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Influence of Native and Processed Cereal Grain Fibers on Gut Health

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by

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INFLUENCE OF NATIVE AND PROCESSED CEREAL GRAIN FIBERS ON GUT HEALTH

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Cereal fibers that can be metabolized by gut microbiota have been shown to promote the growth of beneficial bacteria in the gut. Increased consumption of cereal fibers may improve host / gut microbiota interactions in obesity and other metabolic diseases by normalizing gut dysbiosis. The present dissertation describes four research projects to assess the impact of cereal dietary fibers on gut microbiota and host metabolism. In the first study, we determined the treatment temperatures for production of soluble, non-digestible, feruloylated oligo- and polysaccharides (FOPS) from maize bran and wheat bran, and determined the fermentation properties of partially purified FOPS from maize bran and wheat bran. *In vitro* fermentation revealed that wheat FOPS were more bifidogenic than maize FOPS. However, maize FOPS led to continual production of short-chain fatty acids (SCFA), resulting in the highest SCFA and butyrate production at the end of the fermentation. In addition, maize FOPS showed significantly higher antioxidant activity than wheat FOPS. The study showed FOPS from maize bran may exhibit enhanced benefits on gut health compared to those of wheat bran. In the second study, we further determined whether the colonic fermentation of FOPS could counteract the deleterious metabolic effects of a high-fat (HF) diet through modulating the gut microbiota using a mouse model. Our results suggest that colonic fermentation of FOPS plays an important role in preventing metabolic disorders in HF-fed mice, and that these metabolic improvements depend on specific alterations of the gut microbiota through FOPS fermentation. *Blautia* and *Akkermansia* might be considered potential therapeutic targets for improving body and adipose tissue weights, while SCFA production seems linked to improvements in glucose metabolism. In the third study, by obtaining long-term dietary records from fecal donors, we aimed to determine the correlations between dietary intake variables and dietary fiber degradation and short-/branched-chain fatty acid (BCFA) and ammonia production during *in vitro* fecal fermentation. We found that butyrate production was correlated with fecal donor intake of many nutrients, of which principal component analysis revealed were mostly

contributed by grain-, nut-, and vegetable-based foods. Negative correlations were found for propionate with intake of total carbohydrate, added sugar, and sucrose and for ammonia and BCFA production with intake of unsaturated fats. These results suggest that diets high in plant-based foods and high in unsaturated fats are associated with microbial metabolism that is consistent with host health. In the fourth study, we determined the impacts of long-term dietary pattern on gut microbiota composition and the change in composition of the gut microbiota during fermentation of predigested whole wheat flour. Butyrate production was significantly correlated with the abundance of *Butyricicoccus*, *Coprococcus*, *Dorea*, *Faecalibacterium*, and *Lachnospiracea incertae sedis*. BCFA and ammonia production displayed negative correlations with the abundance of *Roseburia* and *Parasutterella*. *Bifidobacterium* and *Butyricicoccus* were enhanced by pre-digested whole wheat flour. Taken together, these results provide new evidence for modulating the gut microbiota through dietary treatment and indicate its contribution to host metabolism.

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Preface

This dissertation is organized as follows: a literature review (Chapter 1) followed by manuscripts describing four research projects (Chapters 2, 3, 4, and 5). Chapter 1 provides a review of the current literature on the impacts of native and processed cereal grain fibers on the gut microbiota. This chapter has been formatted using the guidelines for *Journal of Food Science*; Chapter 2 describes our study on production and *in vitro* evaluation of soluble, non-digestible, feruloylated oligo- and polysaccharides (FOPS) from maize bran and wheat bran. This chapter has been formatted for *Journal of Agriculture and Food Chemistry*; Chapter 3 describes the results on the effect of maize-derived FOPS on the metabolic disorders and gut microbiota using an *in vivo* mouse model. This chapter has been formatted for *Molecular Nutrition & Food Research;* Chapter 4 describes results on the correlations between dietary intake variables and dietary fiber degradation and short-/branched-chain fatty acid (BCFA) and ammonia production during *in vitro* fecal fermentation. Chapter 5 describes results on the impacts of long-term dietary pattern on gut microbiota composition and the change in composition of the gut microbiota during fermentation of predigested whole wheat flour. Chapters 4 and 5 have been formatted for *Nutrition Research*. It is noted that materials presented in Chapters 2 and 4 have already been published.

Hypotheses:

Chapter 2: Because the parent polymer of FOPS, maize heteroxylan, ferments more slowly with higher production of SCFA than other arabinoxylans, we hypothesized that FOPS from maize would be more bifidogenic and support bacterial fermentation the longest and produce the highest SCFA compared with FOPS from wheat, thus possibly contributing to a colonic environment that is less susceptible to disease.

Chapter 3: We hypothesized that unique structural diversity coupled with the presence of antioxidants in FOPS would aid in the improvement of insulin response and lipid profile, alleviating the inflammation syndromes, sustaining prolonged bacterial fermentation, and altering the gut microbiotia to a healthier state.

Chapter 4: Because diet influences gut microbiota, we hypothesized that diet would impact the extent of dietary fiber utilization and the types of metabolic end-products produced by the microbiota during *in vitro* fecal fermentation.

Chapter 5: We hypothesized that long-term dietary pattern and the fermentation of whole grain wheat would influence the gut microbiota composition.

Objectives:

Chapter 2: To determine treatment temperatures for production of FOPS from maize bran and wheat bran and determine the fermentation properties of partially purified FOPS from these substrates.

Chapter 3: To determine if consumption of maize-derived FOPS could counteract the deleterious effects of high-fat (HF) feeding and modulate the gut microbiota in mice.

Chapter 4: To determine the correlations between dietary intake variables and dietary fiber degradation and short-/branched-chain fatty acid (BCFA) and ammonia production during *in vitro* fecal fermentation.

Chapter 5: To determine the influence of long-term dietary pattern and the fermentation of whole grain wheat on the gut microbiota composition.

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Chapter 1 . Modulating the gut microbiota using native and processed cereal grain fibers

1. Abstract

The human gut is colonized by trillions of microorganisms, which form a complex microbial system. This super organ has important effects on human physiology, such as metabolizing food and xenobiotic compounds, contributing to the pathogenesis of metabolic diseases, and regulating colonic resistance against pathogenic bacteria. Dietary interventions with cereal-based foods have been proposed to modulate the gut microbiota. Considering the unique chemical and physiological properties of cereal fibers and their attached antioxidants, these compounds may serve as important candidates for dietary intervention to elicit beneficial effects by altering the gut microbiota to a healthier state. However, the rigid structure and poor colonic fermentability of these non-digestible components in cereal grains may limit the utilization of these fibers by the gut microbiota. A variety of novel processing techniques, including enzymatic treatment, hydrothermal treatment, extrusion, chemical extraction, supercritical CO_2 treatment, and ozonolysis treatment, may be considered to increase the utilization of cereal fibers.

2. Gut microbiota

The human gut harbors a vast array of microorganisms that form a complex microbial system. The commensal bacteria of the human microbiota typically include five microbial phyla: *Firmicutes*, *Bacteriodetes, Antinobacteria,* and *Proteobacteria*. *Firmicutes* and *Bacteroidetes* typically representing over 90% of the total gut microbiota for most individuals (Prakash and others 2011). Most of the gut microbiota are non-culturable, but this notion has recently challenged by Goodman et al., (Goodman and others 2011) who reported that 99% of the bacteria that were characterized in the phylum, class, and order levels could be detected in a cultured fecal sample.

The colonization of the gut mcirobiota starts at birth, and the microbial composition changes through different stages of life. The infant acquires a gut microbiota from his or her mother and the environment. The most commonly isolated microorganisms from newborn feces are *Staphylococcus* spp., *Enterobacteriaceae,* and *Streptococcus* spp (Di Gioia and others 2014), but the infant gut microbiota has a relatively simple structure and is unstable over time. Even different feeding strategies may affect the gut microbiota significantly. For instance, breast-fed infants have a less complex gut microbiota that is

dominated by *Bacteroides* spp. and *Bifidobacteria* (over 90% of the total) compared with formula-fed infants (Di Gioia and others 2014). Ecological and evolutionary forces lead to a more stable microbiota in adults by shaping the microbial diversity. Later, a set of age-related shifts in the composition and function of the gut microbiota may occur, for instance, a significant decrease in *Bifidobacteria* and a decline in immune system responses, occur after the age of 60 years (Nicholson and others 2012).

