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In vitro Fermentation Patterns of Rice Bran Components by Human Gut Microbiota

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In vitro Fermentation Patterns of Rice Bran Components by Human Gut Microbiota

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

by

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McNeese State University
Bachelor of Science in Nutrition and Food Sciences, 2014

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This thesis is approved for recommendation to the Graduate Council.

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Abstract

The prevalence of colorectal cancer (CRC) has been increasing in recent years worldwide. In the United States, it is ranked the second leading cause of cancer death. Risks of CRC increase with age and are associated with several lifestyle factors such as diet, drinking and smoking habits, and levels of physical activity. There is an abundance of scientific literature demonstrating the protective roles of several dietary components including fibers and polyphenolic compounds. These compounds have been shown to be able to positively modulate gastrointestinal ecology by increasing the production of short-chain fatty acids (SCFA) and promoting the population of beneficial bacteria. Whole grain cereals such as wheat, corn, sorghum, and rice are rich sources of these components. However, they are more abundant in the cereal bran layer which is typically removed during polishing. Research have shown that feruloylated arabinoxylan oligosaccharides (FAXO) isolated from cereals such as wheat, corn, and sorghum exerted prebiotic-like properties by increasing SCFA production and selectively promote microbiota population. Polyphenolic compounds have also been demonstrated to be able to modulate gut microbiota ecology. However, rice bran FAXO and rice bran polyphenolics (RBPP) have not been studied for such properties. Therefore, two rice bran components including FAXO and RBPP are hypothesized to have positive impacts on human gut microbiota. In this study, prebiotic-like properties of FAXO and RBPP were assessed by determining the fermentation patterns of FAXO and RBPP to increase SCFA production and by evaluating the impacts of FAXO and RBPP on the composition of human gut microbiota.

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Finally, I would like to take this opportunity to express my profound gratitude to my beloved parents, my relatives in Florida whom I did not know I had until 3 years ago, and my Louisiana family, especially Darlin and Mimi, for their love and continuous support.

Dedication

This thesis is dedicated to the memory of my cousin Thu Luong. Although you are no longer with us, there is not one day that goes by that I do not think about you. Your love and your strength will always be my motivation in life.

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Introduction

Awareness about colon health has been on a rise. In 2014, National Cancer Institute reported that colorectal cancer (CRC) is the third prevalent non-skin cancer in the United States. Moreover, it has been rated as the second most fatal type of cancer. Individuals with digestive disorders such as inflammatory bowel disease, ulcerative colitis, and polyps are most prone to CRC. Lifestyle factors including diet, levels of physical activity, smoking, alcohol consumption, and body composition have been associated with CRC (Doll and Peto 1981).

Dietary components such as dietary fiber, probiotics and prebiotics, and phenolic compounds have been shown to confer positive colonic health effects. This is achieved by modulating gut microbiota and enhancing production of short-chain fatty acids (SCFA) (Macfarlane and Macfarlane 2003).

Prebiotics are defined as “selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health” (Gibson et al. 2010).

Currently, *Bifidobacterium* and *Lactobacillus* population are considered beneficial bacteria and are often targets for dietary intervention (Manning and Gibson 2004). Fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and lactulose are compounds that are currently considered true prebiotics since they have been reported to stimulate *Bifidobacterium* and *Lactobacillus* population (Gibson et al. 2010). In addition, other dietary materials have also been proposed to be candidate prebiotics. Non-digestible oligosaccharides are the primary new candidates since they appear to confer certain degrees of fermentation selectivity (Gibson et al. 2010). Among these oligosaccharides, feruloylated arabinoxylan oligosaccharides (FAXO) isolated from cereal bran such as wheat, barley, and corn

have gained much attention as they have been shown to exert prebiotic-like properties. FAXO have been implicated for having prebiotic-like activities because of their ability to pass through the upper gastrointestinal tract undigested to the colon where they are hydrolyzed and subsequently fermented by gut microbiota to produce SCFA (Englyst and Cummings 1985, Courtin et al. 2009).

Another dietary component that can also positively affect the intestinal ecology is polyphenolics and their derived products. The intake of flavanol-rich foods has been shown to modify the composition of the gut microbiota by selectively inhibiting pathogen growth and stimulate the growth of beneficial bacteria, thus influencing the microbiota composition (Tzonu et al. 2008)

As mentioned above, FAXO isolated from cereal bran such as wheat, corn, and barley have been assessed for their prebiotic properties. However, FAXO and RBPP isolated from rice bran have not been studied for their potential colon health promoting properties including prebiotic activities. In addition, the synergistic activities of non-digestible oligosaccharides, particularly FAXO, and RBPP in improving colonic health has not been well studied. Therefore, the hypotheses of this study were that fermentation of rice bran components by colonic microbiota increases production of SCFAs, particularly butyric acid and that beneficial gut microbiota differentially increases after incubation with FAXO and RBPP. The objectives of the present study were to determine (1) the SCFA production of FAXO and RBPP by human gut microbiota, (2) the ability of FAXO and RBPP to stimulate beneficial human gut microbiota, and (3) the relationship of human gut microbiota and SCFA production.

Chapter 1: Literature Review

1. Rice Bran

Rice (*Oryza sativa*) is one of the major cereal crops and a staple food for half of the world's population (Childs 2014). In the United States, Arkansas is the leading rice-producing state, accounting for about 50% of total U.S. rice production (Childs 2014). Rice bran is a byproduct from rice milling process to produce polished rice. Rice bran is defined as the brown outer layer of rice kernel comprising pericarp, aleurone layer and embryo (Figure 1), which account for 10-15% of rough rice by weight (Champagne et al. 2004). Rice bran has been intensively studied for its nutritional values. It has been reported to contain significant amounts of nutrients such as protein, unsaturated fat, vitamins, minerals, dietary fiber, and polyphenolic compounds, which have been shown to exhibit beneficial effects in various *in vitro*, animal, and human studies (Kahlon et al. 1992, Kahlon and Chow 2000, Qureshi et al. 2002). Although it is used primarily as animal feed, rice bran and its components are now finding major applications as value-added products (Jariwalla 2001, Ryan 2011).

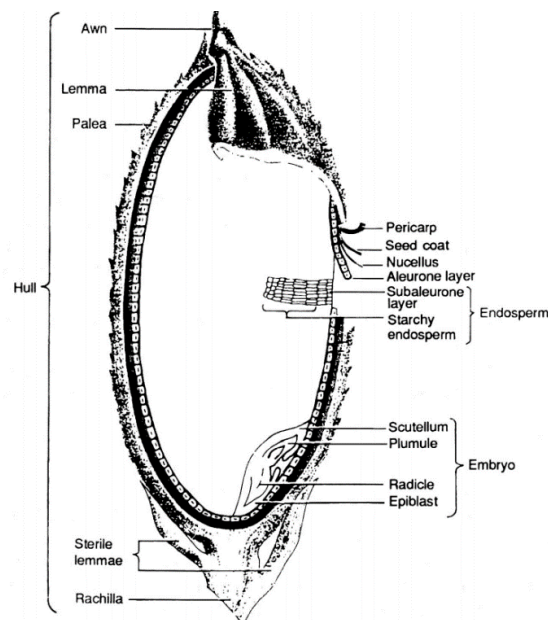


Figure 1. Structure of the rice grain. (Adapted from Juliano and Aldama 1937)

2. Feruloylated Arabinoxylan Oligosaccharides (FAXO)

Arabinoxylans (AX) are the most abundant structural polysaccharides present in most cereals. They were first identified in wheat by Hoffman and Gortner (1927). Since then, similar polysaccharides have been found in other cereals such as rice, barley, rye, and corn (Izydorczyk and Biliaderis 1995). They are composed of a xylan backbone linked together by $\beta(1\rightarrow4)$ glycosidic bonds that are variably substituted with α -L-arabinofuranoside at either C-(O)-2 or C-(O)-3 (Saulnier et al. 2007). Ferulic acid is present as substituents as well and they are generally linked to the C-(O)-5 position of arabinose units (Figure 2) (Izydorczyk and Biliaderis 1995), hence the name feruloylated arabinoxylans. AX together with other polysaccharides build up the cell walls of grain tissues and thus become part of the skeletal framework that maintains tissue (Rattan et al. 1994). The frequency and nature of substituents differ greatly amongst AX from different origins (Rattan et al. 1994). Specifically, AX from rice, maize, and sorghum bran contain more arabinose substituents than those from wheat, rye or barley bran (Rao and Muralikrishna 2007). Molecular mass of AX is difficult to determine since extraction process could lead to degradation of the polymers. However, it is generally estimated that they contain from 1,500 to 15,000 residues (Izydorczyk and Biliaderis 1995, Ebringerova and Heinze 2000). A small portion of AX in cereals is water-extractable (Maes and Delcour 2002). The majority is water-unextractable due to non-covalent interactions such as hydrogen linkages and covalent bonds with neighboring AX molecules through dehydrodiferulic acid bridges and with other cell wall components such as proteins, cellulose, and lignin (Iiyama et al. 1994, Izydorczyk et al. 1998, Maes and Delcour 2002).

