Gibson et al., 2017), and the ability of polyphenols to enhance oligosaccharide utilization by *Lactobacillus* would provide a new avenue of prebiotic supplementation. As human clinical trials have attributed several strains of *Lactobacillus* with anti-obesogenic

actions (Crovesy et al., 2017), this mode of supplementation may be especially beneficial in body weight maintenance. In addition, previous studies have observed stimulation of *Lactobacillus* by red wine polyphenols and anthocyanin malvidin-3-glucoside (Dolara et al., 2005; Hidalgo et al., 2012). Often associated with long-term high carbohydrate diets, *Prevotella* is a PA-producing genus with potential impact on human health (Wu et al., 2011). *Prevotella* was significantly stimulated by BSE, SSE, FOS+BSE, and FOS+SSE compared to NC, but not by FOS alone (**Figure 9**). There were no significant differences between sorghum polyphenols with and without FOS, indicating that sorghum polyphenols were responsible for the increases in *Prevotella*. At 24 h in all treatments, the O/O group had significantly higher levels of *Prevotella* than the NW group. While *Prevotella* was the 3rd most abundant genus in the O/O group after 24 h, it was not within the top 15 most abundant in NW (**Figure 5**). Previously, *Prevotella* dominant microbiota were associated with enhanced production of BA from FOS (Chen et al., 2017). These findings may explain why BA production tended to be higher in O/O samples in the present study. To our knowledge, this is the first report of polyphenols enhancing *Prevotella* in the human microbiota. A recent study found that in 62 obese subjects, individuals with a high *Prevotella:Bacteroides* ratio lost significantly more weight in response to a high-fiber diet than individuals with lower levels of *Prevotella* (Hjorth et al., 2018). Enhancing the ratio of *Prevotella:Bacteroides* may assist in weight-loss strategies that are based on increased fiber intake. In the present study, *Bacteroides* was more abundant than *Prevotella* in the NC at 24 h, however in O/O samples all treatments resulted in increased *Prevotella:Bacteroides* with higher levels of *Prevotella* after 24 h (**Table 4**).

In addition to enhancing beneficial bacteria, downregulation of potentially harmful genera is a positive outcome of nutritional interventions to modulate the microbiota. *Dorea* has been found in increased prevalence in irritable bowel disease (Rajilic-Stojanovic et al., 2011; Saulnier et al., 2011), multiple sclerosis (Chen et al., 2016) and non-alcoholic liver disease (del Chierico et al., 2017). Compared to the negative control, *Dorea* was decreased by all FOS-containing treatments as well as by BSE alone (**Figure 9**). The ability of black sorghum polyphenols to inhibit *Dorea* is consistent with the findings of Pham et al. (2017) of suppressed *Dorea* by red rice bran polyphenols. As *Dorea* has been seen as a biomarker for dysbiosis in multiple diseases, decreasing its abundance through nutritional means may help restore harmonious gut ecology, and our results indicate that black sorghum polyphenols may have these effects.

Additional targets of prebiotic supplementation include BA-producing bacteria. BA is not only the main energy source of colonocytes, but research has purported numerous roles in colon-cancer antagonism, inflammation suppression, and colonic barrier function (Hamer et al., 2008). In the present study, we found increased BA production with FOS, FOS+BSE, and FOS+SSE in both weight classes. This trend was paralleled by increased abundance of *Anaerostipes* in response to FOS-containing treatments, though in individual weight groups the increase was only significant for FOS+BSE and FOS+SSE (this may be due to decreased sample size) (**Table 4**). *Anaerostipes* produces BA by metabolizing lactate produced by other species or utilizing AA through the butyryl CoA: acetate CoA transferase pathway (Louis and Flint, 2016), and our results suggest that this genus was responsible for much of the BA production during this *in vitro* fermentation.

Faecalibacterium is another BA-producing genus which during the current study was decreased by all treatments with FOS, BSE, and FOS+BSE significantly lower than the NC at 24 hours. In contrast, it was previously reported that *Faecalibacterium* was increased in response to FOS, and to a greater extent, red rice bran polyphenols during a similar experiment (Pham et al., 2017). These conflicting results are likely due to the presence of different polyphenol compounds. The differential responses of *Faecalibacterium* to FOS may also reflect different patterns of crosstalk between the bacterial species present in each experiment, and underscore the complexity of gut microbial fermentation.

Roseburia is a novel bacterium which also has the ability to produce BA through the butyryl CoA: acetate CoA transferase pathway (Duncan et al., 2002). Though little research has been done with this genus, reduced abundance of *Roseburia* has been a marker of dysbiosis in both ulcerative colitis (Machiels et al., 2014) and colorectal cancer (Wang et al., 2012). Previous studies have identified stimulation of *Roseburia* by various carbohydrate sources (Neyrinck et al., 2011, 2012), but it was not significantly impacted by FOS-containing treatments in the present study. SSE, on the other hand, caused significant increases compared to both NC and FOS (**Figure 10**), indicating utilization of sumac sorghum polyphenols by *Roseburia*. This is not the first report of *Roseburia* stimulation by polyphenols, as this genus also increased in response to red rice bran polyphenols (Pham et al., 2017). When weight class groups were analyzed separately NW followed a similar pattern, however in O/O there were no significant differences between treatments. NW also had a significantly higher response to SSE than O/O (**Table 4**). These findings suggest that the microbial environment present in O/O individuals is less conducive to colonization by *Roseburia*.

5. Conclusions and Next Steps

Black and sumac sorghum brans are significant sources of polyphenols with unique polyphenol profiles and differing radical scavenging capabilities. Although sorghum extracts did not significantly impact SCFA production from FOS, they enhanced proliferation of *Prevotella* and the butyrate producing genera *Roseburia*. Sorghum polyphenols and FOS worked synergistically to enhance *Bifidobacterium* and especially *Lactobacillus*, a probiotic genus that has been difficult to stimulate through prebiotic supplementation. We observed differential responses to treatment in NW and O/O microbiota for *Lactobacillus*, *Bifidobacterium*, and *Roseburia*, supporting the theory that gut microbial metabolism is altered in O/O. In addition, BA concentrations in response to FOS tended to be higher in O/O individuals. Sorghum polyphenols may help modulate gut microbial populations, especially in concert with other prebiotic substances such as FOS. Further research is needed to examine the potential prebiotic activities of sorghum bran extracts. Human intervention trials would help determine if these alterations in bacterial populations would lead to long-term enhancement of SCFA production. Next steps include testing of the major polyphenol compounds in these extracts. Effects of polyphenols on individual bacteria should also be confirmed by fermentations with isolated strains. Determining which individual polyphenol compound is responsible for beneficial effects on the gut microbiota would enable the optimization of nutraceutical products aimed towards improvement of gut health.

Tables and Figures

Table 1. Subject Characteristics

	All (n=22)	Normal Weight		Overweight/Obese	
		Male (n=5)	Female (n=6)	Male (n=5)	Female (n=6)
Age (years)	29.0 ± 1.4	29.4 ± 2.1	24.2 ± 0.9	28.2 ± 1.6	34.2 ± 4.0
BMI (kg/m ²)	27.1 ± 1.2	23.0 ± 0.3	22.2 ± 0.5	30.9 ± 1.3*	32.2 ± 2.2*
FBG (mg/dL)	92.1 ± 1.1	92.9 ± 1.4	90.6 ± 2.0	92.6 ± 3.1	96.3 ± 1.4

Data are expressed as Mean ± SEM. *Indicates significant difference at P <0.01. FBG: Fasting blood glucose.