In addition to the mother's impact at birth, other factors have been suggested to influence the gut microbiota. First, the host genetic background may contribute to the individuality of the microbiota. Studies performed using mouse models indicated the linkage of quantitative trait loci (QTL) with specific microbiota composition, confirming the importance of genetic control in shaping the diversity of the microbiota (Benson and others 2010). Second, environmental factors, such as diet and exercise, have also been proposed as a driving force for the gut microbiota (Petriz and others 2014; Clarke and others 2014).

The gut microbiota has a huge metabolic capacity and can complement functions that have not developed within the human body (Round and Mazmanian 2009), making the microbiota a "super organ" that is responsible for the digestion of complex food/drug components, metabolism and energy harvesting, colonization resistance and other functions (Round and Mazmanian 2009; O'Keefe 2008).

3. The role of the gut microbiota in human physiology

3.1. Gut microbiota and food/xenobiotic metabolism

The gut microbiota can either produce harmful metabolites associated with human diseases or beneficial components that protect against diseases, depending on dietary intake (O' Keefe and others 2015). For instance, the toxic effect of melamine in infant milk is mediated by the biotransformation of this compound by certain gut microbiota (Zheng and others 2013). Moreover, the gut microbiota can metabolize dietary L-carnitine from red meat to trimethylamine-N-oxide, which is a proatherogenic compound (Koeth and others 2013), while the production of short chain fatty acids (SCFA) from carbohydrate fermentation, vitamins and conjugated linoleic acid may have beneficial health effects. Although we still have a limited understanding of the metabolism of different compounds by the gut microbiota, carbohydrate and polyphenol metabolism have been well studied.

Fruit, vegetables, and cereals are the major dietary components that provide dietary fibers for the microbiota. Thanks to at least 81 different glycoside hydrolase families, these fibers can be metabolized to sugars by the gut microbiota; these sugars are fermented to SCFA, including acetate, propionate, and butyrate. These SCFA can trigger the secretion of the hormone GLP-1, resulting in increased of insulin secretion and decreased feeling of hunger (Tolhurst and others 2012). Specifically, butyrate, which is a preferred energy source of gut epithelial cells, has been shown to promote energy expenditure and improve insulin sensitivity in a mouse model (Gao and others 2009). Saccharolytic fermentation is important because it may create a beneficial gut environment, in contrast, when carbohydrates are in short supply, the gut microbiota will switch to the fermentation of amino acids, resulting in the release of harmful metabolites including branched chain fatty acids (BCFA) and ammonia (Van Nuenen and others 2004).

In addition to the complex carbohydrates, polyphenols are other non-digestible components in our daily diet than can be metabolized to a large extent by the gut microbiota, yielding products such as hydroxyphenylacetic, phenylpropionic, and phenylbutyric acids, which have potential health benefits (Saura-Calixto 2010). For instance, the 3, 4-dihydroxyphenylacetic and 4-hydroxyphenylacetic acid metabolites have shown higher inhibition of platelet aggregation than their precursors (rutin or quercetin) (Kim and others 1998). The hydroxyphenylacetic and phenylpropionic derivatives from the microbial degradation of flavonoids have been linked to inhibited endothelial dysfunction (Schewe and others 2008).

3.2. Gut microbiota and metabolic diseases

Metabolic syndrome (including insulin resistance, hyperglycemia, hypertension, and dyslipidemia) is a cluster of obesity-related disorders that affects 35% of adult Americans (Jialal and others 2014). The increasing metabolic syndrome epidemic results from an energy imbalance that involves the consumption of more calories than expended and is characterized by increased risk of type-2 diabetes and other metabolic diseases. Recent studies have suggested the existence of a strong association between the gut microbiota and metabolic syndrome (D'Aversa and others 2013; Festi and others 2014).

3.2.1. Gut microbiota and obesity

The first study to link obesity with the gut microbiota profile was conducted in genetically obese mice and reported that obesity is associated with different microbiota at the phylum level, with an increased

prevalence of *Firmicutes* and a decreased prevalence of *Bacteroidetes* in obese mice in comparison to their lean counterparts (Turnbaugh and others 2008). Consistent with the mouse data, the first human study also showed a higher proportion of *Bacteroidetes* in normal weight individuals than in obese individuals (Ley and others 2006). However, some subsequent studies have reported differing results. For instance, Collado et al. reported a significantly higher level of *Bacteroidetes* in overweight women than in normal-weight individuals (Collado and others 2008). The failure of the Human Microbiome Project to find an association between the *Firmicutes*/*Bacteroidetes* ratio and obesity also raise the questions about whether this ratio should be associated with obesity (Moran and Shanahan 2014).

Compositional changes in the gut microbiota in response to weight loss have been reported, but the results are also inconsistent. For instance, Schwiertz et al. reported a decrease in *Bacteroidetes* after weight loss (Schwiertz and others 2010), while Duncan et al. reported no change in *Bacteroidetes* after a 4-week carbohydrate-reduced diet (Duncan and others 2008). These mixed results may be explained by the complexity of the gut microbiota and the existence of large individual differences.

Rather than compositional differences at the phylum level, species-level bacterial differences are more likely to be responsible for the association between diseases, such as obesity, and the gut microbiota, because the function of gut microbiota diverges at the species level (Tuohy and Del Rio 2014). For instance, the consumption of *Bifidobacterium longum* decreased the expression of genes that encode inflammatory cytokines, but the ingestion of *Bifidobacterium. animalis subsp. lactis* elicited a different effect, resulting in an increase in anti-inflammatory cytokines (Furrie 2006; Arunachalam and others 2000). Moreover, *Akkermansia muciniphila*, which is a mucin-degrading bacterial species, has been inversely correlated with body weight in rodents and humans (Everard and others 2013). A previous study also demonstrated the specific function of *A. muciniphila*, including the reversal of high fat-induced weight gain, adipose inflammation, and insulin resistance (Everard and others 2013).

Although recent studies have suggested the core microbiota composition is associated with different health or disease states, such as obesity, the problem of whether an altered gut microbiota causes the obese state or the obese state results in an altered gut microbiota is of interest. The applicability of the results to humans is in debate because the physiology and metabolic pathways of these animals are quite different from those of animals with conventional gut flora. An emerging view suggests that the gut

microbiota is characterized by a core metabolic function rather than by certain types of bacteria; thus, even distantly related bacteria share similar key metabolic functions and these key metabolic functions are directly related to health or disease (Tuohy and Scott 2015). For instance, the gut microbiota may impact the genesis of obesity through metabolic products or between microbiota-host signaling.

Potential mechanisms that link the gut microbiota with obesity have been proposed. First, through the digestion of complex dietary carbohydrates, the gut microbiota can produce SCFA, including acetate, propionate, and butyrate. These organic acids not only represent an energy source, but also trigger cell-specific signaling cascades, regulating energy harvest. For instance, G-protein coupled receptors (GPCRs), GPR41 and GPR43 have been shown to be activated by SCFA, eliciting cascade reactions. The activation of GPR 41 can enhance the secretion of peptide YY (PYY) from intestinal L cells, inhibiting gastric emptying and food intake, whereas the activation of GPR43 is reported to inhibit fat accumulation in the adipose tissue and trigger the secretion of GLP-1, which is a peptide that inhibits gastric emptying and food intake (Everard and Cani 2014; Kimura and others 2014). Second, the gut microbiota can regulate host metabolism by regulating bile acids. Bile acids reach the colon, where they can affect the composition of the gut microbiota and are also modified by the gut microbiota (Joyce and others 2014). The primary function of bile acids is to emulsify dietary lipids and fat-soluble vitamins, but these acids can also act as signaling molecules for two different receptors in the host: cellular farnesoid X receptor (FXR), which controls the transcription of genes that affect glucose and lipid metabolism, and TGR5, which increases GLP-1 secretion (D'Aversa and others 2013). Another study suggested that the bile salt hydrolase activity (BSH) of gut microbiota plays an important role in energy homostasis. Significant reductions in weight gain, serum cholesterol, and liver triglycerides were observed in mice with elevated BSH activity (Joyce and others 2014). Third, by acting on the mucus thickness or tight junction proteins, the gut microbiota may regulate the gut permeability, resulting in the genesis of low-grade inflammation that is characteristic of obesity and many obesity-associated disorders (Cani and others 2009). Cani et al. has suggested that lipopolysaccharide (LPS), which is a constituent of gram-negative gut bacteria, may act as a trigger for this inflammation, a condition known as metabolic endotoxemia (Cani and others 2009). The altered gut microbiota associated with increased epithelia permeability resulted in the easy translocation of LPS to the blood and increased plasma levels of LPS. The LPS may subsequently be transported by a mechanism

involving lipoproteins and trigger the secretion of proinflammatory cytokines (e.g., TNF-a, IL-1, and IL-6) after binding to the CD14 and TLR4 receptors on innate immune cells (D'Aversa and others 2013). The proposed mechanism by which LPS causes inflammation and obesity was established using a mouse model. For instance, LPS treated mice developed symptoms, such as increased body, liver, and adipose tissue weight gain to a similar extent as high fat-fed mice (Cani and others 2007).