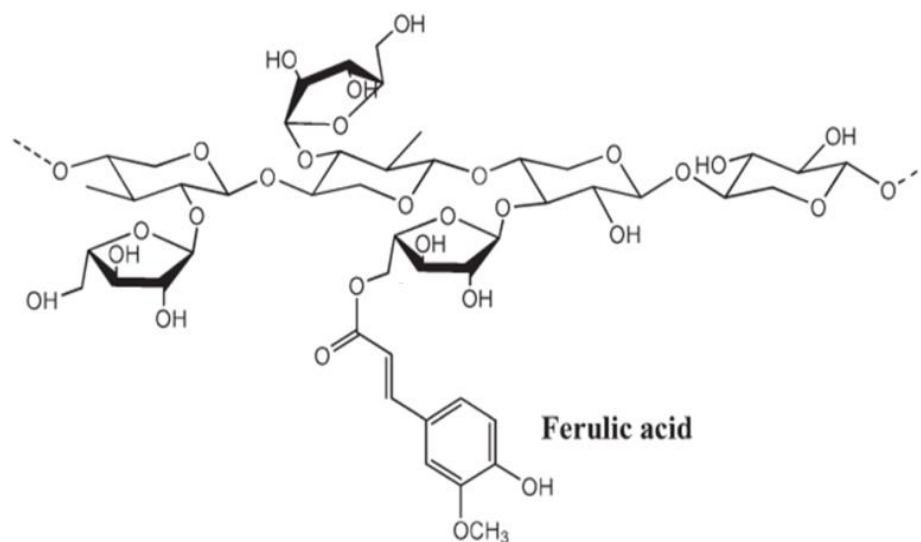


Figure 2. Structure of feruloylated arabinoxylans (Adapted from Buanafin 2009)

Feruloylated arabinoxylan oligosaccharides (FAXO) are hydrolyzed products of AX of high degree of polymerization. Degradation of AX into FAXO can be achieved by using degrading enzymes, acid hydrolysis, or hydrothermal treatment (Vázquez et al. 2000).

3. Pigmented Rice and Rice Bran Polyphenolics (RBPP)

Whole grain rice products, especially those from pigmented varieties, provide a rich reservoir of polyphenolic compounds that have been suggested to have many health benefits (Min et al. 2012, Chen et al. 2012, Deng et al. 2013). Many epidemiological studies have shown that consumption of whole grain cereal is highly correlated with reduced incidence of chronic diseases (Jacobs et al. 1998, Liu et al. 1999, Anderson et al. 2000), of which free radicals are thought to play a critical role in the onset and progression (Sayre et al. 2008, Sen and Chakraborty 2011). Their protective effects are due to the scavenging of free radicals by polyphenolics that are rich in whole grain cereals (Sen and Chakraborty 2011).

According to the USDA National Small Grains Collection, pigmented rice can be classified into seven color classes: white, light-brown, speckled brown, brown, red, variable purple, and purple. Among them, light-brown rice is most commercially grown and has been the primary focus of rice polyphenolics-related studies. Other pigmented varieties such as red and purple bran rice have recently gained attention because they contain high amounts of phenolic compounds, including anthocyanins and proanthocyanidins, which have been reported to have health-promoting potentials (Biswas et al. 2011, Sompong et al. 2011, Min et al. 2012, Chen et al. 2012).

The majority of polyphenolics in rice grains is present in the bran fraction which is a by-product of the milling process. Lipophilic, hydrophilic, and insoluble forms of these compounds have been identified (Hudson et al. 2000, Cicero and Gaddi 2001, Pérez-Jiménez and Saura-Calixto 2005). Phenolic compounds such as triclin, ferulic acid, caffeic acid, and methoxycinnamic acid have been identified in rice bran (Hudson et al. 2000). In addition, total polyphenolic content in the bran fraction of red and purple rice variety, which is made up mostly of free phenolics including proanthocyanidins and anthocyanins, is significantly higher than that of light-colored bran rice (Min et al. 2012).

Polyphenolics of high molecular weight such as proanthocyanidins are poorly absorbed in the small intestine and largely remain intact when reaching the colon. Only a small percentage of dietary polyphenolics (5-10%), mainly monomeric and dimeric structures, may be directly absorbed in the small intestine, generally after deconjugation reactions. After being absorbed into the small intestine, these less complex polyphenolic compounds may be subjected to extensive Phase I (oxidation, reduction and hydrolysis) and particularly Phase II (conjugation) biotransformations in the enterocytes and then the hepatocytes, resulting in a series of water-

soluble conjugate metabolites (methyl, glucuronide and sulfate derivatives) rapidly liberated to the systemic circulation for further distribution to organs and excretion in urine (Manach et al. 2005). In the large intestine, remaining unabsorbed polyphenols are enzymatically hydrolyzed by colonic microbiota, subsequently resulting in polyphenolic compounds with lower degrees of polymerization (Aura et al. 2005). These polyphenolics are also further metabolized by microbiota through the cleavage of glycosidic linkages and the breakdown of the heterocyclic backbone (Aura et al. 2005). For example, the microbial catabolism of proanthocyanidins results in the production of lactones and aromatic and phenolic acids with different hydroxylation patterns and side-chain lengths, depending on the precursor structures (Manach et al. 2004, Saura-Calixto et al. 2007). All these microbial-derived phenolic metabolites may be absorbed in the intestine or excreted in feces. When absorbed, they reach the liver through the portal vein where they may be further subjected to extensive first-pass Phase II metabolism (including glucuronidation, methylation, sulfation or a combination of these) until they finally enter the systemic circulation and are distributed to the organs or eliminated in urine.

Polyphenolic compounds are well known for their antioxidant properties, the ability to scavenge reactive oxygen species, which have been shown to be effective in preventing chronic diseases including cancer, hypertension, neurodegenerative diseases, and aging (Sen and Chakraborty 2011, Biswas et al. 2011). However, it has been suggested that health benefits of whole grain polyphenolics are more site-specific, being more effective in the colon (Adom and Liu 2002). This could be due to the fact that most polyphenolics in cereal bran are bound and may not be released by gut microbiota until they reach the colon (Adom and Liu 2002). This unique property may explain the reduced risk of colon cancer associated with increased consumption of whole grains and whole grain products (Thompson 1994, Jacobs et al. 1995).

Polyphenolics and their derived products can also positively affect the intestinal ecology. For example, the intake of flavanol-rich foods has been shown to modify the composition of the gut microbiota (Tzonuis et al. 2008). Polyphenolics and their metabolites selectively inhibit pathogen growth and stimulate the growth of beneficial bacteria, thus influencing the microbiota composition. For instance, resveratrol, a potent antioxidant found in wine, increased *Bifidobacterium* and *Lactobacillus* populations (Larrosa et al. 2009) and abolished the expression of virulence factors of *Proteus mirabilis* to invade human urothelial cells (Wang et al. 2006). In addition, phenolics and flavonoids may also reduce the adhesion ability of *Lactobacillus rhamnosus* to intestinal epithelial cells (Parkar et al. 2008). Tea catechins have also been shown to modify mucin content of the ileum in rat model which could modulate bacterial adhesion and colonization (Ito et al. 2006). Therefore, polyphenols appear to have potential to confer health benefits via modulation of the gut microbiota.

4. Gut Microbiota and Human Health

Although the human digestive tract is colonized by microorganisms, the distal gut (including cecum, large intestine or colon, and rectum) is the most densely populated part of the gut with 10^{12} - 10^{14} microorganisms including bacteria, archaea and yeast per gram feces (Whitman et al. 1998). Bacteria comprise about 40-55% of solid stool in people having typical Western diets (Stephen and Cummings 1980). Bacteroidetes and Firmicutes are the two dominant divisions accounting for almost 95% phylotypes. On the lower taxonomic levels, more than 50 genera and over 400 species have been identified in human gut (Eckburg et al. 2005). However, the composition and activity of the gut microbiota is extremely complex and unique for each individual as it is driven by many different factors (Nicholson and Wilson 2003).

Although the gut is essentially sterile at birth, it is rapidly populated with bacteria through environmental exposure (Palmer et al. 2007). Subsequently, the shaping of microbial landscape depends on a series of complex and dynamic interactions throughout life including diet, lifestyle, disease, and antibiotic use (Nicholson and Wilson 2003).

The gut microbiota plays very important roles in health and disease in humans. It is involved in energy harvest and storage, as well as in a variety of metabolic functions such as fermenting and metabolizing undigested carbohydrates. More importantly, the gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions (Chow et al. 2010). The importance of the gut microbiota is unquestionable and it is sometimes referred to as the “forgotten organ” (O’Hara et al. 2006). Thus, it is important to maintain a healthy, or balanced, gut microbiota. Aberrations in the gut microbiota, or often referred to as dysbiosis, have been shown to correlate with many metabolic diseases such as colon cancer, inflammatory bowel disease, type 2 diabetes, and obesity (Ley et al. 2006, Peterson et al. 2008). However, the underlying roles and functionality of specific bacteria relative to disease still remain unclear (Gerritsen et al. 2011).

4.1. Obesity

The prevalence of obesity in the United States has increased by more than 75% over the past 25 years. Currently, nearly two-thirds of the U.S. population is overweight and 1 in 3 adults are clinically obese (Ogden et al. 2007). Obesity can lead to serious health consequences, including increased risk for type 2 diabetes, cardiovascular disease, pulmonary hypertension, and mortality (Hensrud and Klein 2006, Ogden et al. 2007). Although diet plays an important role in the development of obesity, interest has been focused on other factors that might contribute to

this disease. Particularly, the composition of gut microbiota has been implicated as a critical determinant in the development of obesity (DiBaise et al. 2012).

The association between obesity and gut microbiota composition was first observed with mouse models by Ley et al. (2005). In their study, genetically obese mice showed 50% fewer Bacteroidetes accompanied by a proportional increase in Firmicutes compared to wild type counterparts (Ley et al. 2005). Consistent with the mouse model data, evidence also suggests that differences in the gut microbiota exist between obese and lean humans (Ley et al. 2006, Schwartz et al. 2009). Changes in the gut microbiota upon weight loss in obese individuals also have been reported but results are not consistent. Schwartz et al. (2009) showed that the proportion of Bacteroidetes was increased by weight loss. In contrast, Duncan et al. (2008) reported no relationship between percentage of Bacteroidetes in feces and amount of weight lost (kg) after a period of 8 weeks, comprising 4 weeks on each of two diets, either high-protein, low carbohydrate, ketogenic (LC) or high-protein, moderate-carbohydrate, non-ketogenic (MC). However, there was a significant reduction in a group of butyrate producing Firmicutes in the obese group after diet intervention (Duncan et al. 2008). Thus, although diet changes undoubtedly have impacts on the gut microbiota, and in turn, on colonic health and function, the differences between the gut microbiota in lean and obese individuals remain incompletely understood and further investigation in the area is needed.