Table 2. Polyphenol profile of black and sumac sorghum bran

	BGSB	SGSB	BSE	SSE
Polyphenols (mg gallic acid equiv/g)	27.5 ± 1.5	43.0 ± 2.0*	321.5 ± 2.7	571.7 ± 6.0*
Proanthocyanidins (mg catechin equiv/g)	0.4 ± 0.1	7.8 ± 0.5*	8.4 ± 1.4	86.9 ± 1.1*
3-Deoxyanthocyanins (mg/g)	1.2 ± 0.0*	0.1 ± 0.0	10.1 ± 0.3*	2.0 ± 0.4
Luteolinidin	1.0 ± 0.0*	0.1 ± 0.0	9.3 ± 0.4*	2.0 ± 0.4
Apigeninidin	0.2 ± 0.0*	ND	0.9 ± 0.1*	ND
Radical Scavenging EC₅₀ (µg gallic acid equiv/g)	54.1 ± 2.9*	79.9 ± 3.2	274.0 ± 20.3	269.5 ± 2.1

Data are expressed as Mean ± SD. *Indicates significant difference between black and suman for brans and extracts. P <0.01. BGSB: black grain sorghum bran, SGSB: sumac grain sorghum bran, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract, ND: None Detected.

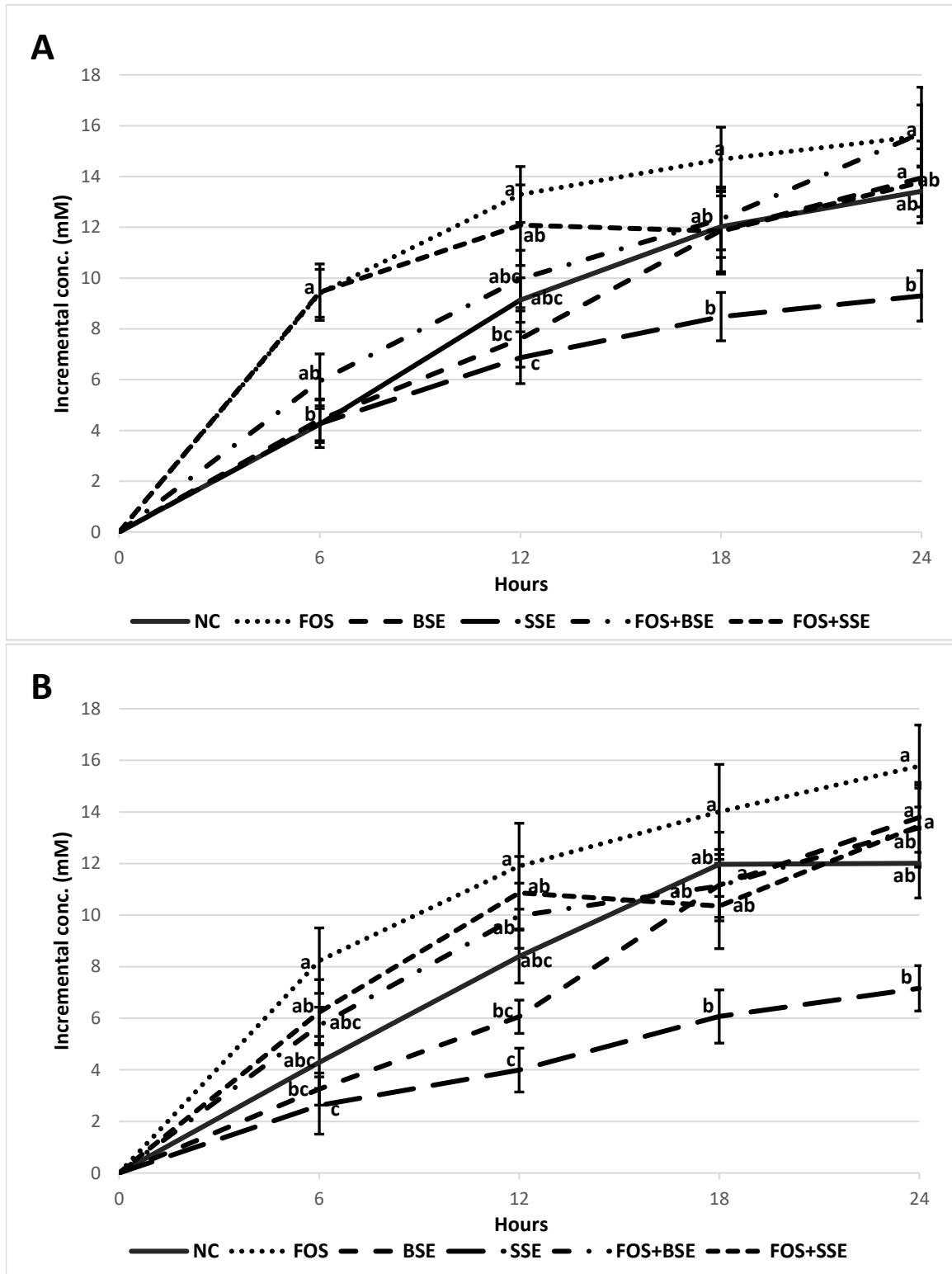


Figure 1. Total SCFA production during fermentation with fecal samples from (A) NW and (B) O/O subjects. Different letters indicate significant differences between treatments at the same time point. $P < 0.05$. $n = 11/\text{group}$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