3.2.2. Gut microbiota and type-2 diabetes

The rapid increase in the prevalence of type-2 diabetes in the recent decades makes this disease a worldwide health concern. Approximately 20-25% of the world's population has the problem of metabolic syndrome, including obesity, insulin resistance, hyperglycemia, and hyperlipidemia. This population is also at high risk for type-2 diabetes (Alberti and others 2006). The gut microbiota plays a vital role in these pre-diabetes metabolic syndromes, such as obesity and insulin resistance. Cani et al. established the association between the gut microbiota, inflammation, and pre-diabetes metabolic syndromes and suggested the bacterial LPS/CD14 system may regulate insulin sensitivity. CD14 has been identified as a pattern recognition receptor (PRR) that binds LPS. In a four-week high fat diet, wild-type mice had increased LPS accompanied by alternations in gut microbiota composition and insulin resistance (Cani and others 2007). In contrast, when CD14 knockout mice were subjected to the same high-fat diet, the genetically modified mice gained less weight and exhibited increased insulin sensitivity and reduced systemic inflammation compared with the wild-type mice. In contrast, the reinfusion of bacterial LPS to the CD14 knockout mice abolished all of these metabolic improvements (Cani and others 2007). In another experiment, obese mice treated with an antibiotic that altered the gut microbiota and decreased endotoxemia exhibited improvements in insulin resistance, fasting glycemia, and inflammatory parameters compared with control ob/ob mice (Membrez and others 2008; Cani and others 2008). Another study linked increased bacterial translocation with the onset of insulin resistance. Bacterial translocation is used to describe the migration of viable bacteria from the gastrointestinal tract to extraintestinal sites. For instance, increased *E.coli* translocation into the blood contributed to the establishment of diabetic status, whereas a probiotic treatment that decreased translocation could reverse the diabetic status (Amar and others 2011). These results may be associated with gut permeability as discussed in the obesity section, because obesity is one of the most important risk factors for type-2 diabetes.

3.3. Gut microbiota and colonization resistance

Colonic resistance against intestinal pathogens is conferred primarily by the commensal gut microbiota, resulting in the protection of the host intestine. For instance, fecal transplants of healthy gut microbiota to reconstitute the gut microbiota of a patient with *Clostridium difficile* are highly effective in treating this disease (Youngster and others 2014). This phenomenon can be explained from an ecological perspective. The establishment of a healthy gut microbiota allows commensal organisms to occupy specific ecological niches according to specific nutritional states or metabolic functions. Once stability is established, the resilience of the gut system may protect it from infection with pathogenic bacteria. The colonization of commensal bacteria contributes to the homeostasis of the gut ecosystem by regulating the immune system and stimulating the production of antimicrobial factors (Buffie and Pamer 2013). This phenomenon is of interest because with increased antibiotic therapy, antibiotic-resistant bacteria in the gut may proliferate in the intestinal lumen, posing a serious health threat.

4. Impacts of cereal fibers on gut microbiota

Due to the important physiological influence of the gut microbiota and its metabolic products, the gut microbiota is an important target, especially in the management of obesity and related metabolic diseases. Diet is a critical driving force that contributes to the diversity of the human gut microbiota. Numerous studies have focused on the influence of long-term diet on the gut microbiota composition. For instance, the fecal microbiota from people with high fiber consumption is enriched for *Prevotella* and *Xylanibacter* (De Filippo and others 2010). Long-term intake of protein and animal fat resulted in the enrichment in *Bacteroides*, whereas long-term intake of carbohydrates was associated with *Prevotella* (Wu and others 2011). In addition to the long term diet, David et al. suggested that even a short-term drastic dietary change has impacts on the gut microbiota. Ten subjects were recruited and consumed either a plant-based diet or an animal based diet for five days. In response to the plant diet, the gut microbiota became better at breaking down carbohydrates and producing SCFA, while in response to the meat diet, the gut microbiota changed their metabolism by achieving more genes for breaking down harmful chemicals in meat and producing the products of amino acid fermentation (David and others 2013).

Two mechanisms are proposed regarding the impact of diet on the gut microbiota. First, different bacteria vary in the capacity to utilize substrates, which is determined by their genomes. The competition

for different substrates decides the relative abundance of the bacteria (Flint and others 2014). This principle makes it possible to manipulate the gut microbiota using probiotics or prebiotic-related substances. Second, dietary factors may change the gut environment, and different bacteria vary in their tolerance to this environment, such as the pH or mineral concentrations (Flint and others 2014).

Evidence continues to mount concerning the manipulation of the gut microbiota using prebiotics (Delzenne and others 2011). For instance, fructans and galactooligosaccharides have attracted great interest. However, the intake of these oligosaccharides in most diets (1-4 g/d) [17] is small in comparison with the quantities of cereal fiber that are the typical substrates for gut bacteria (11-20 g/d) [18].

Epidemiological studies have associated the intake of whole grains with a reduced risk of many metabolic diseases. Most studies also demonstrated that whole grains can protect against weight gain and obesity. For instance, in three studies that were based on food frequency questionnaires including 3559 participants, whole grain intake was inversely associated with weight gain (McKeown and others 2002; Newby and others 2003). In a study that examined adults in Great Britain from 1986-87 and 2000-2001, an inverse association between whole grain intake and obese population was observed (Thane and others 2007). Other studies have linked whole grain consumption with reduced risk of metabolic diseases. In a meta-analysis of prospective cohort studies, an inverse association between whole grain intake and type 2-diabtes was reported (de Munter and others 2007). In a cross-sectional study of 2941 subjects, increased intake of whole grain was associated with reduced risk factors for metabolic syndrome, such as fasting insulin (McKeown and others 2002).

However, intervention studies that have focused on the physiological effects of consuming a whole grain diet are inconsistent. Giaco et al. examined 60 women and men with metabolic disease who received a whole grain-based diet or a refined-cereal diet and found significant decreases in postprandial insulin and triglycerides in the whole grain group (Giacco and others 2014). A randomized controlled trial reported decreased satiety and improved blood glucose response after the consumption of cereal based food with enriched wheat and pea fiber compared with the refined bread (Gonzalez-Anton and others 2015). Another randomized, controlled, single-blind, cross-over study showed that insulin sensitivity improved after increased insoluble oat fiber intake (31.2g/d) for just 3 days (Weickert and others 2006). However, other studies reported no significant effects of whole grain consumption. For instance, Andersson et al.

found no difference between obese subjects who consumed a whole grain-based diet and obese subjects who consumed a refined wheat diet with respect to insulin sensitivity or markers of inflammation (Andersson and others 2007). In another study of 102 healthy individuals, no differences in plasma Low-density lipoprotein, cholesterol, and glucose were observed between subjects on a refined wheat diet and subjects on an oat fiber-rich diet (Chen and others 2004).

Attention has been focused on the physiological effects of the cereal polysaccharide moiety (i.e., prebiotic effects) and the antioxidant compounds associated with the polysaccharides.