4.2. Type 2 Diabetes

Type 2 diabetes, a metabolic disease which primary cause is insulin resistance, has also been shown to be associated with changes in gut microbiota composition (Membrez et al. 2008) although it is far left behind as the focus of research compared to obesity. Larsen et al. (2008)

showed that the proportions of phylum Firmicutes and class Clostridia were significantly reduced in the diabetic group compared to the control group. Furthermore, they also found that the ratios of Bacteroidetes to Firmicutes were positively correlated with the severity of diabetes and plasma glucose concentration (Larsen et al. 2008). In addition, *Bifidobacterium* and *Faecalibacterium prausnitzii* abundance appeared to be lower in type 2 diabetic patients than in lean subjects (Furet et al. 2010, Wu et al. 2010). Interestingly, *Bifidobacterium* and *Faecalibacterium prausnitzii* are suggested to have anti-inflammatory effects by producing metabolites able to block NF- κ B activation and the secretion of proinflammatory mediators (Furet et al. 2010, O'Mahony et al. 2010).

Furthermore, other studies have demonstrated that the gut microbiota played a major role in the onset of insulin resistance and type 2 diabetes by triggering low-grade inflammation, which is also a common feature characterizing obesity and several metabolic disorders (Backhed et al. 2007, Cani et al. 2007, Shen et al. 2013). According to Cani et al. (2007), gut microbiota can control the host metabolism and contribute to the development of low-grade inflammation through several potential mechanisms. They proposed that disruption in the gut microbiota-host symbiotic relationship could cause increases in gut permeability by altering the expression, localization and distribution of tight junction proteins, overactivating the CB1 receptors, and decreasing intestinal alkaline phosphatase activity leading to a decrease in lipopolysaccharide detoxification. Gut barrier alterations are responsible for metabolic endotoxaemia leading to low-grade inflammation and metabolic disorders (Everard and Cani 2013).

Altogether, these data reveal an existing association between gut microbiota and the pathology of obesity and type 2 diabetes in humans. However, numerous questions still remain debatable or unanswered such as whether gut microbiota alterations are associated with the diet

or with the pathology of obesity and type 2 diabetes themselves and whether the gut microbiota changes observed in obesity and type 2 diabetes are a cause or a consequence of the pathology. Therefore, more detailed studies in humans are needed to further investigate the gut microbiota-host relationship.

5. Fermentation Patterns of Gut Microbiota and Colonic Health

As discussed above, human gut microbiota is highly diverse with over 50 genera and over 400 species of bacteria that have been identified (Eckburg et al. 2005). The dominant organisms in terms of numbers are anaerobes including bacteroides, bifidobacteria, eubacteria, streptococci, and lactobacilli (Topping and Clifton 2001). Undigested organic food components such as carbohydrates and peptides are often metabolized by the gut microbiota through fermentation to produce energy for microbial growth and maintenance and other metabolic end products. Fermentation is a complex process and involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter. Along with heat, major products of fermentation include gas such as H₂, CH₄, and CO₂, and short-chain fatty acids (SCFA) (acetic, propionic, butyric acid - Figure 4) (Topping and Clifton 2001). Amino acid breakdown products including branched-chain fatty acids and ammonia are also found but in much smaller amounts (Roberfroid 2005).

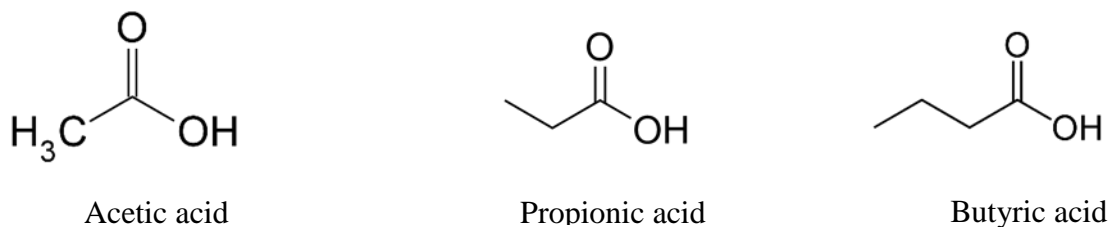


Figure 3. Acetic acid, propionic acid, and butyric acid structure

SCFA, fermentation end-products of carbohydrates by the gut microbiota, are strongly associated with colonic health in humans (Roediger 1980, Jenkins 1999, Smith et al. 2013). They are organic fatty acids with 1 to 6 carbon atoms and are the principal end products which arise from bacterial fermentation of undigested food components such as polysaccharides, oligosaccharides, peptide, amino acids, and glycoprotein precursors in the colon (Miller and Wolin 1979, Cummings and Macfarlane 1991). Linear SCFA, with acetic acid, propionic acid, and butyric acid (Figure 3) being the major products, in the fermentation process of undigested carbohydrates, have been the focus of interest in regards of colonic health (Macfarlane and Macfarlane 2003). SCFA modulate colonic functions in several different ways. Increases in SCFA result in the decreases of pH of the colonic luminal environment, which supports the growth of beneficial bacteria species while limits the growth of detrimental bacteria species (Jenkins et al. 1987). Decreases in pH also facilitate the absorption of certain vitamins and minerals in distal gut (Tuohy et al. 2005). More importantly, butyrate, the preferred energy source of colonocytes (Della Ragione et al. 2001), has been intensively investigated for its potentially protective activity against colon carcinogenesis, intestinal inflammation, and oxidative stress and for its contribution in improving the intestinal barrier. Although many *in vitro* and animal studies strongly suggest the protective role of butyrate against colon diseases, direct evidence in humans is still lacking (Bornet et al. 2002, Hijova and Chmelarova 2007). Butyrate is also involved in phosphorylation and acylation of histone proteins at molecular level which is thought to be crucial for the ability of butyrate to modulate the expression of numerous genes involved in colonic health (Archer and Hodin 1999).

Butyrate is not only produced directly from fermentable substrates but also produced from acetate by gut microbiota. Diez-Gonzalez et al. (1999) reported that butyrate was formed from two acetyl-CoA molecules that yielded acetoacetyl-CoA and then was converted into butyryl-CoA. Subsequently, butyryl-CoA was converted into butyrate via either butyrate kinase or butyryl-CoA:acetate-CoA transferase. According to Duncan et al. (2002), butyrate could be converted from acetate by butyrogenic bacteria including *Coprococcus* sp., *Roseburia* sp., and *Faecalibacterium prausnitzii*. Among those, six *Roseburia* and *F. prausnitzii* strains were shown to exhibit butyryl-CoA:acetate-CoA transferase but not butyrate kinase activity (Duncan et al. 2002). They also showed acetate net utilization, especially *F. prausnitzii*, for which acetate is a growth requirement (Smith 1985). These findings suggest the complexity of the colonic ecosystem as metabolites can be interconverted into one another.

Health effects of SCFA on gut health, especially butyrate, have been demonstrated in *in vitro* and in animal models. However, evidence in human is still lacking. Thus, further studies are needed to determine the activities of SCFA and to unravel the effects of diet on gut health in human.

5.1. FAXO as Substrates for Gut Microbes

Although many carbohydrates could be fermented by gut microbiota to produce SCFA, only a few are widely recognized as prebiotics. Originally, prebiotics were defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and (or) activity of one or a limited number of bacterial species already resident in the colon, and, thus, improve host health” (Gibson and Roberfroid 1995). However, in 2008, the definition of “dietary prebiotics” was updated as “selectively fermented ingredients 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) to account for modulation of other mixed microbial ecosystems by a prebiotic approach. To be considered a prebiotic, a food must meet all three criteria: (1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (2) fermentation by intestinal microbiota; and (3) selective stimulation of growth and/or activity of beneficial intestinal bacteria (Gibson et al. 2004). Following this description, only a few carbohydrate compounds were considered prebiotics including inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) (Roberfroid et al. 2007). In recent years, many compounds, mostly oligosaccharides and polysaccharides, have been proposed to have prebiotic properties. However, at present, the evidence is not as convincing as for fructans and galactans.

There is a growing body of evidence allowing FAXO to be considered to have prebiotic properties. FAXO from wheat source has been shown not only to be resistant to hydrolysis under gastric conditions (37°C, pH = 2) (Courtin et al. 2009), but also to escape digestion and absorption in small intestine due to the lack of enzymes to degrade these compounds (Englyst and Cummings 1985). A study by Glitso et al. (1999) found that rye AX were recovered as much as 100% in ileum in pigs. Thus, these findings suggest that FAXO remain largely intact and unabsorbed when reached the colon, where they are fermented by the gut microbiota. Many studies have confirmed the fermentability of AX and FAXO isolated from brewery's spent grain and wheat, including *in vitro* and *in vivo* in both animals and humans (Kabel et al. 2002, Grasten et al. 2003, Van Craeyveld et al. 2008). Increases in SCFA production upon fermentation of these compounds also have been corroborated (Grasten et al. 2003, Van Craeyveld et al. 2008). In addition, AX and FAXO appear to be relatively selective substrates. Several *in vitro* studies

have demonstrated that they can be utilized by some *Bifidobacterium* species and, to a lesser extent, some *Bacteroides* species and *Lactobacillus* species, but not by *E.coli* and *Clostridium* species (Yamada et al. 1993, Van Laere et al. 2000, Moura et al. 2007).