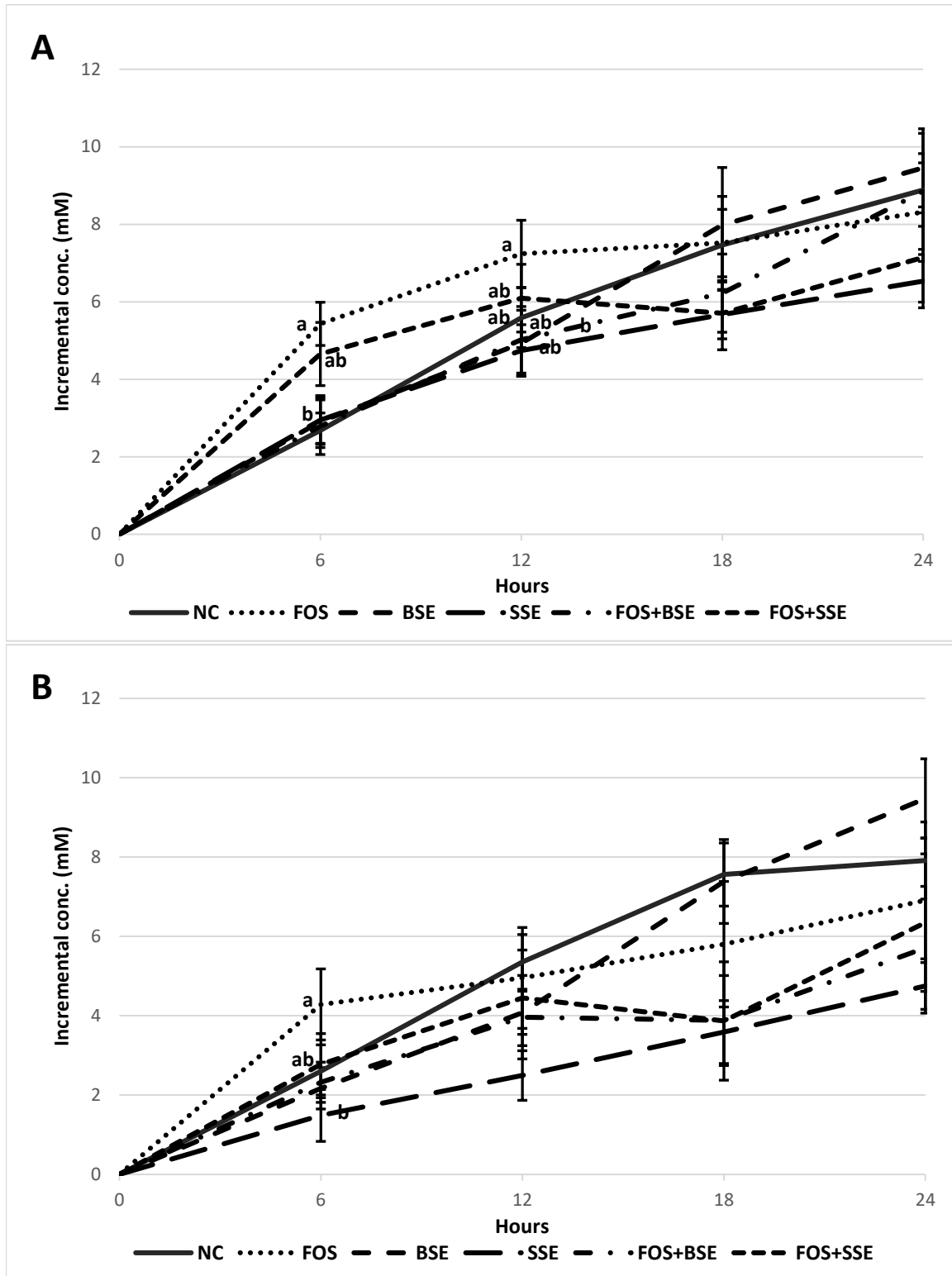


Figure 2. Acetic acid production during fermentation with fecal samples from (A) NW and (B) O/O subjects. Different letters indicate significant differences between treatments at the same time point. $P < 0.05$. $n = 11/\text{group}$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

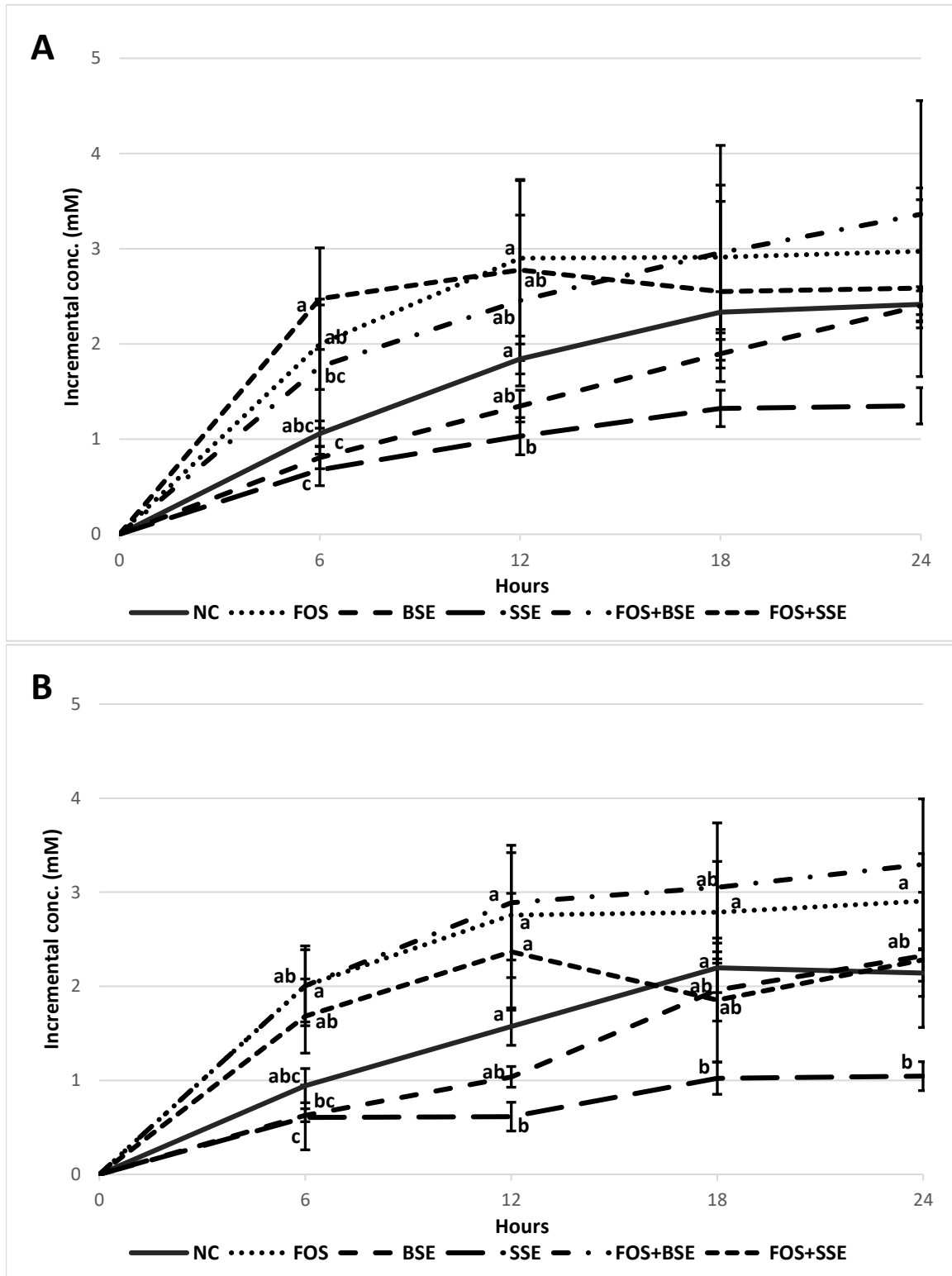


Figure 3. Propionic acid production during fermentation with fecal samples from (A) NW and (B) O/O subjects. Different letters indicate significant differences between treatments at the same time point. $P < 0.05$. $n = 11/\text{group}$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