Cereal polysaccharide moiety has heterogeneous chemical structures and is classified as soluble DF (SDF) and insoluble DF (IDF) based on its water solubility. Typically, the IDF is the predominant form in maize and wheat, while oats has a considerable amount of SDF. The beneficial effects of cereal dietary fiber have been well researched. For instance, administration of barley β-glucan has been shown to induce a strong bifidogenic effects (Mitsou and others 2010). Supplementation of water-extractable wheat arabinoxylan in the diet has been report to exert prebiotic effect and reduce obesity (Neyrinck and others 2011).

The main phenolic compounds in cereal grains are hydroxycinnamic acids, including ferulic acid, diferulic acid, sinapic acid, p-coumaric acid, and caffeic acid (Vitaglione and others 2008). Over 95% of these compounds are cross-linked with the cell walls, forming dietary fiber-phenolic compounds complexes. For instance, ferulic acids substitute the α-l-arabinofuranosyl moieties of arabinoxylan at C(O)-2 and/or C(O)-3 and are involved in oxidative cross-linkages with other arabinoxylan chains and cell wall components (Izydorczyk and Biliaderis 1995). Free phenolic acids have been shown to exhibit strong antioxidant activity and are associated with a broad range of health benefits, including anti-inflammatory, anti-microbial, and anti-carcinogenic activities in *in vitro* and animal studies (Padayachee and others 2013). Compared with free phenolic acids, the effects of bound phenolic compounds on human health are largely undetermined *in vitro* due to their low water solubility. Upon digestion, a lack of release of these bound phenolic compounds was observed in both the stomach and the small intestine (Padayachee and others 2013). However, the colonic gut microbiota has the ability to ferment the dietary fiber fraction and release associated phenolic compounds. The released phenolic compounds are further metabolized by hydrogenation, dehydroxylation, or demethylation to produce new metabolites, including

hydroxyphenylacetic, phenylpropionic, and phenylbutyric acids with potential health benefits (Saura-Calixto and others 2010; Selma and others 2009). For instance, the 3, 4-dihydroxyphenylacetic acid, 2, 3-dihydroxybenzoic acid, and 3-hydroxyphenylpropionic acid generated via microbial metabolism have anti-diabetic potential *in vitro*, as they promote the survival and function of pancreatic beta cells (Fernández-Millán and others 2014). Polyphenols and their metabolites have the ability to stimulate the growth of probiotic bacteria, such as *Lactobacillus acidophilus* (Hervert-Hernández and others 2009), and inhibit the growth of pathogenic bacteria, such as *Escherichia coli* (Cueva and others 2010). However, most data concerning the health benefits of cereal phenol compounds is based on *in vitro* and animal data; more research in humans is needed.

5. Novel processing treatments for cereal grains that might improve the fermentation properties of the dietary fibers

The cereal dietary fiber complex (polysaccharide moiety and antioxidants) can exert many benefits on gut health due to the action of the gut microbiota. However, some bacterial glucosidases and esterases have limited actions in breaking down the highly cross-linked dietary fiber complex. If these fibers are poorly fermented, their functionality, especially with respect to gut health would be limited. Therefore, a number of novel processing technologies have been reported, offering new ways to utilize recalcitrant bran products. These technologies can make cellulose/hemicellulose more accessible to enzymatic breakdown and lead to the increased solubilization of hemicellulosic sugars (Pérez and others 2008), thereby increasing the potential fermentation properties of these compounds. Although some of these treatments have only been studied in cereal straws, it can be speculated that increased susceptibility to enzyme attack (i.e., fermentation properties) would be observed if these treatments were applied to bran because of similar structure (Kahar 2013).

5.1. Enzymatic treatment

Cell wall-degrading enzymes are the most common reagents used to treat insoluble bran materials. Arabinoxylan-oligosaccharides from bran have been produced using xylanase treatment (Katapodis and others 2003; Swennen and others 2006). Depending on the conditions used, this method is efficient in releasing 30-40% of insoluble arabinoxylan (Swennen and others 2006; Rose and Inglett 2010). Bran

treated with xylanase resulted in faster SCFA formation and ferulic acid release compared with native bran (Nordlund and others 2013). However, the action of some types of exo-hydrolyses on insoluble dietary fiber led to the production of undesired monosaccharaides, causing a net decrease in dietary fiber content. Thus, studies have focused on selecting enzymes that act on the insoluble dietary fiber matrix and do not result in monosaccharide production. For instance, Napolitano et al. have reported that treating insoluble dietary fiber matrix with a specific preparation of *Trichoderma* enzymes triples the amount of soluble dietary fiber without significantly reducing the total dietary fiber (Napolitano and others 2006).

5.2. Hydrothermal treatment

Hydrothermal processes have been used to release the recalcitrant bran fiber complex. The hydrothermal treatment of bran fraction can be performed with water (Leppänen and others 2011) using conventional (Rose and Inglett 2010) or microwave heating (Peng and others 2012) or by steam explosion with aqueous ethanol (Hongzhang and Liying 2007). Higher yields in release of insoluble structures have been reported for the hydrothermal compared with enzymatic treatment (Rose and Inglett 2010). However, the hydrothermal process may result in the formation of undesirable byproducts, such as furfural (Otieno and Ahring 2012), which requires the subsequent process of purification.

5.3. Extrusion

Mechanical stress during extrusion is responsible for the breakdown of insoluble dietary fiber, resulting in increases in soluble fiber (Gualberto and others 1997). This process exhibits both high thermal dynamic efficiency and low cost to scale up. The impact of extrusion on the dietary fiber in bran has been reported. For instance, in a recent study, under the conditions of 50, 70 and 100% of the maximum rotations per min with the temperature ranging 161 to 180 ℃, extrusion did not affect the insoluble fiber content in rice and wheat bran, while a significantly increased soluble fiber content was observed in both types of brans (Gualberto and others 1997). Blasting extrusion processing (BEP) is a novel food processing technique compared with the traditional extrusion. BEP treats material with a combination of multiple physical phenomena including high pressure, shear stress, torque, and high temperature, resulting in fast processing and high throughput. BEP has also been applied to soybean residues, resulting in an ~ 11 fold

increase in soluble dietary fiber and ~3 fold decrease in insoluble dietary fiber, with a 30% decrease in the total dietary fiber (Chen and others 2014).

5.4. Chemical extraction

Hemicelluloses can be solubilized by alkali. Oxidizing agents can then be used to remove lignin. Doner et al. reported a novel method for solubilizing and isolating arabinoxylan from destarched corn fiber. Alkaline hydrogen peroxide (AHP) extraction for 2h at 60 ℃ increased the yields of water-soluble arabinoxylan to 42% (Doner and Hicks 1997). Although the chemical method is the cheapest and easiest to perform, the final products are always perceived as unnatural by customers. The production of chemical waste is also an environmental concern.

5.5. Supercritical CO_2 treatment

Supercritical H_2O, ammonia, and carbon dioxide (SC-CO_2) can be used as potential treatment agents. Supercritical CO_2 treatment has been applied to remove fat from rice bran, because the treated rice bran may be more stable for future utilization. Dietary fibers were extracted from the treated rice bran and displayed increased water-holding capacity and water-retention capacity compared with untreated bran (2010). However, the impact of supercritical CO_2 treatment on the specific structure of the dietary fiber in the bran remains unclear. Moreover, when subjecting rice straw to supercritical CO_2 treatment at a low temperature, extensive porosity and lamellar structures were observed. The fiber structure becomes fluffier and softer, perhaps allowing more exposure to enzymatic attack (Gao and others 2010).

5.6. Ozonolysis treatment

Ozone may increase enzymatic hydrolysis extent of potentially fermentable sugars in wheat and rye straw. Increases of ~60% (wheat straw) and 40% (rye straw) in enzymatic hydrolysis yield were observed after ozone treatment in selected conditions (Garcia-Cubero and others 2009). The ozonolysis pretreatment can degrade the lignin polymer and slightly increase soluble hemicellulose content of lignocellulosic biomass by reacting with compounds containing conjugated double bonds and functional groups with high electron densities (Garcia-Cubero and others 2009) .