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Chapter 2: Publication Manuscript

Abstract

Rice bran is a rich source of bioactive components that have potentials to promote gastrointestinal health but are typically removed with the bran during polishing. Feruloylated arabinoxylan oligosaccharides (FAXO) isolated from rice bran fiber and rice bran polyphenolics (RBPP) isolated from red rice bran are hypothesized to have positive impacts on human gut microbiota. The objectives of this study are to determine the prebiotic-like properties of FAXO and RBPP by determining the fermentation patterns of FAXO and RBPP and production of short-chain fatty acid (SCFA) and by evaluating their impacts on the composition of human fecal microbiota. Fresh fecal samples collected from healthy adults ($n = 10$, 5M, 5F) with no signs or symptoms of bowel diseases or conditions were diluted with anaerobic medium. Each sample received 5 treatments separately: CTRL (control, no substrates), FOS (fructooligosaccharides), FAXO, RBPP, and MIX (FAXO with RBPP). Samples were prepared inside an anaerobic chamber and then incubated at 37°C. An aliquot of 1.5 mL was withdrawn from each treatment tube at 0, 4, 8, 12, and 24 hours and stored immediately at -80°C. SCFA concentrations were measured quantitatively using gas chromatography. Microbial populations were determined by 16S rRNA gene sequences via Illumina MiSeq platform and analyzed with QIIME (Quantitative Insights Into Microbial Ecology). Results showed that SCFA production was significantly increased with FAXO and was comparable to fermentation with the well-established prebiotic FOS. The synergistic effects of FAXO and RBPP in terms of SCFA production were not observed since RBPP alone and in combination with FAXO did not increase SCFA productions. Changes in microbiota population profiles were found, especially in *Bacteroides*, *Prevotella*, and

Dorea population, indicating that FAXO might be differently fermented, modulating microbiota profiles. Synergistic effects of FAXO and RBPP were also observed as they increased *Coprococcus* and *Roseburia* abundance. Results from this study suggested that FAXO and RBPP isolated from rice bran can potentially promote colon health.

1. Introduction

Colorectal cancer (CRC) is the third common non-skin cancer and ranks second most common in mortality in the United States (National Cancer Institute, 2014). CRC is more prevalent in individuals with digestive disorders or gastrointestinal diseases such as inflammatory bowel disease, ulcerative colitis, and polyps. It has also been found to be associated with many lifestyle factors including diet, levels of physical activity, smoking, alcohol consumption, and body composition (Doll and Peto 1981). In recent years, there has been a growing interest in functional foods, such as prebiotics and probiotics, dietary fibers, and other dietary components that target the colon and improve colonic and systemic health by modulating gut microbiota and enhancing production of short-chain fatty acids (SCFA), which have been proved to confer positive colonic health benefits (Macfarlane and Macfarlane 2003).

Prebiotics are defined as “selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health” (Gibson et al. 2010). Currently, only a few carbohydrates are considered true prebiotics, including fructooligosaccharides (FOS) and galactooligosaccharides (GOS). These compounds have been reported to stimulate *Bifidobacterium* and *Lactobacillus* population, which are considered beneficial bacteria and are often targets for dietary intervention (Manning and Gibson 2004).

Many other dietary materials have been proposed to be candidate prebiotics. However, non-digestible oligosaccharides are mainly new candidates since they appear to confer the degree of fermentation selectivity (Gibson et al. 2010). Among these oligosaccharides, feruloylated arabinoxylan oligosaccharides (FAXO) isolated from cereal bran such as wheat, barley, and corn have gained much attention as they have been shown to exert prebiotic-like properties. FAXO

have been implicated for having prebiotic-like activities because of their ability to pass through the upper gastrointestinal tract undigested to the colon where they are hydrolyzed and subsequently fermented by gut microbiota to produce short-chain fatty acids (SCFA), which play important roles in regards of colon health (Englyst and Cummings 1985; Courtin et al. 2009).

Polyphenolics and their derived products can also positively affect the intestinal ecology. The intake of flavanol-rich foods has been shown to modify the composition of the gut microbiota by selectively inhibiting pathogen growth and stimulate the growth of beneficial bacteria, thus influencing the microbiota composition (Tzonuis et al. 2008)

FAXO and polyphenolics are present in abundance in whole grain cereals and their products, including whole grain rice. However, these components, which are present mostly in the bran, are typically removed from rice during polishing. FAXO and RBPP isolated from rice bran have not been studied for their potential colon health promoting properties including prebiotic activities. In addition, the synergistic activities of non-digestible oligosaccharides, particularly FAXO, and RBPP in improving colonic health has not been well studied. Therefore, the objectives of the present study were to determine (1) the SCFA production of FAXO and RBPP by human gut microbiota, (2) the ability of FAXO and RBPP to stimulate beneficial human gut microbiota, and (3) the relationship of human gut microbiota and SCFA production.

2. Materials and Methods

2.1. Substrates and Standards

Feruloylated arabinoxylan oligosaccharides (FAXO) were obtained from Dr. Savary, Arkansas State University (Jonesboro, AR, USA). Rice bran polyphenolic (RBPP) fraction isolated from red rice bran was obtained from Dr. Chen, Agricultural Research Service, USDA (Stuttgart, AR, USA) (Chen et al. 2012). The polyphenolic profile of RBPP is shown in Figure 1. Fructooligosaccharides (FOS) were purchased from Megazyme International Ireland Ltd. (Bray Business Park, Wicklow, Ireland). All materials were tightly sealed and stored at -20°C until use. SCFA standards including acetic acid, propionic acid, and butyric acid were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Subjects, Dietary Records, and Fecal Sample Collection

The study protocol was approved by University of Arkansas Institutional Review Board (IRB #13-09-080). All participants were recruited from University of Arkansas (Fayetteville, AR, USA) and surrounding area (Appendix A). Thirty-two volunteers (15 males and 17 females), 21-45 years of age, participated in a screening session to sign a consent form and a screening form. Only participants who were generally healthy (BMI < 25) with no digestive diseases, non-smokers, not currently taking any medications, and had not taken antibiotics in the last 6 months were recruited. During screening sessions, height and weight of subjects were recorded for Body Mass Index (BMI) calculation. Medical history and bowel movement habits were also recorded to confirm eligibility of participants. In addition, Food Frequency Questionnaires (FFQ) were completed by recruited participants.

Ten eligible subjects (5 males and 5 females) were recruited for the study. Each participant received a stool collection kit (Commode Specimen Collection System; Fisher Scientific, Pittsburgh, PA, USA) one or two days before the day of experiment. Subjects were instructed to deliver a tightly sealed fecal sample within one hour of defecation. Fecal samples were immediately transferred to an anaerobic chamber upon delivery to perform the experiment.

2.3. *In vitro* Fermentation

In vitro fermentation of substrates with the fecal inocula was carried out following the method described by Yang et al. (2013). In short, 50 mg each of FAXO, RBPP, their combination (MIX - FAXO with RBPP), and FOS used as control were mixed in 10 mL of sterile fermentation medium consisting of (per liter) peptone (2 g; Fisher Scientific, Waltham, MA, USA), yeast extract (2 g; Alfa Aesar, Ward Hill, MA, USA), bile salts (0.5 g; Oxoid, Hampshire, UK), NaHCO₃ (2 g), NaCl (0.1 g), K₂HPO₄ (0.08 g), MgSO₄·7H₂O (0.01 g), CaCl₂·6H₂O (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma-Aldrich, St. Louis, MO, USA), bovine hemin (50 mg; Sigma-Aldrich, St. Louis, MO, USA), Tween 80 (2 mL), vitamin K (10 µL; Sigma-Aldrich, St. Louis, MO, USA), and 0.025% (wt/vol) resazurin solution (4 mL). Fecal slurry was prepared by vortexing 1 g of fecal sample with 10 mL of sterile phosphate-buffered saline until homogenized then filtering through four layers of cotton gauge. Test tubes containing fermentation medium and treatments were then inoculated with 0.2 mL of fecal slurry. All steps for fermentation were conducted in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA). Test tubes were then capped, tightly sealed, and vortexed for 5 seconds to mix. Subsequently, test tubes were transferred to the incubator set at 37°C. Immediately before incubation, 1.5 mL of the mixture was taken out from each test tube using a sterile syringe for

time point 0 into a 2-mL centrifuge tube containing 0.1 mL of 2 M KOH stop solution.

Subsequent aliquots were obtained in the same manner at 6, 12, and 24 hours and stored at -80°C until analysis.

2.4. Short-Chain Fatty Acid Analysis

Fermentation samples were thawed at room temperature and mixed with a vortex mixer. An amount of 225 µL was withdrawn from each aliquot and was treated with 25 µL of a mixture containing 5% meta-phosphoric acid and 5% copper sulfate with 50 mM 4-methyl-valeric acid added as an internal standard. After 10 min of reaction time, the mixture was centrifuged at 12,000 x g for 2 min. A supernatant of 200 µL was transferred into a labeled tube and stored at -20°C until analysis.

SCFA standards were prepared using 1:2 serial dilution with the stock solution containing 10% v/v of each SCFA (acetic acid, propionic acid, and butyric acid) in Milli-Q water. SCFA standards were also treated with the same mixture containing meta-phosphoric acid, copper sulfate, and 4-methyl-valeric acid as with fermentation samples.

SCFA contents were analyzed using a gas chromatograph (GC) equipped with a flame ionization detector (FID) (Shimadzu Corp., Kyoto, Japan) and a fused silica capillary column (ID-BP21; SGE, L: 30 m, I.D: 0.25 mm, film: 25 µm, Ringwood, Victoria, Australia). Temperature ramp was as following: 4°C/min from 100°C (2 min) to 120°C (1 min), then 3°C/min until 150°C. One µL of treated sample (thawed and homogenized) was injected in split mode (30:1). Nitrogen was used as a carrier gas. Data were recorded and processed using the integrated Shimadzu database. Concentrations of acetic acid, propionic acid, and butyric acid

were determined using a standard curve of each SCFA with concentration ranging from 0 to 30 mM.