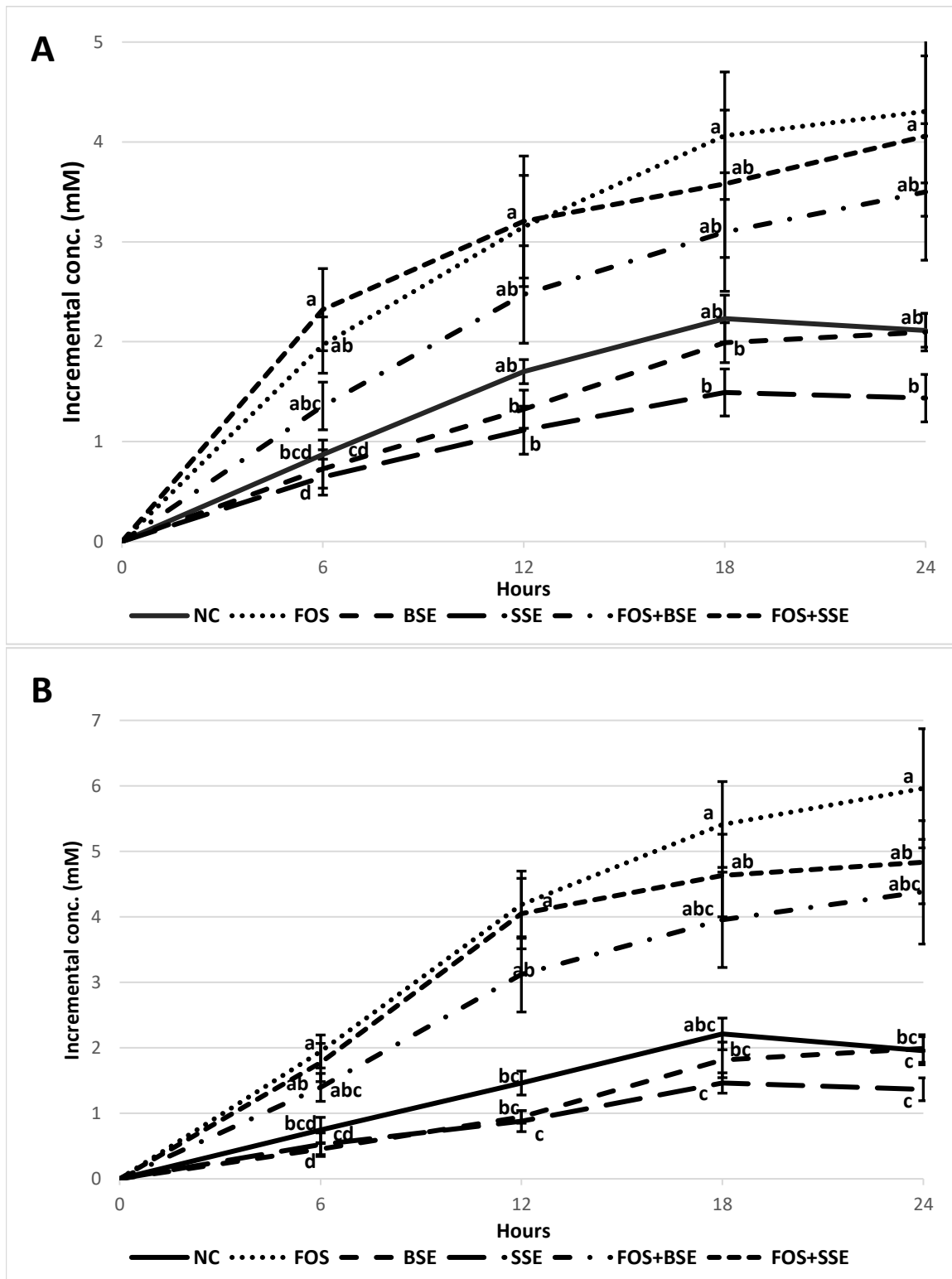


Figure 4. Butyric acid production during fermentation with fecal samples from (A) NW and (B) O/O subjects. Different letters indicate significant differences between treatments at the same time point. $P < 0.05$. $n = 11/\text{group}$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

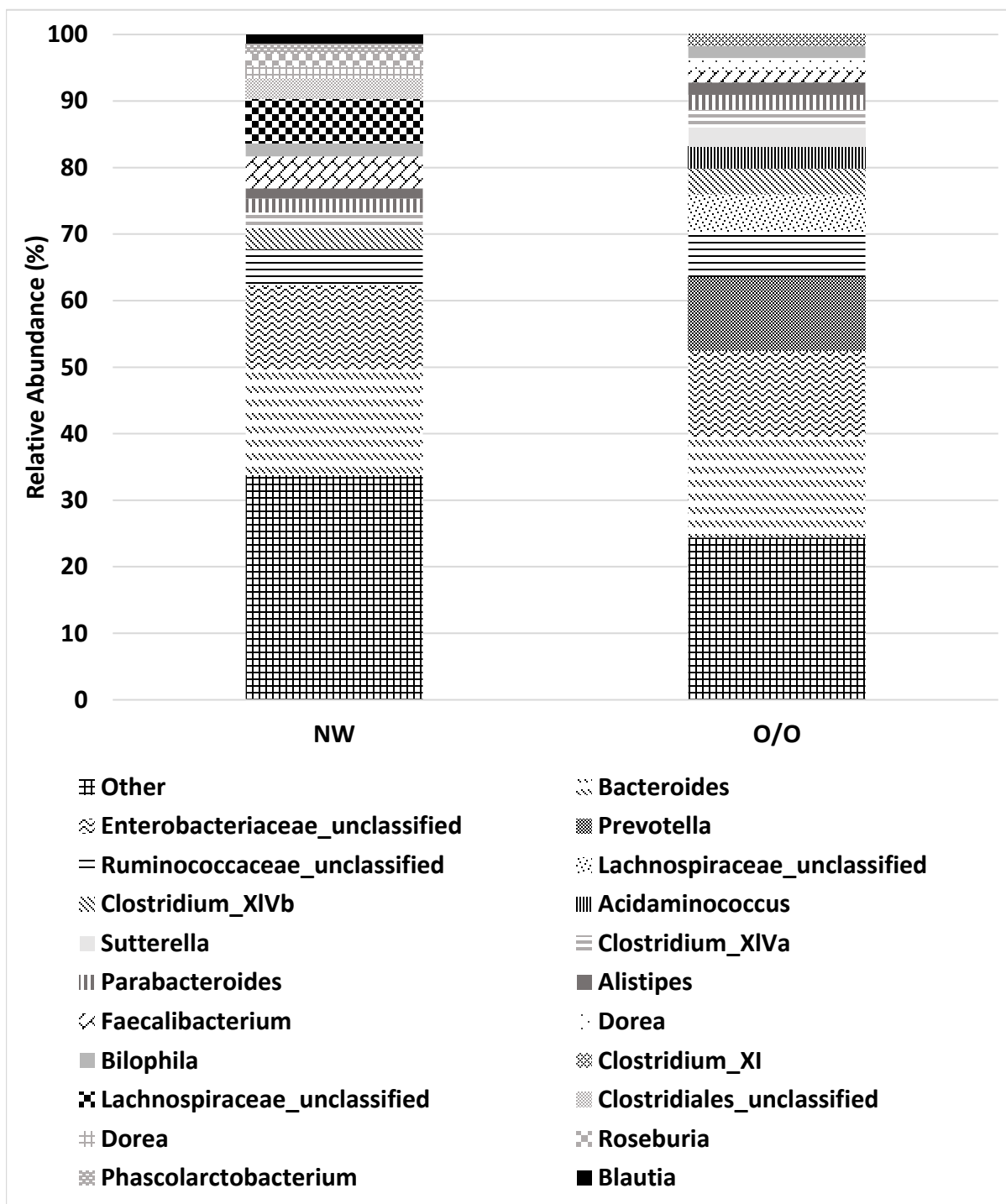


Figure 5. Abundant Genera in NW and O/O. N=11/group.