6. Conclusions

Recent mechanistic studies in animal and human models have provided insight into the proposed contributory role of the gut microbiota in metabolizing food /xenobiotic compounds, contributing to the pathogenesis of metabolic diseases, and regulating colonic resistance against pathogenic bacteria. Modulation of the gut microbiota composition or its biochemical capacity may be facilitated by dietary intervention. Considering the unique chemical and physiological properties of cereal fibers, these compounds may serve as important candidates for dietary intervention to elicit beneficial effects by altering the gut microbiota to a healthier state. However, the rigid structure and poor colonic fermentability may limit the utilization of theses fibers and further research may be focused on increasing the utilization of cereal fibers through novel processing techniques, including enzymatic treatment, hydrothermal treatment, extrusion, chemical extraction, supercritical CO_2 treatment, and ozonolysis treatment. The choice should depend on weighing up the advantages and disadvantages of each techniques and considering the following factors, including equipment, cost, and nature of cereal fibers.

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Chapter 2 . Production and *in vitro* fermentation of soluble, non-digestible, feruloylated oligo-and polysaccharides from maize and wheat brans

1. Abstract

High-pressure hydrothermal treatment of cereal bran results in fragmentation of the cell wall, releasing soluble, non-digestible, feruloylated oligo- and polysaccharides (FOPS), which may be beneficial to gut health. The objectives of this study were to (1) determine treatment temperatures for production of FOPS from maize bran and wheat bran and (2) determine the fermentation properties of partially purified FOPS from maize bran and wheat bran. FOPS were produced by heating bran and water (10%, w/v) in a high-pressure stirred reactor until the slurry reached 160–200 °C (in 10 °C increments). Final temperatures of 190 °C for maize bran and 200 °C for wheat bran resulted in the highest release of FOPS (49 and 50% of starting non-starch polysaccharide, respectively). Partial purification with ion exchange and dialysis resulted in a final product containing 63 and 57% total carbohydrate and 49 and 30% FOPS, respectively (other carbohydrate was starch). Following *in vitro* digestion (to remove starch), *in vitro* fermentation revealed that wheat FOPS were more bifidogenic than maize FOPS. However, maize FOPS led to continual production of short-chain fatty acids (SCFA), resulting in the highest SCFA and butyrate production at the end of the fermentation. In addition, maize FOPS showed significantly higher antioxidant activity than wheat FOPS. This study identified a process to produce FOPS from maize bran and wheat bran and showed that, considering the overall beneficial effects, FOPS from maize bran may exhibit enhanced benefits on gut health compared to those of wheat bran.

2. Introduction

The basic structure of arabinoxylan, the predominant non-starch polysaccharide in grains, is a (1,4)-linked β-d-xylopyranosyl backbone substituted at C(O)-2 and/or C(O)-3 with α-l-arabinofuranosyl moieties, some of which are themselves substituted with ferulic acid that can be involved in oxidative cross-linkages with other arabinoxylan chains and other cell-wall components.(1) Beyond these basic structures, arabinoxylan can also contain oligosaccharide side chains comprised of pentose or hexose sugars or uronic acids. Dependent upon botanical source and location within the kernel, arabinoxylan can differ in distributions and types of side chains, molecular weight, ferulate content, and degree of cross-

linking.(1, 2) Because of its complex nature, arabinoxylan is sometimes referred to as glucuronoarabinoxylan or heteroxylan.(3-5) For simplicity, arabinoxylan will be used in this paper to refer to this complex class of polysaccharides.

Two sources of divergent arabinoxylan structures are those from maize and wheat. Enzymatic or dilute acid degradation accompanied by partial methylation analysis of alkali-solubilized arabinoxylan from maize bran and wheat bran has revealed greater branch density and complexity in maize arabinoxylan compared to that of wheat. (4, 6, 7)

In its native form, arabinoxylan is mostly insoluble and cross-linked, making it a poorly fermentable substrate for gut bacteria.(8-10) However, partial hydrolysis of arabinoxylan from wheat bran with xylanase releases arabinoxylan oligosaccharides (AXOS), which are highly fermentable in the gut and stimulate the growth of bifidobacteria and other beneficial bacteria.(11) AXOS have also been shown to reduce blood serum triglyceride and cholesterol levels, facilitate diabetic weight loss, and enhance antioxidant capacity in the liver.(11)

Arabinoxylan may also be released from its insoluble matrix by autohydrolysis,(12, 13) but the structure of the carbohydrates released are different from those released by enzyme. For instance, autohydrolysis breaks down the arabinoxylan into many size pieces ranging from monosaccharides up to polysaccharides with a degree of polymerization (DP) of 750 or more,(12, 13) while enzymatic treatment releases mostly shorter chain oligosaccharides.(14) Additionally, autohydrolysis can release arabinoxylan structures that are resistant to enzymatic hydrolysis, as evidenced by increased yields with autohydrolysis (ca. 50% from maize bran and wheat bran) (12, 13) compared to enzymatic release (ca. 30% in wheat bran(10, 14) and <3% in maize bran (15)).

Although the fermentation properties of AXOS have been reported extensively,(11) utilization of the soluble, non-digestible, feruloylated oligo- and polysaccharides (FOPS) released by autohydrolytic processing have only been studied in a few instances, and in these cases, the hydrolytic product was further treated with xylanase.(16-18) Because maize bran and wheat bran contain vastly different arabinoxylan structures(4, 6, 7) and the parent arabinoxylans from these two sources have been shown to be used very differently by gut bacteria,(8) the objectives of this study were to (1) determine treatment temperatures for

production of FOPS from maize bran and wheat bran and (2) determine the fermentation properties of partially purified FOPS from these substrates.

3. Materials and Methods

3.1. Safety

The high-pressure processing followed the safety portions of the instrument manual (4848, Parr Instrument Co., Moline, IL). The chemicals used in this study required no special safety considerations.

3.2. Production of FOPS

Maize bran was obtained from Bunge Milling (Crete, NE), and wheat bran was obtained from Horizon Mills (Newton, KS) and milled with a cyclone sample mill (UDY, Boulder, CA) equipped with a 1 mm screen. A total of 150 g of milled bran and 1350 mL of water were combined in a 2 L high-pressure reactor (model 4848, Parr, Moline, IL). The slurry was heated to 170, 180, 190, or 200 °C at full power (ca. 4 °C/min) under constant stirring at a speed of 400 rpm. The slurry was then cooled to 80 °C using an internal serpentine cold water cooling coil (ca. 15 min). Following the treatment, the slurries were centrifuged at 10000g for 10 min and the supernatant was retained. The supernatant was referred to as the autohydrolysate liquor (Figure 1). Each treatment was performed in duplicate.

Total carbohydrate, total starch, free monosaccharides, free and esterified ferulic acid, furfural, hydroxymethylfurfural (HMF), total starch, and protein were determined in all autohydrolysate liquors (see Compositional Analyses). FOPS were calculated according to the following equation:

$$FOPS\ (\%) = Wara + Wxyl + Wman + Wgal + Wglc + Wfa$$

where Wara is total arabinan (%), Wxyl is total xylan (%), Wman is total mannan (%), Wgal is total galactan (%), Wglc is total non-starch glucan (%), determined as the difference between the total glucan content after acid hydrolysis and the starch content, and Wfa is total esterified ferulate. Autohydrolysate liquors corresponding to treatment temperatures that resulted in the highest release of FOPS were also analyzed for molecular weight distribution and antioxidant activity (see Compositional Analyses).

The autohydrolysate liquors from treatment temperatures corresponding to the highest release of FOPS were pooled and dialyzed (molecular weight cut-off of 500, Spectrum Laboratories, Rancho Dominguez, CA). The retentate was mixed with ion-exchange resin (AG501-X8, Bio-Rad, Hercules, CA)

at a rate of 1 g/20 mL and stirred gently overnight. After ion-exchange resin was removed by filtration, the sample was treated under partial vacuum at 80 °C to concentrate the solids and remove volatile compounds. The material, referred to as partially purified FOPS, was then freeze-dried and assayed for total carbohydrate, free monosaccharides, free ferulic acid and esterified ferulate, furfural, HMF, starch, protein, antioxidant activity, and molecular weight distribution (see Compositional Analyses).