2.5. DNA Extraction and Sequence Analysis

Bacterial proliferation capability of each treatment was assessed by DNA sequence analysis of time 0 and 30 hours. Bacterial DNA was extracted from sample aliquots using QIAamp Fast DNA Stool Mini Kit (Qiagen, Gaithersburg, MD, USA). DNA concentrations were measured using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, WA, USA). All samples were diluted with DNase- and RNase-free water to achieve concentrations of 10 ng/ μ L. DNA samples were then mixed with AccuPrime Pfx SuperMix (Thermo Fisher Scientific, Waltham, WA, USA) and primers and were amplified via polymerase chain reaction (PCR) using Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany). Amplification of DNA samples were confirmed by agarose gel electrophoresis. Amplified DNA samples were normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, WA, USA) before pooling to make DNA sample library. Sequencing based on 16S-rRNA V4 region was performed using an Illumina MiSeq platform with the method developed by Kozich et al. (2013). Raw sequencing data acquired from Illumina BaseSpace were processed with a bioinformatics tool QIIME pipeline (Caporaso et al. 2010).

2.6. Statistical Analyses

All statistical analyses were carried out by JMP software (version 12; SAS Institute, Cary, NC, USA), using one-way ANOVA for comparing three or more data sets or paired t-test

for comparing two data sets. Data are presented as Mean \pm SEM (Standard Error of Mean) unless specified as standard deviation (SD). Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Subject Characteristics

In the present study, 10 subjects (5 males, 5 females) were recruited. Participant information including age, height, weight, and body mass index (BMI) is shown in Table 2. BMI of all subjects were within normal range (19.6 – 24.6).

3.2. Short-Chain Fatty Acid Analysis

SCFA concentrations were quantified based on peak areas using a standard curve for each SCFA (Figure 1). Total SCFA concentrations were measured at different time points from 0 hr to 24 hr (Table 3). FAXO appeared to be a preferred substrate by the microbiota as evidenced by the increase in total SCFA. Compared to FOS, a widely-recognized prebiotic, total SCFA production of FAXO was very comparable at later time points of 12 hr and 24 hr as no significant differences were found. However, at time point 4 hr and 8 hr, SCFA production of FOS was significantly higher compared to FAXO ($P < 0.05$), indicating different fermentation patterns of FOS and FAXO. Specifically, FAXO appeared to have slower and steadier fermentation rates throughout the incubation period compared to FOS, which were rapidly fermented in the beginning (time point 4 and 8 hr) but then slowed down over time.

RBPP were not fermented by the microbiota as no significant SCFA were produced. Furthermore, there was no significant difference in total SCFA production between FAXO and MIX (FAXO with RBPP) at any time points, indicating that RBPP also did not affect the SCFA production from FAXO.

Individual SCFA production (acetate, propionate, and butyrate) was also determined (Figure 2). Acetate (Figure 2A) showed to be the predominant SCFA produced as its

concentration at time point 24 hr was 3-4 times higher compared to propionate and butyrate (Figure 2B and C), which were produced at similar levels. Acetate production of all treatments also exhibited similar trends as observed in total SCFA production. In particular, acetate production of FOS were significantly higher than that of FAXO at time point 4 hr and 8 hr but not at time point 12 hr and 24 hr. However, for propionate and butyrate production, no significant differences were found at time point 4 hr and 8 hr in when comparing FOS and FAXO. No synergistic effects between FAXO and RBPP were observed as there was no significant difference between FAXO and MIX at any time point for all three individual SCFA.

3.3. Microbiota Analysis

The 16S rRNA sequencing data were analyzed to investigate the changes in microbiota composition after 24-hour incubation with FAXO, RBPP, and MIX compared with CTRL (control, no substrate) and FOS. At phylum level, four major phyla were identified in all samples including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figure 3). After 24 hours of incubation, different substrates appeared to be able to modulate the microbiota composition significantly. FAXO appeared to increase Bacteroidetes and decrease Firmicutes abundance significantly compared to CTRL ($P < 0.05$). Proteobacteria population was also suppressed significantly in FAXO compared to CTRL ($P < 0.05$). On the other hand, FOS, did not significantly affect the abundance of Bacteroidetes and Firmicutes compared to CTRL. FOS also showed a significant increase in Actinobacteria population and a significant decrease in Proteobacteria compared to CTRL ($P < 0.05$). RBPP alone and in combination with FAXO (MIX) only affected the Proteobacteria population as evidenced by a significant decrease in the

abundance of this phylum compared to CTRL ($P < 0.05$). However, RBPP seemed to decrease Proteobacteria to a lesser extent compared to FAXO.

Relative abundance of representative genera was also investigated. Results showed that FOS increased *Bifidobacterium* abundance dramatically which indicated FOS were utilized by *Bifidobacterium* species ($P < 0.05$) (Figure 4A). An increase in *Lactobacillus* was observed; however, it was not significant compared to CTRL (Figure 4B). No significant changes were observed in *Bifidobacterium* and *Lactobacillus* with FAXO treatment. Similarly, RBPP and MIX did not appear to significantly affect the abundance of *Bifidobacterium* and *Lactobacillus*. Overall, FAXO, RBPP, and MIX did not seem to exert any effects on *Bifidobacterium* and *Lactobacillus*, two genera that are often targets for prebiotic action as they are commonly associated with many health benefits.

However, considering other genera, significant differences were observed. Specifically, *Bacteroides* was increased significantly in FAXO and RBPP compared to both CTRL and FOS ($P < 0.05$) (Figure 4C). MIX treatment, however, did not affect *Bacteroides* abundance. FAXO also appeared to increase *Prevotella* abundance significantly compared to CTRL ($P < 0.05$) (Figure 4D). Population of *Dorea* was also affected by FOS, FAXO, and RBPP with significant decreases compared to CTRL ($P < 0.05$) (Figure 4E). In MIX, no significant difference in *Dorea* were found compared to CTRL. *Akkermansia*, a mucus-degrading genus which has gained significant attention in recent years because of its correlation to gut health, was also detected. However, no significant differences in *Akkermansia* population were found between treatments. The average abundance of *Akkermansia* ranged from 0.20% to 0.41% for all treatments. *Faecalibacterium*, a butyrate-producing genus, was increased significantly in abundance with RBPP and MIX compared to CTRL and FOS ($P < 0.05$) (Figure 4F). FAXO also seemed to

increase *Faecalibacterium* but no significant difference was observed. Taken together, these results suggested that although FAXO and RBPP did not alter *Bifidobacterium* and *Lactobacillus*, they appeared to be able to modulate other gut bacteria populations that could also play important roles in host health.

3.4. Relationship of Human Gut Microbiota and SCFA Production

The production of butyrate has been getting much attention because of its anti-inflammatory and anti-neoplastic effects on colonocytes (Segain et al. 2000, Rosignoli et al. 2001). Butyrate is not only produced directly from carbohydrate sources by butyrate-producing bacteria but can also be produced from acetate. As reported by Duncan et al. (2002), butyrate could be converted from acetate by butyrogenic bacteria including *Coprococcus* sp., *Roseburia* sp., and *Faecalibacterium prausnitzii*. The abundance of these bacteria groups was also assessed to evaluate the relationship of gut microbiota and SCFA production.

As shown in Figure 5, synergistic stimulative effects of FAXO and RBPP were observed in *Coprococcus* and *Roseburia* as evidenced by a significant increase in MIX compared to CTRL in these two genera ($P < 0.05$) while both FAXO and RBPP did not differ from CTRL (Figure 5A and B). *F. prausnitzii*, the most abundance species in *Faecalibacterium* genus, also exhibited similar trends as in its genus shown above. Comparing to CTRL, both RBPP and MIX appeared to increase the abundance of this particular species significantly ($P < 0.05$) (Figure 5C).

4. Discussion

In recent years, FAXO have gained particular interest because of their prebiotic-like activities. Prebiotics, by definition, are selectively fermented ingredients 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). Fermentation of prebiotics by gut microbiota results in production of SCFA and a shift in the composition colonic microbiota that is associated with improved over health.

In the present study, FAXO were shown to be fermented by gut microbiota to produce SCFA. The fermentation patterns of FAXO were characterized by slower rates at time point 4 hr and 8 hr and faster rates at later time points compared to FOS. Rumpagaporn et al. (2015) also reported similar findings in fermentation patterns of cereal arabinoxylans isolated from wheat, corn, sorghum, and rice compared FOS (a positive control). As described in their study, fecal samples were collected from 3 healthy subjects and pooled. An amount of 50 mg of each substrate and 1 mL of pooled fecal slurry were used for the experiment. Arabinoxylans from different bran sources including corn and sorghum and hydrolyzed arabinoxylan products including corn, wheat, and rice were used in their study. In their study, total SCFA production in hydrolyzed rice bran arabinoxylan treatment were shown to have significant higher levels compared to FOS at time point 24 hr. In the present study, although total SCFA of FAXO was higher than that of FOS, no significant differences were found at time point 24 hr when comparing FAXO and FOS.

Acetate was the major SCFA produced during fermentation of FAXO. Similar findings were also reported in wheat arabinoxylans (Hughes et al. 2007), arabinoxylan oligosaccharides from brewery spent grain (Kabel et al. 2002), and hydrolyzed rice arabinoxylans (Rumpagaporn

et al. 2015). Acetate production of FAXO and FOS also showed similar patterns as in total SCFA production. In colonic fermentation, acetate is considered the primary SCFA and is often used to monitor colonic events. In the colon, unlike propionate and butyrate, acetate is less metabolized and is readily absorbed. The presence of acetate also decreases colonic pH, which results in increased bio-availability of calcium and magnesium and inhibition of pathogenic bacteria (Teitelbaum and Walker 2002, Wong et al. 2006). Also, acetate can also be converted to butyrate. Two mechanisms that have been reported for the production of butyrate in the colon are acetate utilization and lactate fermentation (Diez-Gonzalez et al. 1999).