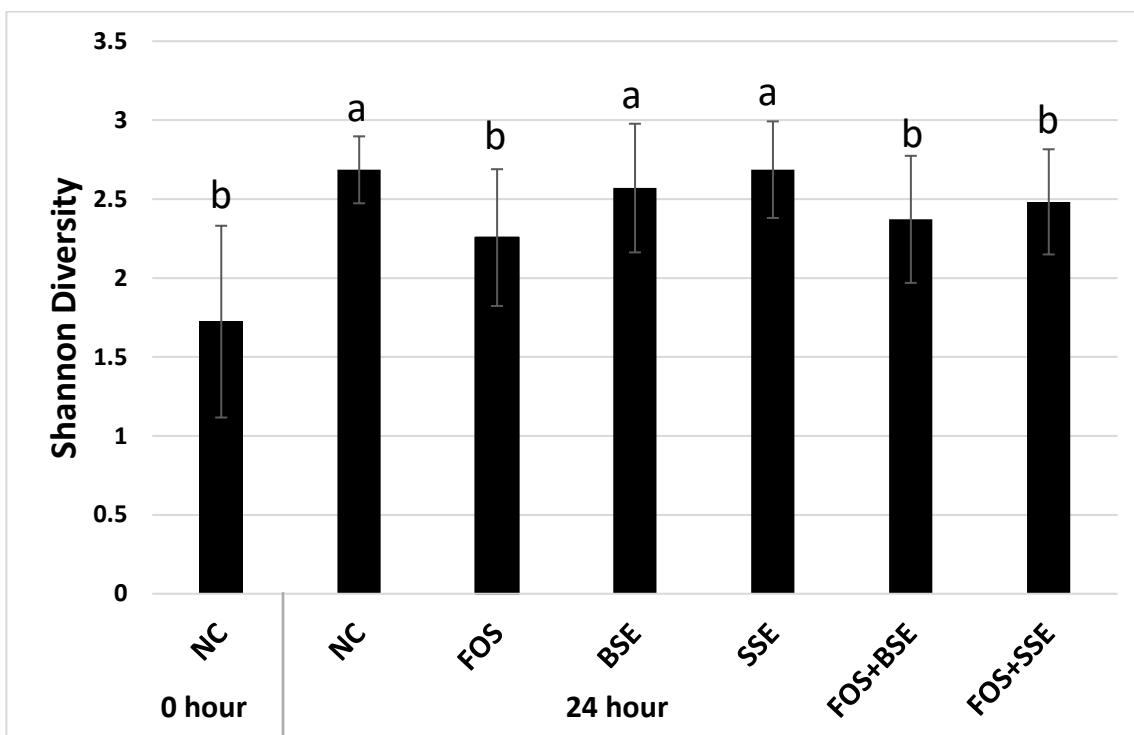


Figure 6. Species diversity after a 24 hour fermentation. Different letters indicate significant differences. $P < 0.05$ ($n=22$). NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

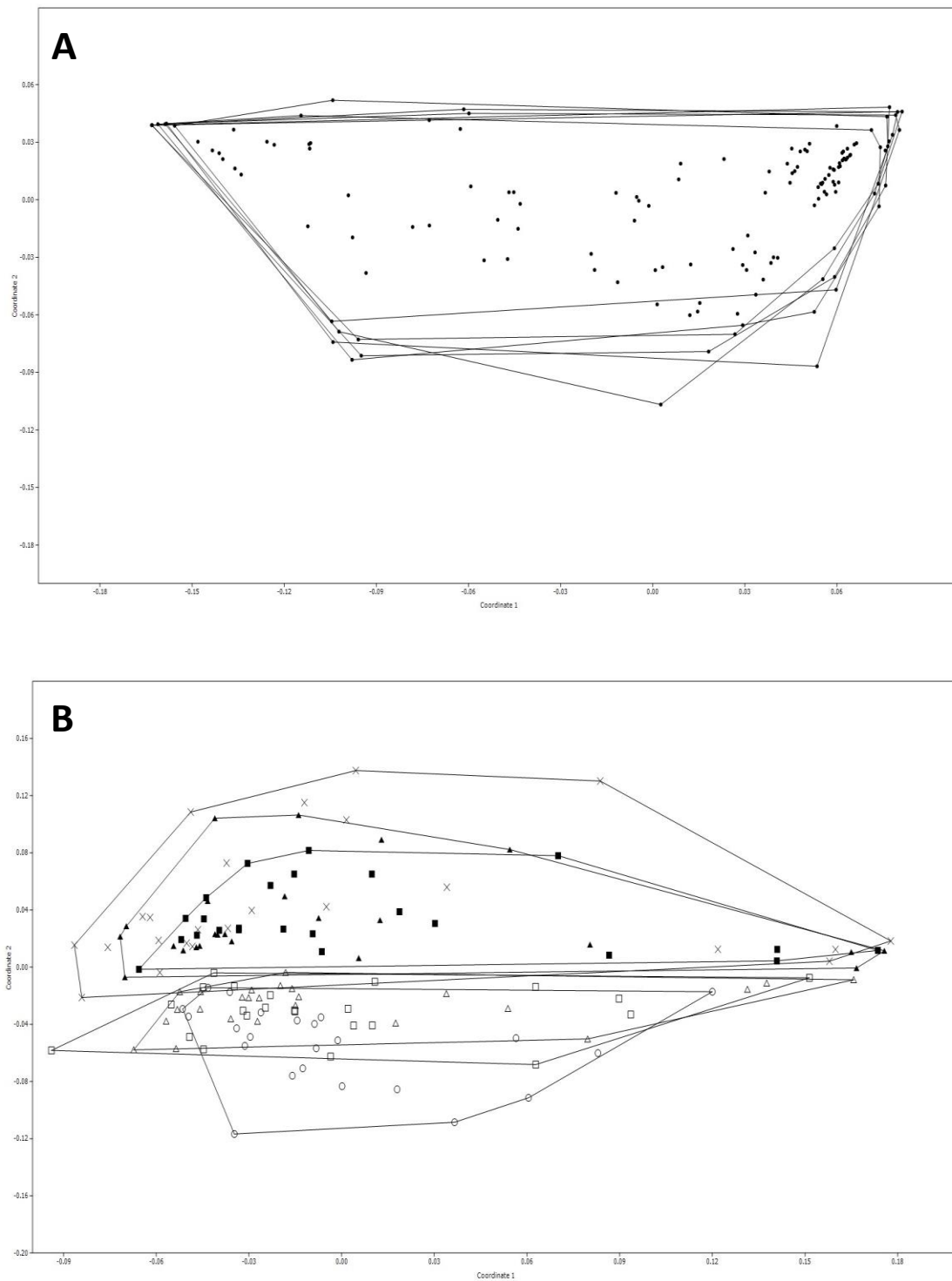


Figure 7. NMDS plot comparing bacterial communities between treatments. $P < 0.05$ ($n = 22$). (A) 0 hour and (B) 24 hour. ○: negative control, x: fructooligosaccharides (FOS), △: black sorghum bran extract (BSE), □: sumac sorghum bran extract (SSE), ▲: FOS+BSE, ■: FOS+SSE