3.3. Fermentation of FOPS

To prepare for fermentation, *in vitro* digestion of partially purified FOPS was performed according to Yang et al.(19) The digested content was dialyzed (molecular weight cut-off of 500). Dialyzed material was freeze-dried and analyzed for total carbohydrate, free monosaccharides, free ferulic acid and esterified ferulate, starch, furfural, HMF, protein, antioxidant activity, and molecular weight distribution (see Compositional Analyses). This material was referred to as digested FOPS.

In vitro fermentation of digested FOPS was performed according to Hartzell et al.,(20) except that the sample size was 70 mg of carbohydrate instead of 100 mg. Fructooligosaccharides (FOS, Beneo, Morris Plains, NJ) were included as a positive control, and tubes containing no substrate were used as blanks. Samples (1 mL each) were taken at predetermined time points and stored at −80 °C. At times 0, 12, and 24 h, samples were assayed for short-chain fatty acids (SCFA), *Bifidobacterium*, and antioxidant activity (see Fermentation Analysis and Compositional Analyses). At other time points, samples were only assayed for SCFA.

3.4. Compositional analyses

Dietary fiber content in maize brain and wheat bran were determined with AACCI method. (21) Total carbohydrate and free monosaccharides were determined according to Rose et al.(13) Briefly, 1 mL of liquid sample or 30 mg of solid sample were analyzed in tandem for carbohydrate content with or without chemical hydrolysis.(21) With hydrolysis constituted total carbohydrate, and without hydrolysis gave free monosaccharides. Oligo- and polymeric carbohydrates were determined as the differences between total carbohydrate and free monosaccharides.

Free ferulic acid and esterified ferulate were measured using methods described by Yuan et al. (22) and Carole et al.(23) For bran samples, 10 mg of sample was weighed into a tube. If the sample was a

liquid (such as autohydrolysate liquors), 0.2 mL of sample was used. If sample was a freeze-dried powder, it was dissolved in water at 3% (w/v) and 0.2 mL of sample was used. Two tubes were prepared for analysis per replicate. To one of the tubes, which represented the total ferulic acid content, 0.2 mL of 0.4 M sodium hydroxide was added. The tube was shaken for 2 h at room temperature, and then 0.3 mL of 0.4 M phosphoric acid was added, followed by 0.2 mL of water. Free ferulic acid was assayed in the other tube in the same manner, except that sodium hydroxide was replaced by water. Each tube was then extracted with 1 mL of ethyl acetate 3 times. The pooled ethyl acetate extracts were evaporated using a partial vacuum, and the extract was dissolved in 2 mL of 50% aqueous methanol. Samples were filtered through a 0.45 μm membrane, and 15 μL was injected into a high-performance liquid chromatograph (HPLC, 1260, Agilent, Santa Clara, CA) equipped with a C18 column (C-18-4E, Skowa Denko K.K., Tokyo, Japan) and ultraviolet (UV) detector at 320 nm. Elution was performed using the gradient described by Carole et al.(23) Quantification was by means of external calibration with a ferulic acid standard (MP Biomedicals, Solon, OH). Esterified ferulate content was calculated by the difference between the total ferulic acid and the free ferulic acid.

Samples were analyzed for furfural and HMF by means of external calibration with authentic standards (Sigma-Aldrich, St. Louis, MO) following Gomis et al., (24) except that the mobile phase was 96:4 (v/v) water/acetonitrile rather than 92:8. If the sample was a liquid, it was analyzed directly or after appropriate dilution (in samples containing high furfural or HMF concentration). If the sample was a freeze-dried powder, it was dissolved in water at 3% (w/v) for analysis.

Total starch was measured in solid samples using a kit (K-TSTA, Megazyme, Bray, Ireland). In liquid samples, the total starch content was measured following the method from Rose et al.(12) The protein content of solid samples was obtained by a FP528 nitrogen/protein determinator (LECO Corporation, St. Joseph, MI) after using a conversion factor of 6.25 for percent nitrogen to percent protein.(25) In liquid samples, the protein content was measured using the Bradford method with bovine serum albumin (Pierco, Rockford, IL) used to create a standard curve.(26)

To determine the molecular weight distribution, 10 mg of freeze-dried powder was dissolved in 2 mL of water. Samples were filtered through a 0.45 μm membrane, and 100 μL was injected into a HPLC (Agilent) equipped with Shodex SB-G, SB-804 HQ, and SB-802 HQ (Skowa Denko K.K., Tokyo, Japan)

columns connected in series followed by a refractive index detector (Agilent). The mobile phase was aqueous 0.02% sodium azide at 0.5 mL/min. Molecular weight calibration was accomplished by means of external pullulan (P-82, Shodex) and glucooligosaccharide (4-7265, Sigma-Aldrich, St. Louis, MO) standards.

Antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ATBS) methods. The DPPH assay was performed by reacting 0.2 mL of appropriately diluted sample with 1 mL of DPPH as described by Sensoy et al.,(27) except that, after incubating for 30 min, the tubes were centrifuged for 10 min at 3000g to remove the cloudiness. The ATBS assay was conducted according to Re et al.(28) Antioxidant activity was expressed relative to the antioxidant activity of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Adrich, St. Louis, MO).

3.5. Fermentation analysis

Samples taken during fermentation were thawed and centrifuged (10000g for 5 min). The supernatant was used for analysis of SCFA after Hartzell et al. (20) and for antioxidant activity (see Compositional Analyses). The pellet was used for analysis of *Bifidobacterium* by quantitative real-time polymerase chain reaction (PCR) as described.(20)

4. Statistical analysis

For autohydrolytic processing, two replications at each temperature were performed. Each replicate was analyzed twice. Differences in chemical compositions were analyzed using analysis of variation (ANOVA) in combination with Fisher's protected least significant difference. The replicates from autohydrolysis were pooled for the in vitro fermentation stage. Differences in SCFA production between treatments were analyzed with three replicates using ANOVA with repeated measures followed by Fisher's protected least significant difference using SAS (version 9.2, SAS Institute, Cary, NC). Differences among substrate treatment on the population of *Bifidobacterium* and antioxidant activity were computed using ANOVA in combination with Fisher's protected least significant difference.

5. Results and discussion

5.1. Production of FOPS

Maize bran and wheat bran contained high concentrations of total dietary fiber (Table 1), most of which was water-insoluble.(29) Higher ferulic acid content was observed in maize bran compared to wheat bran. Maize has the highest ferulic acid content among major cereal brans, which exhibits high antioxidant activity and displays potential health benefits.(29)

The composition of the autohydrolysate liquors differed depending upon the treatment temperature (Figure 2A). The release of FOPS was increased in autohydrolysate liquor from maize bran as the temperature increased from 170 to 190 °C and then showed a marked decrease from 190 to 200 °C. The highest yield represented 49% of the starting non-starch polysaccharide content and 44% of the initial ferulic acid content. With wheat bran, an increased final temperature resulted in a steady increase in the concentration of FOPS in the autohydrolysate liquor, ultimately releasing 50% of the initial non-starch polysaccharide content and 82% of the ferulic acid content at a final treatment temperature of 200 °C. These yields agree well with the percentage of cell-wall polysaccharides released by hydrothermal treatment of other agricultural byproducts (30) and were substantially higher than the AXOS released by enzymatic hydrolysis.(14) Thus, 190 and 200 °C were selected as the desirable temperatures to release FOPS from maize bran and wheat bran, respectively.

The autohydrolysate liquors also contained high concentrations of non-FOPS material (Figure 2B). The release of arabinose and xylose was largely affected by the treatment temperature, with more arabinose being released (mainly side chain) than xylose (mainly backbone), suggesting that arabinoxylan was partially debranched during autohydrolysis (data not shown). Furfural and HMF increased as the treatment temperature increased. These compounds are one of the thermal degradation compounds of pentoses and hexoses, respectively. (31) Starch and protein were also present in autohydrolysate liquors (10.9–15.3 and 0.180–2.01 g/L, respectively), but the concentration did not vary as a function of the treatment temperature. Dialysis, ion-exchange, and vacuum concentration were used to partially remove non-FOPS material from autohydrolysate liquors. The partially purified material from maize bran contained a higher percentage of FOPS compared to wheat bran (49 versus 30%, respectively; see Table 2). Nearly 4-fold higher antioxidant activity was observed on partially purified maize FOPS compared to wheat when using the ATBS radical;

when using the DPPH radical, maize FOPS showed 2-fold higher antioxidant activity compared to wheat (Table 2).