The propionate production of FAXO was also comparable to FOS in this study. Other studies have shown that arabinoxylans produced relatively high propionate (Rose et al. 2010, Rumpagaporn et al. 2015). Rumpagaporn et al. (2015) also reported that propionate concentration in rice arabinoxylans was significantly higher compared to FOS at time point 24 hr. In this study, propionate production of FAXO at 24 hr tended to be higher compared to FOS; however, the differences were not significant. Upon produced by gut bacteria, propionate is absorbed into bloodstream and transported to liver (Wong et al. 2006). Propionic acid production has been shown to have beneficial health effects including lowering glucose-induced insulin secretion in isolated pancreatic islet cells of rats (Ximenes et al. 2007) and anti-proliferative effects on liver cells (Bindels et al. 2012).

Among all SCFA, butyrate has been of greatest interest due to its protective effects of colonocytes against cancer. FOS has been known for the ability to increase butyrate production, hence the butyrogenic effects (Campbell et al. 1997, Djouzi and Andrieux 1997, Rycroft et al. 2001). Fermentation of arabinoxylans was shown to generate lesser butyrate compared to FOS in a study by Rumpagaporn et al. (2015). However, in the present study, no significant differences

were found between FAXO and FOS. Butyrate has been shown to be the preferred energy source for colonocytes (Della Ragione et al. 2001) and inhibit the growth of colonic carcinoma cells. As discussed above, the production of butyrate also comes from the conversion of acetate. Therefore, the production of each individual SCFA depends on other SCFA and SCFA concentrations in anaerobic fermentation can be changed interdependently.

Beside SCFA production upon fermentation, another criterion that a prebiotic must meet is the ability to selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being. Bifidobacteria and lactobacilli were thought to be the targets of prebiotic effects since they have been the focus of research, especially bifidobacteria as they are more abundant in human gut microbiota than lactobacilli. However, in recent years, many studies have reported the potential health effects of different groups of bacteria other than bifidobacteria and lactobacilli.

FAXO appeared to be a relatively selective substrate as demonstrated in several studies. Pure cultures of different *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* species were shown to efficiently utilize FAXO (Yamada et al. 1993, Van Laere et al. 2000, Moura et al. 2007). Another study conducted by Vardakou et al. (2007) found that *in vitro* fermentation of arabioxylan oligosaccharides significantly raised *Bifidobacterium* sp. and reduced *Bacteroides* levels. In the present study, FAXO did not appear to stimulate the growth of *Bifidobacterium* and *Lactobacillus* genus. However, when assessing populations of other bacteria genera, FAXO seemed to confer certain positive health effects. Specifically, FAXO increased *Bacteroides* and *Prevotella* abundance while reduced *Dorea* abundance.

Bacteroides is one of the most abundant genera in the gut microbiota. Aside from its correlation with increased propionate production, *Bacteroides* has also been shown to have

protective effects against the invasion of exogenous bacteria in the colon by producing antagonistic substances including bacteriocins (Nakano et al. 2006). This bacterial characteristic might play an important role in establishing and maintaining the intestinal ecosystem.

It was also interesting to see an increase in *Prevotella* sp. with FAXO. This genera is often associated with people with diets high in carbohydrates and fiber (Wu et al. 2011). However, the variation between subjects in FAXO is quite large and comparing to FOS, FAXO did not differ significantly (Figure 4D). A study conducted by De Filippo et al. (2010) revealed the differences in changes in gut microbiota of European and African children when solid food was introduced. European children's diet was rich in fat and low in fiber while African children's diet was rich in fiber and low in fat and animal proteins. During breast-milk feeding period, no significant differences in gut microbiota were found between two groups of children. However, when solid food was introduced, differences in bacteria populations were observed. There was a significant enrichment of *Prevotella* genus in gut microbiota of African children compared to that of European counterparts. The differences were explained by the ability to produce cellulases and xylanases of this genus. Therefore, an increase in *Prevotella* with FAXO was expected.

Compared to other genera, *Dorea* was far less studied. However, correlation between *Dorea* population and disease has been demonstrated. Specifically, it has been shown that irritable bowel syndrome (IBS) was characterized by an increase in *Dorea* population and a decrease in *Bifidobacterium* and *Faecalibacterium* (Rajilić-Stojanović et al. 2011; Saulnier et al. 2011). These findings suggest that there might be a link between gut microbiota and IBS which could potentially be used for therapeutic treatments.

Another genus that has gained attention in recent years is *Akkermansia*. Isolated in 2004, *Akkermansia* has been known for its mucus-degrading abilities (Derrien et al. 2004). As a result of mucus degradation, *Akkermansia* sp. also have the ability to produce SCFA (Derrien et al. 2004). Since *Akkermansia* sp. are often localized within the mucus layer close to the epithelial cells, these SCFA are more readily available to the host. Furthermore, recent studies also found that the abundance of *Akkermansia* sp. might be correlated with gut health. Specifically, *A. muciphila* was shown to be significantly more abundant in healthy controls compared to patients with IBD (Png et al. 2010). Other studies found that amount of *Akkermansia* sp. was inversely proportional to the severity of appendicitis and obesity (Zhang et al. 2009; Swidsinski et al. 2011). These findings suggest that *Akkermansia* could be associated with anti-inflammatory and protective activities. *Akkermansia* has also been shown to be promoted by the administration of prebiotics in animal models (Everard et al. 2011; van den Abbeele et al. 2011). However, in the present study, no significant differences in *Akkermansia* were found between treatments. This could be due to the limitations of *in vitro* models which might not be ideal for this particular genera.

As discussed above, some bacteria are also capable of converting acetate to butyrate, namely *Coprococcus*, *Roseburia*, *R. intestinalis*, and *Faecalibacterium prausnitzii* (Duncan et al. 2002). It is interesting that while both FAXO and RBPP did not appear to modulate *Coprococcus* and *Roseburia* abundance, MIX treatment showed significant increases in both genera, especially in *Roseburia* where FOS also did not affect its population. These results suggested that there might be synergistic effects between FAXO and RBPP in modulating gut microbiota. Although these butyrogenic bacteria were increased in abundance, no significant

differences were observed in neither acetate production nor butyrate production between FAXO and MIX.

The study was conducted using an *in vitro* anaerobic fermentation model which is commonly used for first assessment of impacts of various compounds on metabolic activities of gut microbiota. However, this method certainly has its limitations. First of all, since it is a closed system, metabolites produced are limited by amounts of substrates used. Second, the accumulation of end-products during fermentation period could alter the conditions of fermentation environment and affect the formation of certain metabolites. Third, *in vitro* methods do not fully replicate *in vivo* intestinal conditions which affects the *in vivo* relevance of the study. Moreover, due to limitations of substrates, only 10 subjects were recruited for the study. The small sample size could have also reduced the statistical power and undermined the effects of treatments.

The present study investigated the fermentability of FAXO and RBPP and the changes in gut microbiota during fermentation with these components. The results demonstrated that FAXO had different fermentation patterns compared to FOS. FAXO were also found to be able to modulate several bacteria populations which could contribute to overall host health.

5. Conclusion

Production of SCFA from FAXO extracted from rice bran by gut microbiota was comparable with that from FOS, although the fermentation pattern of FAXO were generally steadier compared to that of FOS. Furthermore, FAXO also showed to have modulating effects on microbiota profiles. Synergistic effects of FAXO and RBPP in modulating certain butyrate-producing bacteria were also observed. This study warrants further investigation to confirm the prebiotic-like properties of these rice bran components.

6. Acknowledgments

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Table 1. Concentration of phenolics in red rice RBPP (Chen et al. 2012).

Phenolic subgroup	Concentration (mg/g extract)	% total phenolics
Benzoic acid	2.466 ± 0.045	5.1%
Cinnamic acid	20.115 ± 0.022	41.9%
Flavonoid	1.955 ± 0.115	4.1%
Proanthocyanidin	23.489 ± 1.904	48.9%
Total phenolics	48.025 ± 2.042	100%

Values are expressed as mean ± SD.

Table 2. Subject Characteristics

Measurements	All (n=10)	Male (n=5)	Female (n=5)
Age	25.8 ± 4.7	26.0 ± 5.1	25.6 ± 4.2
Height (cm)	170.9 ± 5.8	173.6 ± 4.2	168.2 ± 5.9
Weight (kg)	66.6 ± 7.3	69.8 ± 7.5	63.5 ± 5.8
BMI (kg/m ²)	22.8 ± 2.0	23.1 ± 1.8	22.5 ± 2.3

Values are expressed as mean ± SD.

Table 3. Total production of SCFA during in vitro fermentation with human fecal samples.

Time point (hr)	Total SCFA (mM)			
	FOS	FAXO	RBPP	MIX
0	1.5 ± 0.3 ^a	1.5 ± 0.3 ^a	0.6 ± 0.2 ^a	1.3 ± 0.3 ^a
4	4.0 ± 0.8 ^a	2.4 ± 0.7 ^b	0.3 ± 0.1 ^c	2.9 ± 1 ^{ab}
8	10.2 ± 1.1 ^a	6.7 ± 1.8 ^b	0.0 ± 0.2 ^c	4.9 ± 1.7 ^b
12	9.9 ± 1.1 ^a	10.5 ± 2.7 ^a	0.1 ± 0.2 ^b	9.3 ± 3.1 ^a
24	9.3 ± 1.1 ^a	14.0 ± 3.6 ^a	0.8 ± 0.7 ^b	11.7 ± 3.2 ^a

Values are expressed as mean ± SEM. Treatments with different superscripts within the same row are significantly different ($P < 0.05$). FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenolics, MIX: mixture of FAXO and RBPP.