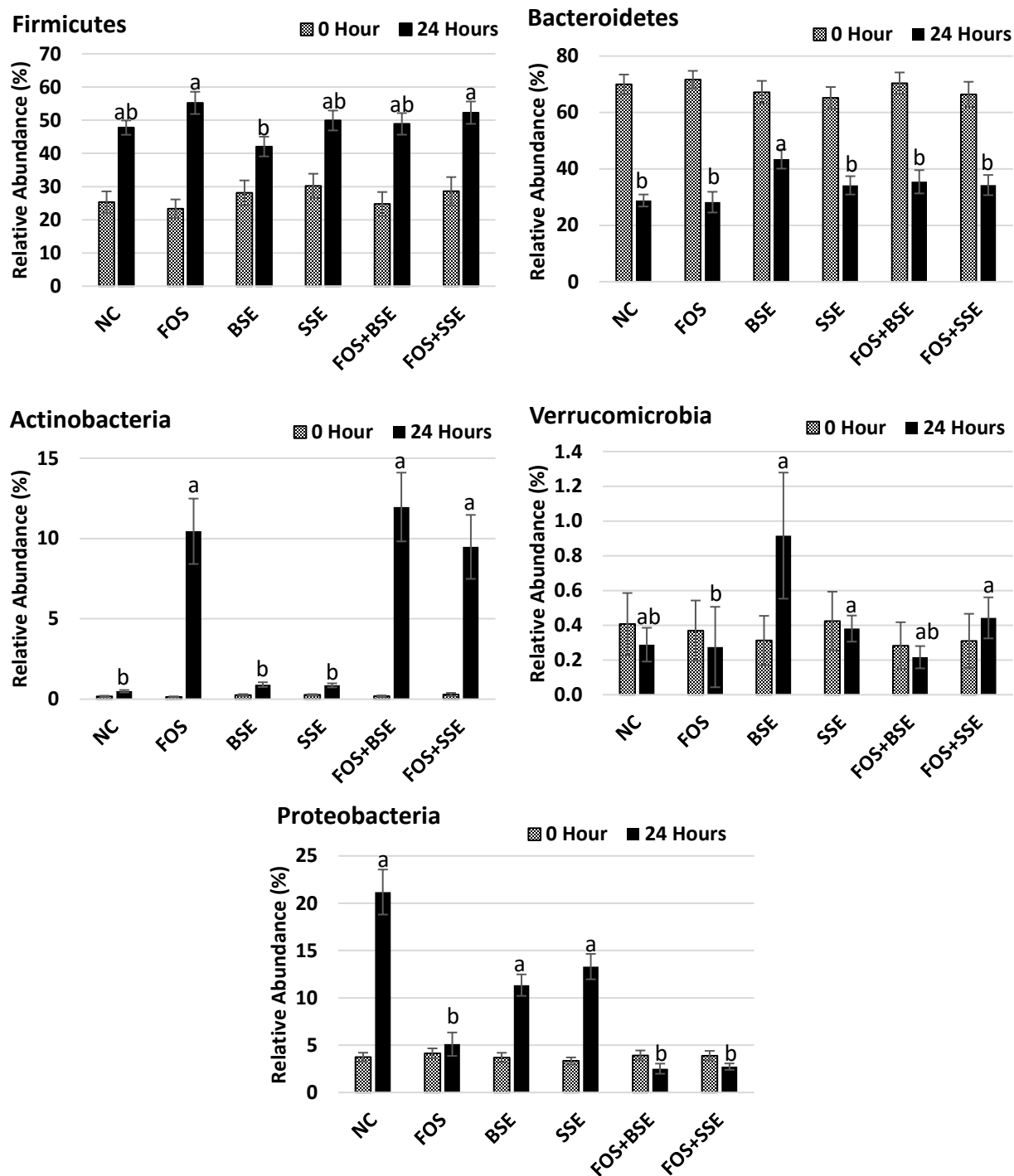


Figure 8. Relative abundance at phylum level in all subjects. Letters indicate significant differences between treatments at 24 hours. $P < 0.05$. $n = 22$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

Table 3. Relative abundance of dominant phyla in NW and O/O

			Relative Abundance (%)					
			NC	FOS	BSE	SSE	FOS+BSE	FOS+SSE
Firmicutes	24	NW	50.3 ± 2.8	59.5 ± 3.6	48.9 ± 3.2*	55.8 ± 3.3	52.2 ± 4.4	52.7 ± 4.5
		O/O	45.2 ± 3.2 ^{ab}	51.0 ± 5.5 ^a	35.2 ± 4.1^b	44.0 ± 4.5 ^{ab}	45.6 ± 4.9 ^{ab}	51.8 ± 5.2 ^a
Bacteroidetes	24	NW	24.8 ± 1.9^{ab}	18.7 ± 2.5^b	34.1 ± 2.3^a	26.0 ± 2.3^{ab}	27.5 ± 4.4 ^{ab}	28.2 ± 3.9 ^{ab}
		O/O	32.8 ± 3.5^{b*}	37.6 ± 5.7^{b*}	52.9 ± 5.1^{a*}	42.3 ± 5.1^{b*}	43.3 ± 6.3 ^{ab}	40.3 ± 5.6 ^b
Actinobacteria	24	NW	0.5 ± 0.1 ^d	13.8 ± 3.8 ^{ab}	1.3 ± 0.2^{bcd*}	1.1 ± 0.2 ^{cd}	15.2 ± 3.5 ^a	14.3 ± 3.3^{abc*}
		O/O	0.5 ± 0.1 ^c	7.1 ± 1.1 ^a	0.5 ± 0.1^c	0.7 ± 0.2 ^{bc}	8.7 ± 2.1 ^a	4.7 ± 1.0^{ab}
Verrucomicrobia	24	NW	0.2 ± 0.1 ^{ab}	0.5 ± 0.5 ^b	1.3 ± 0.7 ^a	0.4 ± 0.1 ^{ab}	0.3 ± 0.1 ^{ab}	0.5 ± 0.1 ^{ab}
		O/O	0.4 ± 0.2 ^{ab}	0.1 ± 0.0 ^b	0.5 ± 0.2 ^a	0.4 ± 0.1 ^{ab}	0.1 ± 0.1 ^{ab}	0.4 ± 0.2 ^{ab}
Proteobacteria	24	NW	21.8 ± 2.4 ^a	6.5 ± 2.2 ^{bc}	12.6 ± 1.6 ^{ab}	14.7 ± 1.6 ^a	3.6 ± 0.9^{c*}	3.4 ± 0.5^{bc*}
		O/O	20.6 ± 4.2 ^a	3.7 ± 1.1 ^{bcd}	10.1 ± 1.6 ^{abc}	11.9 ± 2.1 ^{ab}	1.4 ± 0.3^d	2.1 ± 0.4^{cd}

Data are expressed as Mean ± SEM. Letters indicate significant differences between treatments at 24 hours in each weight class group. * indicates significant difference between weight class groups for a given treatment at 24. P<0.05. n=11/group.