The purification process removed furfural, HMF, and free ferulic acid (i.e., these compounds were not detectable in the partially purified samples; Table 2). More than 90% of the free monosaccharaides present in autohydrolysate liquors from both maize bran and wheat bran were removed with the partial purification process (Table 2).

Because the purification process only concentrated oligo- and polysaccharides and removed non-carbohydrate material, there was still substantial starch contamination in the partially purified FOPS preparations (15.5 and 27.1% in maize bran and wheat bran freeze-dried material, respectively; Table 2). Pretreatments are often employed to remove starch prior to producing FOPS-like materials.(12, 13) However, this step was not performed to simplify the production process because it was anticipated that the digestion phase would remove any contaminating starch prior to determining fermentation characteristics.

Partially purified FOPS showed a broad molecular weight distribution, resulting from the fragmentation of arabinoxylan caused by high-pressure hydrothermal treatment. The majority of polymers ranged from 0.24×10^5 to 1.9×10^5 g/mol (Figure 3). The presence of high-molecular-weight polymers could have been due to the release of polysaccharides or from the release of oligosaccharides that were covalently linked through diferuloyl groups.

5.2. Fermentation of FOPS

Because the partially purified FOPS contained appreciable starch (Table 2), prior to *in vitro* fermentation, in vitro digestion was performed. *In vitro* digestion removed nearly all of the starch and, consequently, increased the percentage of FOPS in the freeze-dried material compared to the partially purified FOPS (Table 2). The digestion process also resulted in concentrating the ferulate content and, subsequently, increasing the antioxidant activity except for wheat bran with DPPH (Table 2). Protein decreased after digestion, but a small percentage persisted (2.37 and 3.27% in maize bran and wheat bran samples, respectively). Some of this protein could have been denatured enzymes from the digestion process as well as enzyme-resistant protein from the starting material. The difficulty in completely removing protein from cereal grain materials has been noted previously. (32)

In vitro digestion removed some of the shortest polymers in wheat FOPS (Figure 3), suggesting that starch may have been fragmented into small dextrins. However, the digested FOPS contained a similar broad molecular weight distribution compared to before *in vitro* digestion.

In vitro fermentation was performed to determine how FOPS from maize bran and wheat bran were metabolized by fecal bacteria. Wheat FOPS resulted in lower SCFA production at 4 h compared to FOS (included as a positive control), and there was no significant change in SCFA production after 4 h (Table 3). Maize FOPS also showed low SCFA production relative to FOS at 4 h but brought about continual production of SCFA thereafter, resulting in the highest SCFA production at the end of the fermentation.

The steadily increasing fermentation profile of maize FOPS suggests that fermentation may persist into the distal part of the colon, where protein fermentation otherwise predominates, resulting in the production of toxic compounds, such as ammonia, phenol, indoles, thiols, amines, and sulfides. (34) A greater increase in SCFA production in the distal colon may result in protective effects by lowering the pH and inhibiting the fermentation of protein.(33)

The difference in chemical structure between maize and wheat arabinoxylan may be responsible for their different fermentation profiles. As mentioned, maize arabinoxylan contains higher branch density and complexity, coupled with higher levels of esterified ferulic acid compared to wheat arabinoxylan.(4, 6, 7) Notably, extended fermentation properties for alkali-extracted maize arabinoxylan(8) as well as its hydrolysate (34) have been shown previously. Thus, slow fermentation appears to be an inherent property of maize arabinoxylan, independent of the DP or method of release from the cell-wall matrix.

Individual SCFA production also differed among substrates. After 24 h of fermentation, microbial butyrate production on maize FOPS was significantly higher than that on other substrates (Table 3). Butyrate has been shown to inhibit inflammatory responses by inhibiting activation of nuclear factor κB.(35) Thus, the high butyrate production of maize FOPS may exert potential health benefits compared to the other substrates.(33, 35, 36) Significantly higher propionate was also observed on maize FOPS compared to wheat FOPS. Propionate may reduce serum lipid as well as the risk of cardiovascular diseases. (37) Propionate has also been shown to have the potential to prevent obesity-related inflammation and associated diseases. (38)

Because the target of many prebiotic studies is to increase *Bifidobacterium*,(33, 39) and because AXOS produced by enzyme treatment have been shown to be particularly bifidogenic,(40-42) the shift in *Bifidobacterium* upon FOPS treatment was investigated. Both maize and wheat FOPS showed a significant increase in *Bifidobacterium* compared to the blank and baseline after 12 and 24 h of fermentation, although the increase was not as great as FOS (except for wheat FOPS after 12 h of fermentation; Figure 4). Wheat FOPS exhibited higher bifidogenicity than maize at the end of the fermentation.

These results are contrary to previous reports demonstrating that AXOS can be a more potent bifidogenic substrate than FOS. (40, 41) This is likely because FOPS (released by autohydrolysis) contained more complex, higher DP structures compared to AXOS (released by enzyme).(14) The higher branch density and complexity of maize arabinoxylan compared to wheat may account for the lower bifidogenicity of maize FOPS, whereas other bacteria may have greater capacity to use these substrates. For instance, the SCFA data suggest that maize FOPS supported butyrate producers (Table 3). The main butyrate producers in the gut include *Eubacterium rectale*/*Roseburia* spp. and *Faecalibacterium prausnitzii*, which have shown beneficial effects on the gut. (43) Other members of the gut microbiota also contribute to the SCFA pool.

Although the gut is an anaerobic environment, the gut epithelia release reactive oxygen species into the lumen of the gut in response to environmental stresses and, in some cases, even commensal bacteria.(44) Numerous diseases, such as inflammatory bowel diseases and colon cancer, are associated with an imbalance in the gut cellular redox system.(45) When the antioxidant activity in the medium was measured, the potential ability of FOPS to prevent oxidative damage in the gut was investigated.

As expected, because of the high ferulate content, at 0 h, the highest antioxidant activity was observed on maize FOPS (Figure 5). During fermentation, antioxidant activities changed depending upon the substrate and radical used for the antioxidant assay. On ATBS, the blank and FOS showed decreasing antioxidant activity as fermentation time progressed. The decrease was delayed when wheat FOPS was used, and maize FOPS showed an increase at 12 h before a decrease was experienced. In contrast to ATBS, radical scavenging ability against DPPH increased in all samples, including the blank and FOS, from 0 to 12 h of fermentation, followed by a significant decrease in all samples, except maize FOPS at 24 h.

The human microbiota can express several enzymes that are capable of releasing and metabolizing ferulic acid, (46, 47) the major antioxidant in FOPS. This has a dramatic effect on biological activity. (46-50) First, gut bacteria probably hydrolyze the ester linkage and release free ferulic acid. (51) This would likely increase the antioxidant activity because free ferulic acid possesses higher antioxidant activity than esterified ferulate.(52) Upon release of the ferulic acid, modification of functional groups by microbial enzymes could further affect the antioxidant activity. Metabolism of ferulic acid first involves hydrogenation of the propen-2-oic acid side chain and demethylation of the ring ether (46) to form 3,4-dihydroxyphenylpropionic acid.(46) This compound has been shown to possess higher antioxidant activity than ferulic acid in edible oil systems;(53) however, this compound is only transient and, subsequently, dehydroxylated to 3-hydroxyphenylpropionic acid and, finally, phenylpropionic acid.(46) These conversions likely decrease antioxidant activity.

Other factors not directly related to the bioconversion of ferulic acid may also contribute to changes in antioxidant activity. For instance, higher antioxidant activity has been observed for free ferulic acid in the presence of AXOS compared to absence AXOS. (52) As mentioned, ferulic acid is initially de-esterified by the gut microbiota, and this could be analogous to free ferulic acid in the presence of AXOS. As fermentation progresses, however, AXOS are removed from the system by microbial metabolism. Microbial metabolism of AXOS also leads to production of SCFA, which lower the pH and could affect antioxidant activity. (54)

This study identified a high-pressure hydrothermal treatment of cereal bran that resulted in fragmentation of the cell wall. Final temperatures of 190 °C for maize bran and 200 °C for wheat bran resulted in the highest release of FOPS (49 and 50% of initial non-starch polysaccharide, respectively) and esterified ferulic acid (44 and 82% of initial ferulic acid, respectively). Maize FOPS displayed a desirable protracted fermentation profile with higher butyrate production compared to wheat FOPS and FOS. Both maize and wheat FOPS exhibited bifidogenic activity, although weaker than FOS. Maize FOPS exhibited high antioxidant capacity during fermentation that could have beneficial properties *in vivo*. Because FOPS can possibly combine beneficial fermentation properties and antioxidant activities, it may have potential to become a functional food ingredient to promote gut health.