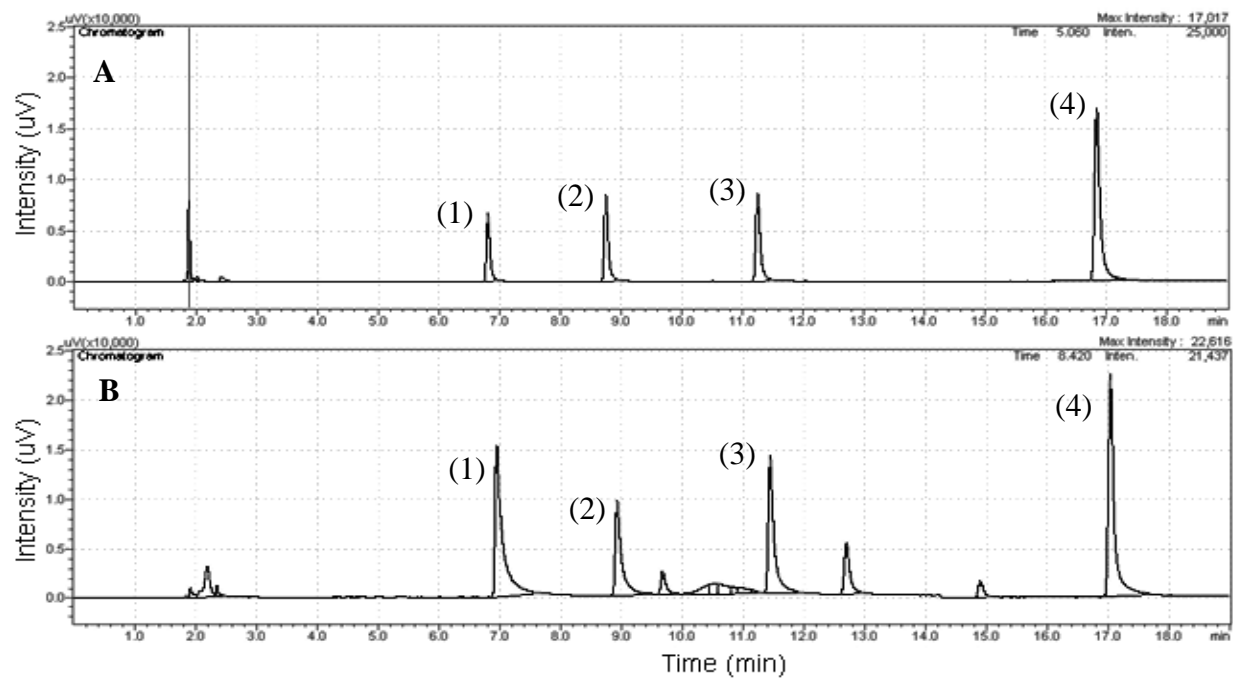


Figure 1. GC chromatogram of (A) SCFA standards and (B) FAXO fermentation sample at time point 24 hr. (1) Acetate; (2) Propionate; (3) Butyrate; (4) Valerate as internal standard.

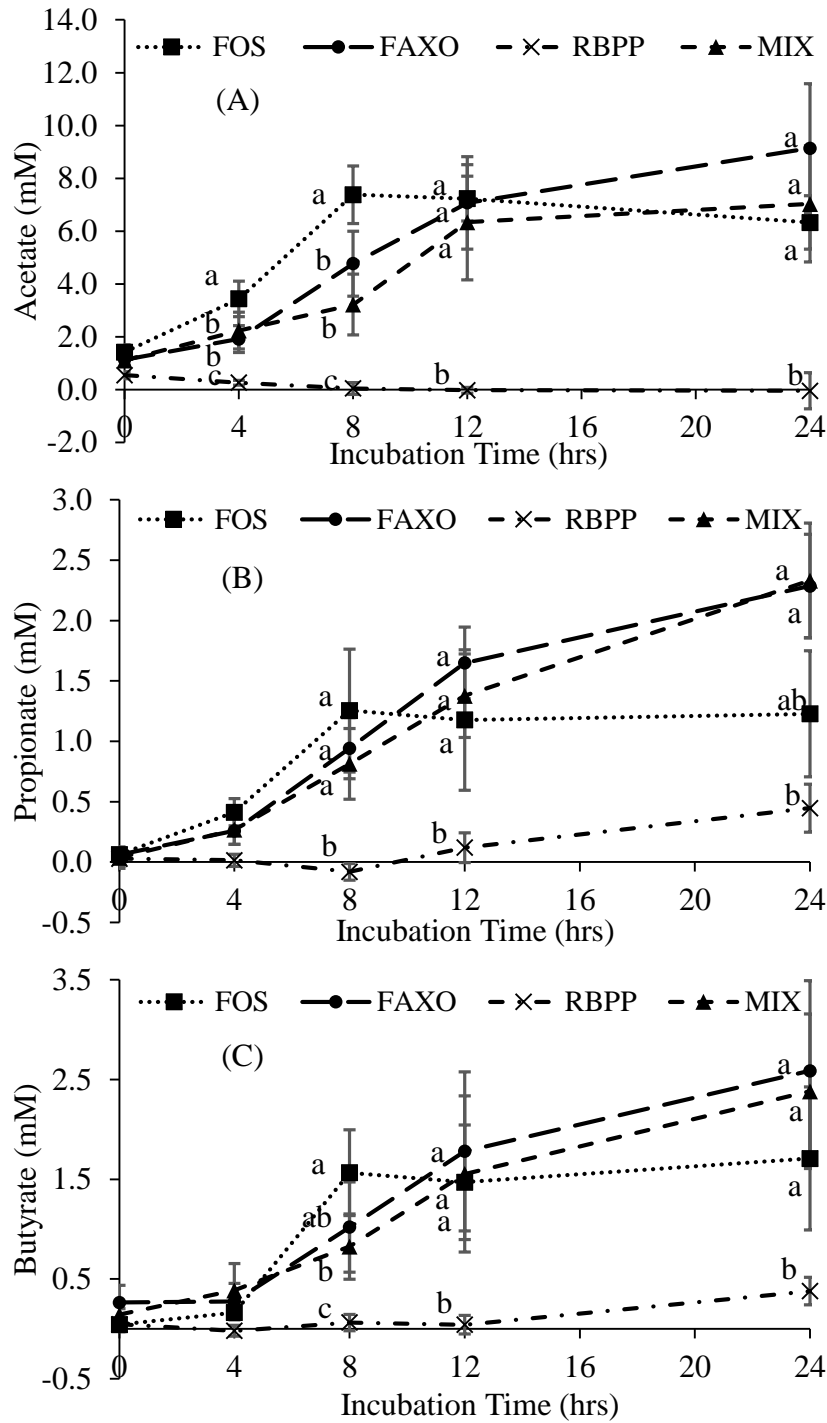


Figure 2. Individual SCFA production during *in vitro* fermentation. (A) Acetate production; (B) Propionate production; (C) Butyrate production; FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenolics, MIX: mixture of FAXO and RBPP. Different letters at the same incubation time denote significant difference ($P < 0.05$)

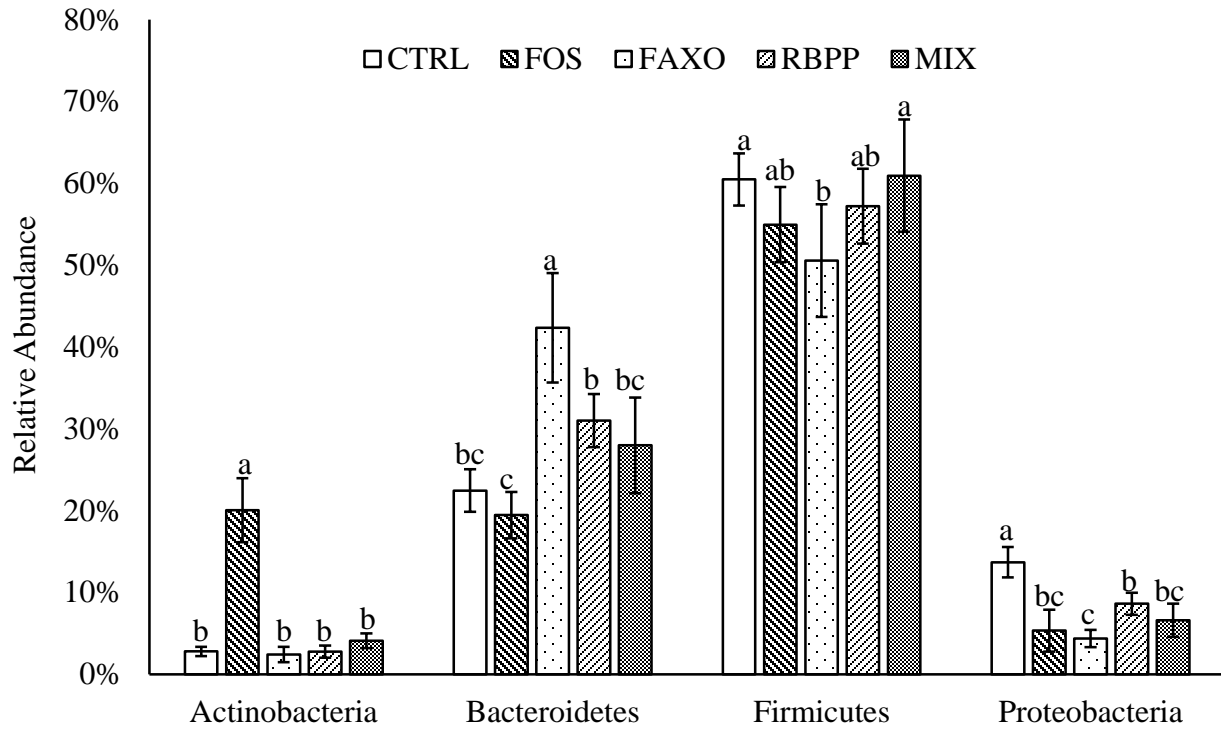


Figure 3. Microbiota composition at phylum level after 24-hr incubation with different substrates. CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenolics, MIX: mixture of FAXO and RBPP. Different letters in the same phylum denote significant difference ($P < 0.05$).