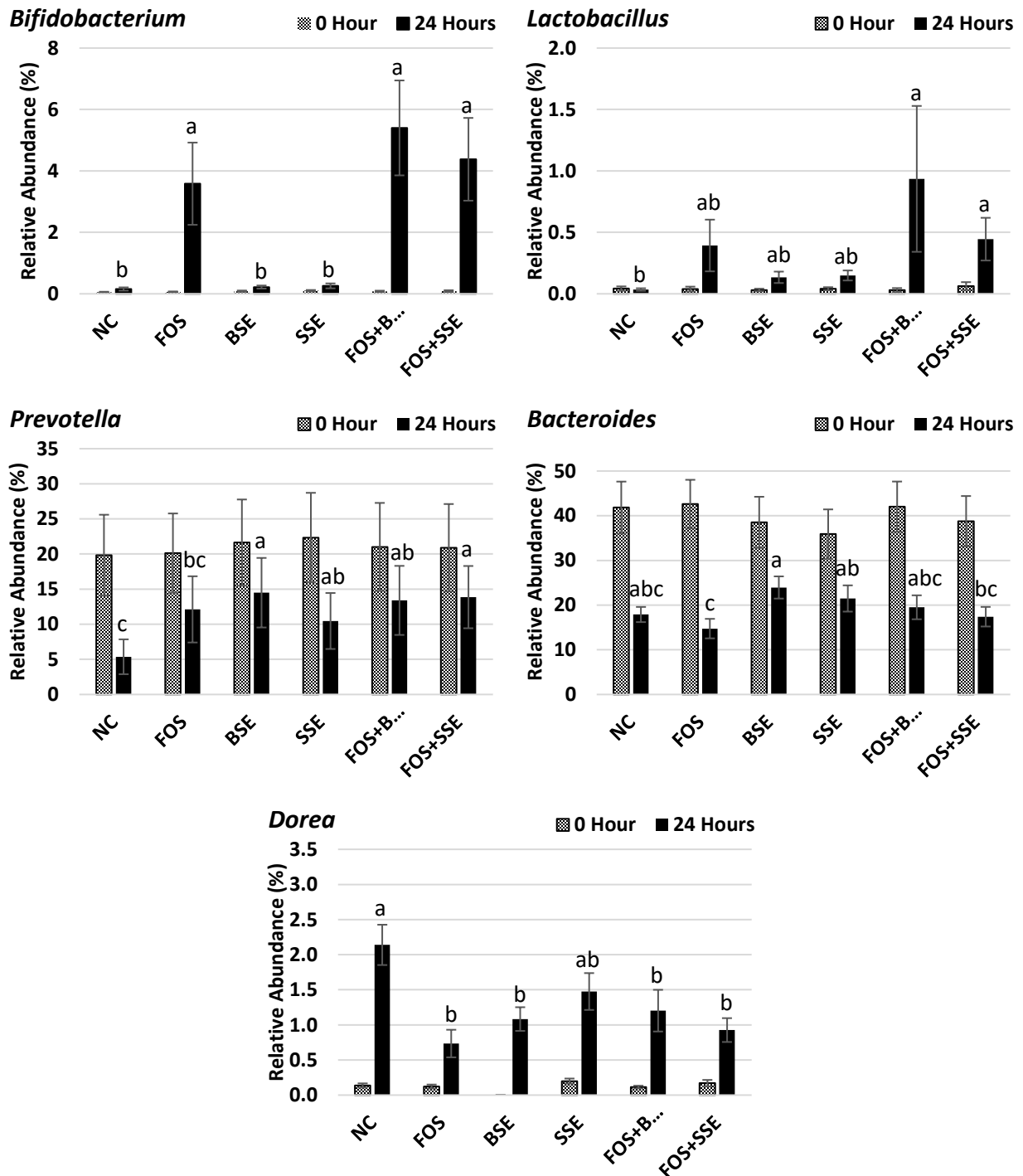


Figure 9. Relative abundance of select genera in all subjects. Letters indicate significant differences between treatments at 24 hours. $P < 0.05$. $n = 22$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

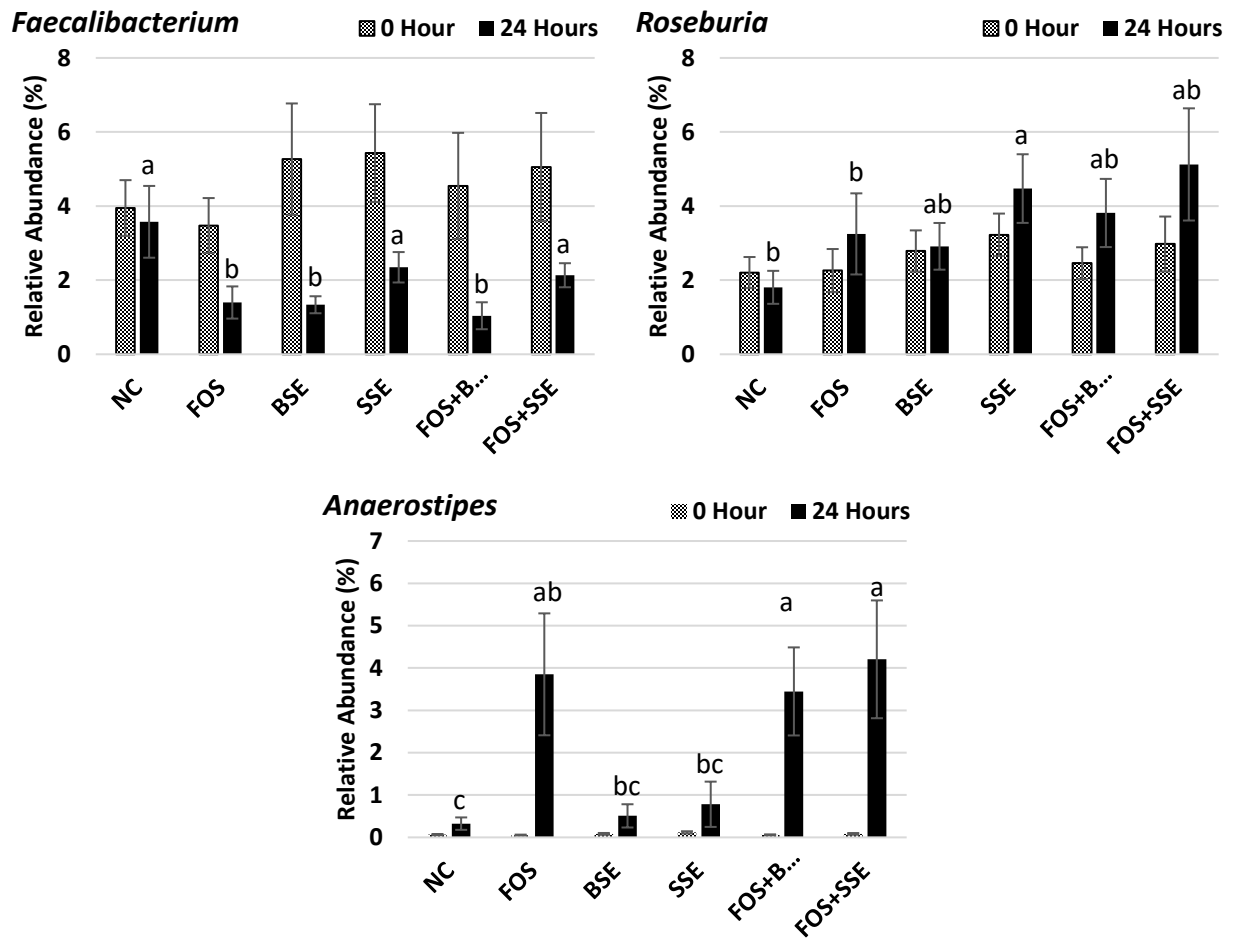


Figure 10. Relative abundance of butyric acid-producing bacteria in all subjects. Letters indicate significant differences between treatments at 24 hours. $P < 0.05$. $n = 22$. NC: negative control, FOS: fructooligosaccharides, BSE: black sorghum bran extract, SSE: sumac sorghum bran extract.

Table 4. Relative abundance at genus level in NW and O/O

			Relative Abundance (%)					
			NC	FOS	BSE	SSE	FOS+BSE	FOS+SSE
<i>Bifidobacterium</i>	24	NW	0.2 ± 0.1 ^c	6.1 ± 2.5^{ab*}	0.3 ± 0.1 ^{bc}	0.3 ± 0.1 ^{bc}	7.6 ± 2.7 ^a	7.2 ± 2.4^{a*}
		O/O	0.1 ± 0.1 ^c	1.1 ± 0.5^{abc}	0.2 ± 0.1 ^{bc}	0.2 ± 0.1 ^c	3.2 ± 1.4 ^a	1.6 ± 0.6^{ab}
<i>Lactobacillus</i>	24	NW	0.0 ± 0.0 ^b	0.4 ± 0.3 ^{ab}	0.2 ± 0.1 ^{ab}	0.2 ± 0.1 ^{ab}	0.4 ± 0.3 ^{ab}	0.5 ± 0.3 ^a
		O/O	0.0 ± 0.0 ^b	0.4 ± 0.3 ^b	0.1 ± 0.0 ^{ab}	0.1 ± 0.0 ^{ab}	1.5 ± 1.1 ^a	0.4 ± 0.2 ^{ab}
<i>Prevotella</i>	24	NW	0.7 ± 0.5^{bc}	1.0 ± 0.6^c	3.6 ± 1.8^a	1.7 ± 1.1^{abc}	2.7 ± 1.1^{ab}	4.6 ± 1.7^a
		O/O	10.1 ± 4.6^{b*}	23.3 ± 8.3^{ab*}	25.4 ± 8.7^{a*}	19.3 ± 7.1^{ab*}	24.1 ± 8.8^{ab*}	23.1 ± 7.9^{a*}
<i>Bacteroides</i>	24	NW	19.4 ± 1.9	16.9 ± 2.9	26.6 ± 3.1	24.9 ± 5.1	22.1 ± 3.4	20.0 ± 3.0
		O/O	16.4 ± 2.8 ^{ab}	12.6 ± 3.3 ^b	21.3 ± 3.9 ^a	18.1 ± 2.9 ^{ab}	16.9 ± 4.2 ^{ab}	14.8 ± 3.2 ^b
<i>Dorea</i>	24	NW	2.4 ± 0.5 ^a	0.6 ± 0.2 ^b	1.3 ± 0.2^{ab}	1.7 ± 0.4 ^{ab}	1.5 ± 0.5 ^{ab}	0.9 ± 0.2 ^{ab}
		O/O	1.9 ± 0.3 ^a	0.9 ± 0.4 ^b	0.8 ± 0.2^{ab*}	1.2 ± 0.3 ^{ab}	0.9 ± 0.3 ^{ab}	0.9 ± 0.3 ^{ab}
<i>Faecalibacterium</i>	24	NW	5.1 ± 1.8^{a*}	2.1 ± 0.8 ^b	1.8 ± 0.4^{ab*}	3.0 ± 0.7^{ab*}	1.7 ± 0.7^{b*}	2.7 ± 0.5^{ab*}
		O/O	1.9 ± 0.6^{ab}	0.7 ± 0.2 ^{ab}	0.9 ± 0.2^{bc}	1.7 ± 0.5^a	0.4 ± 0.2^c	1.6 ± 0.3^{abc}
<i>Roseburia</i>	24	NW	2.3 ± 0.8 ^b	1.8 ± 0.8 ^b	3.6 ± 1.1 ^{ab}	6.1 ± 1.6^{a*}	3.2 ± 1.1 ^{ab}	2.7 ± 0.8 ^{ab}
		O/O	1.3 ± 0.4	4.7 ± 2.0	2.3 ± 0.6	2.8 ± 0.7	4.5 ± 1.5	7.6 ± 2.8
<i>Anaerostipes</i>	24	NW	0.2 ± 0.1 ^c	4.1 ± 2.4 ^{abc}	0.3 ± 0.1 ^c	0.3 ± 0.1 ^{bc}	4.1 ± 1.8 ^a	4.7 ± 2.3 ^{ab}
		O/O	0.4 ± 0.3 ^c	3.6 ± 1.8 ^{abc}	0.8 ± 0.5 ^c	1.2 ± 1.1 ^{bc}	2.8 ± 1.1 ^{ab}	3.8 ± 1.7 ^a

Data are expressed as Mean ± SEM. Letters indicate significant differences between treatments at 24 hours in each weight class group. * indicates significant difference between weight class groups for a given treatment at 24. P<0.05. n=11/group.

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Conclusion

Grain sorghum bran harbors a diverse profile of polyphenols which varies with bran color and has positive implications in cancer, diabetes, and colonic health. In the present study we found sumac sorghum bran to be richer in total polyphenols and proanthocyanidins (condensed tannins) than black bran, while the black bran had much higher levels of 3-deoxyanthocyanins. Marked dysbiosis of the gut microbiota has been observed in obesity and other conditions, and nutrition strategies to improve gut health have been a major topic in nutrition research. We found that polyphenols from black and sumac sorghum brans impacted bacterial distributions, increasing the abundance of several beneficial genera. We observed some differing responses between weight class groups, implicating altered microbial metabolism in overweight/obese individuals. We hypothesized that sorghum bran polyphenols would impact production of SCFA, increase beneficial gut microbiota in both groups, and improve bacterial profile of overweight/obese individuals. While our findings do not supply strong evidence of sorghum polyphenols impacting SCFA production, they supported our hypothesis of increases in beneficial groups of bacteria. We were not, however, able to conclude whether or not the overall bacterial profile was improved in O/O. Although sorghum polyphenols did not significantly impact SCFA production during this 24-hour fermentation, it is possible that the impacts on bacterial species could lead alterations in SCFA production during long-term consumption. Further research needed includes human intervention trials and isolation of major phenolic components of sorghum brans for individual testing.

Appendix A: IRB Approval Letter



Office of Research Compliance
Institutional Review Board

February 17, 2017

MEMORANDUM

TO: Sun-Ok Lee
Danielle Ashley
Inah Gu
Wing Shun Lam

FROM: Ro Windwalker
~~Windwalker~~
IRB Coordinator

RE: New Protocol Approval

IRB Protocol #: 17-02-433

Protocol Title: *Does Sorghum Bran Play an Important Role in Improving Colon-Specific Health?*

Review Type: ☐ EXEMPT ☒ EXPEDITED ☐ FULL IRB

Approved Project Period: Start Date: 02/14/2017 Expiration Date: 02/13/2018

Your protocol has been approved by the IRB. Protocols are approved for a maximum period of one year. If you wish to continue the project past the approved project period (see above), you must submit a request, using the form *Continuing Review for IRB Approved Projects*, prior to the expiration date. This form is available from the IRB Coordinator or on the Research Compliance website (<https://vpred.uark.edu/units/rscp/index.php>). As a courtesy, you will be sent a reminder two months in advance of that date. However, failure to receive a reminder does not negate your obligation to make the request in sufficient time for review and approval. Federal regulations prohibit retroactive approval of continuation. Failure to receive approval to continue the project prior to the expiration date will result in Termination of the protocol approval. The IRB Coordinator can give you guidance on submission times.

This protocol has been approved for 120 participants. If you wish to make any modifications in the approved protocol, including enrolling more than this number, you must seek approval *prior* to implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

If you have questions or need any assistance from the IRB, please contact me at 109 MLKG Building, 5-2208, or irb@uark.edu.

Appendix B: IBC Approval Letter



June 29, 2017

MEMORANDUM

TO: Dr. Sun-Ok Lee

FROM: Ines Pinto, Biosafety Committee Chair

RE: Protocol Modification

PROTOCOL #: 17036

PROTOCOL TITLE: Effect of sorghum bran extracts on fecal fermentation

APPROVED PROJECT PERIOD: Start Date June 8, 2017 Expiration Date June 7, 2020

The Institutional Biosafety Committee (IBC) has approved your request, dated June 28, 2017, to modify Protocol # 17036, "Effect of sorghum bran extracts on fecal fermentation" to add laboratory personnel.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Appendix C: IBC Personnel Form

Form 1: GENERAL INFORMATION, confid.

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name: (first and last) - POSITION (Title, academic degrees, certifications, and material field of expertise)

QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE
Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels)

Example:

Bob Biohazard - Associate Professor, PhD Microbiology

14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.

Sun-Ok Lee - Associate Professor, PhD Human Nutrition	18 years of experience in conducting and managing research involving human feces and urine samples at BL2
Danielle Ashley - MS student, Nutrition	No experience. Get a training from Dr. Lee
Inah Gu - MS student, Nutrition	3 months of experience in Fecal samples
Wing Shun Lam - MS student, Nutrition	No experience. Get a training from Dr. Lee
Laura Fassler- Undergraduate student	No experience. Get a training from Dr. Lee

Additional Personnel Information (if needed):

All personnel took the EH&S Online training including bloodborne pathogens, hazardous waste, biosafety, fire safety, and autoclave safety.
All personnel are provided with the hepatitis A & B immunizations. The use of departmental cost center is allowable. It will keep a log as whom have received it and whom have declined.
PI, Sun-Ok Lee, will give a training to all personnel to prevent an unexpected events in the process of research.