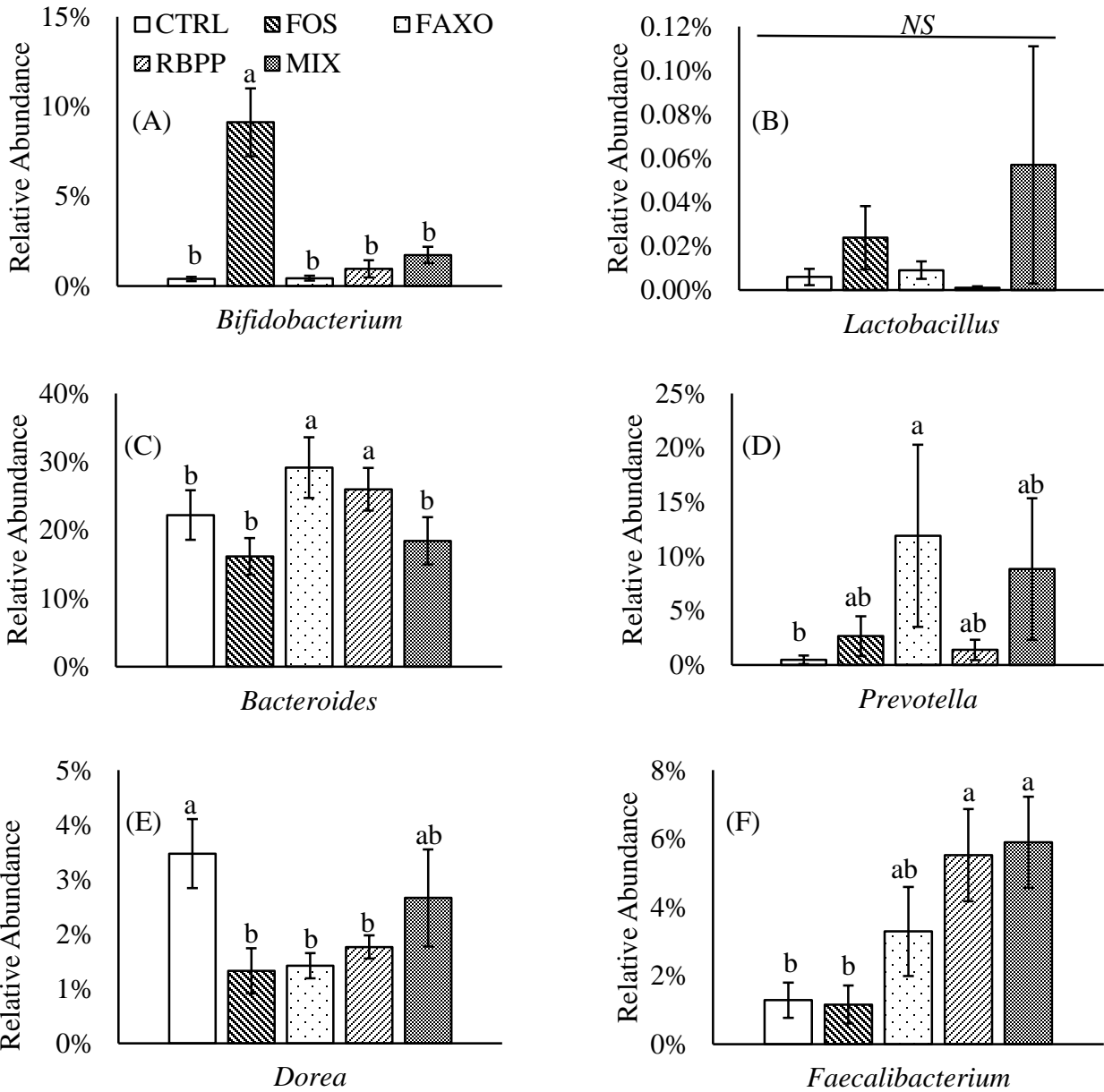


Figure 4. Changes in abundance of different genera after 24-hr incubation with different substrates. CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenolics, MIX: mixture of FAXO and RBPP. Different letters denote significant difference ($P < 0.05$).

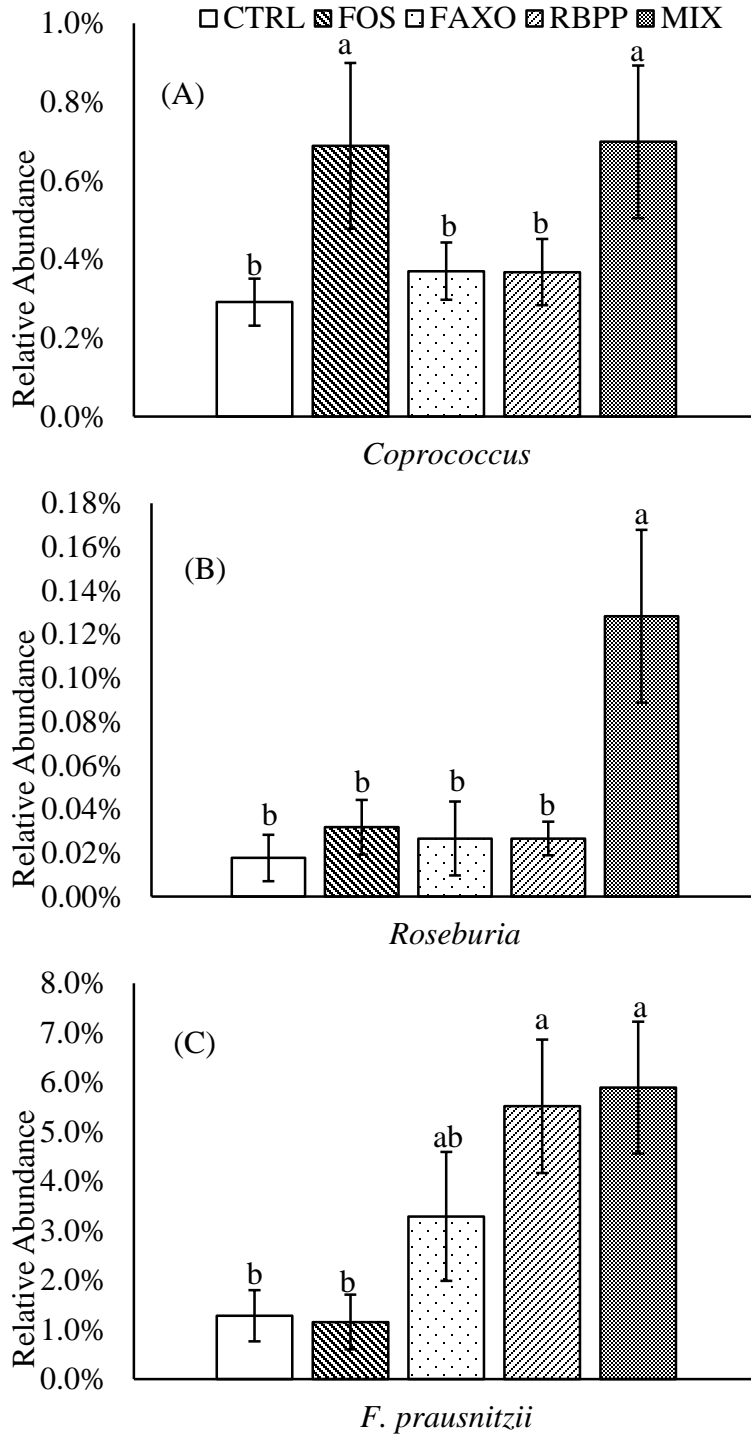


Figure 5. Changes in abundance of different butyrogenic bacteria after 24-hr incubation with different substrates. CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenolics, MIX: mixture of FAXO and RBPP. Different letters denote significant difference ($P < 0.05$).

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Chapter 3: Overall Conclusion

The present study added to the growing body of evidence on prebiotic-like properties of rice bran components such as FAXO and RBPP. Compared to FOS, FAXO tended to be fermented at slower yet steadier rates. RBPP did not appear to affect the production of SCFA from FAXO. In terms of microbiota composition, both FAXO and RBPP had significant impacts on bacteria abundance to a certain degree. At phylum level, FAXO appeared to significantly increase Bacteroidetes and increase Firmicutes abundance, which are two major phyla in gut microbiota. Although FAXO and RBPP did not seem to affect bifidobacteria and lactobacilli population, they appeared to be able to modulate bacteria populations of other genera. FAXO significantly increase *Bacteroidetes* and *Prevotella* population and decrease *Dorea* population. RBPP was found to significantly increase *Faecalibacterium* abundance. Synergistic effects of FAXO and RBPP were observed in butyrogenic bacteria population *Coprococcus* and *Roseburia* by significant increases compared to FAXO and RBPP. Results from this study reaffirm that FAXO had potentials to be prebiotics. However, further investigation is needed to clarify the prebiotic functions of these rice bran components.

Appendix A: IRB Approval Letter



Office of Research Compliance
Institutional Review Board

September 8, 2014

MEMORANDUM

TO: Sun-Ok Lee
Tung Pham
Xuan Gu
Ellen Pottgen

FROM: Ro Windwalker
IRB Coordinator

RE: PROJECT CONTINUATION

IRB Protocol #: 13-09-080

Protocol Title: *Establishing the Function and Availability of Bioactive Components from Whole-Grain Rice Varieties for Colon-Specific Health Benefits*

Review Type: EXEMPT EXPEDITED FULL IRB

Previous Approval Period: Start Date: 09/24/2013 Expiration Date: 09/19/2014

New Expiration Date: 09/19/2015

Your request to extend the referenced protocol has been approved by the IRB. If at the end of this period you wish to continue the project, you must submit a request using the form *Continuing Review for IRB Approved Projects*, prior to the expiration date. Failure to obtain approval for a continuation on or prior to this new expiration date will result in termination of the protocol and you will be required to submit a new protocol to the IRB before continuing the project. Data collected past the protocol expiration date may need to be eliminated from the dataset should you wish to publish. Only data collected under a currently approved protocol can be certified by the IRB for any purpose.

This protocol has been approved for 80 total 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 210 Administration Building, 5-2208, or irb@uark.edu.

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