6. References

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Table 1. Selected composition of corn and wheat bran (% dry basis)[a]

Component	Corn bran	Wheat bran
Starch	10.6±0.2	14.4±0.3
Total dietary fiber	64.6±1.4	47.2±1.4
Arabinan	12.5±0.5	9.8±0.2
Xylan	24.7±1.6	13.9±1.0
Mannan	0.772±0.060	0.691±0.000
Galactan	2.82±0.03	0.440±0.020
Glucan	16.2±1.7	12.1±1.9
Uronic acid	3.23±0.04	0.988±0.031
Klason lignin	4.38±0.11	9.25±0.11
Ferulic acid	2.51± 0.05	0.150 ±0.011
Protein	5.33±0.01	17.1±0.0
Other (by difference)	17.0	21.2

[a]Mean values ± SD (n=2); total dietary fiber expressed as the sum of neutral sugars, uronic acids, and Klason lignin; glucan refers to non-starch glucan.

Table 2. Composition of freeze-dried partially purified FOPS and digested FOPS from corn bran and wheat bran.[a]

	Corn bran		Wheat bran	
Component	Partially purified FOPS	Digested FOPS	Partially purified FOPS	Digested FOPS
Total carbohydrate (%)	63.2±0.5	66.1±0.9*	57.0±1.1†	61.0±1.1*†
FOPS (%)	48.6±0.5	68.0±0.9*	29.7±1.1†	57.1±0.8*†
Arabinan (%)	7.38±0.20	10.7±0.2*	6.40±0.20†	10.3±0.8*
Xylan (%)	31.8±0.5	44.4±0.8*	16.1±0.5†	36.4±2.0*†
Mannan (%)	0.16±0.18	0.34±0.03	0.13±0.08	0.41±0.03*
Galactan (%)	3.12±0.9	5.50±0.31*	1.03±0.11†	1.49±0.49†
Glucan (%)	4.40±0.56	4.29±0.24	5.88±0.73	8.22±1.85†
Esterified ferulate (%)	1.76±0.06	2.68±0.24*	0.16±0.03†	0.221±0.003*†
Free monosaccharides (%)	0.83±0.15	0.84±0.09	0.34±0.11	3.28±0.77*†
Arabinose (%)	0.33±0.07	0.54±0.03*	0.11±0.10	1.70±0.18*†
Xylose (%)	0.19±0.03	trace*	0.08±0.06†	trace*
Mannose (%)	0.13±0.03	trace*	0.13±0.04	trace*
Galactose (%)	0.17±0.09	0.02±0.01*	0.015±0.01†	trace
Glucose (%)	0.02±0.00	0.29±0.03	trace	1.57±1.02*†
Starch (%)	15.5±0.4	ND*	27.1±5.8†	0.64±0.04*†
Free ferulic acid (%)	ND	ND	ND	ND
Protein (%)	4.21±0.18	2.37±0.31*	4.87±0.27	3.27±0.62*†
Furfural (%)	ND	ND	ND	ND
HMF (%)	ND	ND	ND	ND
Antioxidant activity				
ATBS (μmol Trolox/g)	27.9±2.1	36.6±2.6*	7.17±0.30†	14.1±2.1*†
DPPH (μmol Trolox/g)	70.7±5.3	83.3±0.9*	31.6±1.8†	27.1±5.1†

[a]Values are reported as mean (dry basis) ± SD (n=2); feruloylated oligo- and polysaccharides (FOPS) is the sum of arabinan, xylan, mannan, galactan, glucan, and esterified ferulate; hydroxymethylfurfural (HMF) ; ND = not detected.;*significantly different from partially purified FOPS of the same bran type; †significantly different from the corresponding FOPS sample made from corn bran ($p<0.05$).

Table 3. Short chain fatty acid (SCFA) production (μmol/100mg initial carbohydrate) during *in vitro* fermentation of blank, fructooligosaccharides (FOS), and feruloylated oligo- and polysaccharides (FOPS) from corn bran and wheat bran.[a]

Metabolite/	Fermentation time (h)			
Sample	4	8	12	24
Acetate				
Blank	213±18c	198±21b	203±31c	300±23c
Corn FOPS	370±26b	541±52*a	530±40a	886±173*a
Wheat FOPS	433±20b	472±75a	485±45a	454±29b
FOS	560±30a	517±74a	495±98a	563±30b
Propionate				
Blank	45.5±5.2d	61.6±6.0c	62.8±7.1c	85.2±8.7*b
Corn FOPS	71.7±6.7c	106±11*ab	111±11b	179±34*a
Wheat FOPS	95.4±3.8b	90.8±10.9b	100±9b	91±6b
FOS	137±10a	121 ±14a	155±19*a	164±9a
Butyrate				
Blank	58±6d	57.0±3.2c	52.7±15.4c	77.3±3.4*c
Corn FOPS	104±7c	146±8*b	181±13*b	244±6*a
Wheat FOPS	150±5b	182±15*a	209±9*a	206±10b
FOS	181±19a	183±16*a	207±17*a	216±6*b
Total SCFA				
Blank	316±26e	317±28b	318±52b	462±31d
Corn FOPS	546±26cd	795±74*a	822±43a	1040±218*a
Wheat FOPS	679±32bc	745±100a	794±58a	751±30c
FOS	878±46a	821±82a	715±131a	943±131*b

[a]Values are reported as mean± SD of 3 replicates; units are μmol/100mg initial carbohydrate; means followed by different letters with each column and SCFA type are significantly different; *significantly different from the previous time point ($p<0.05$).

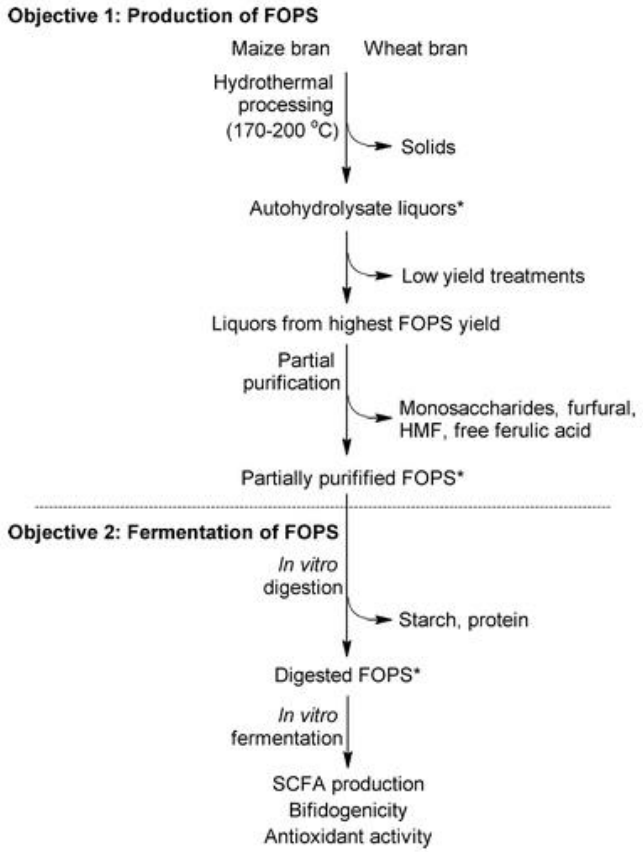

Figure 1. Flowchart of the protocol, which consisted of two objectives: (1) production of FOPS and (2) fermentation of FOPS. (∗) Compositional analysis was performed at these stages, including: total FOPS, monosaccharides, furfural, HMF, free ferulic acid, total starch, protein, antioxidant activity, and molecular weight distribution.

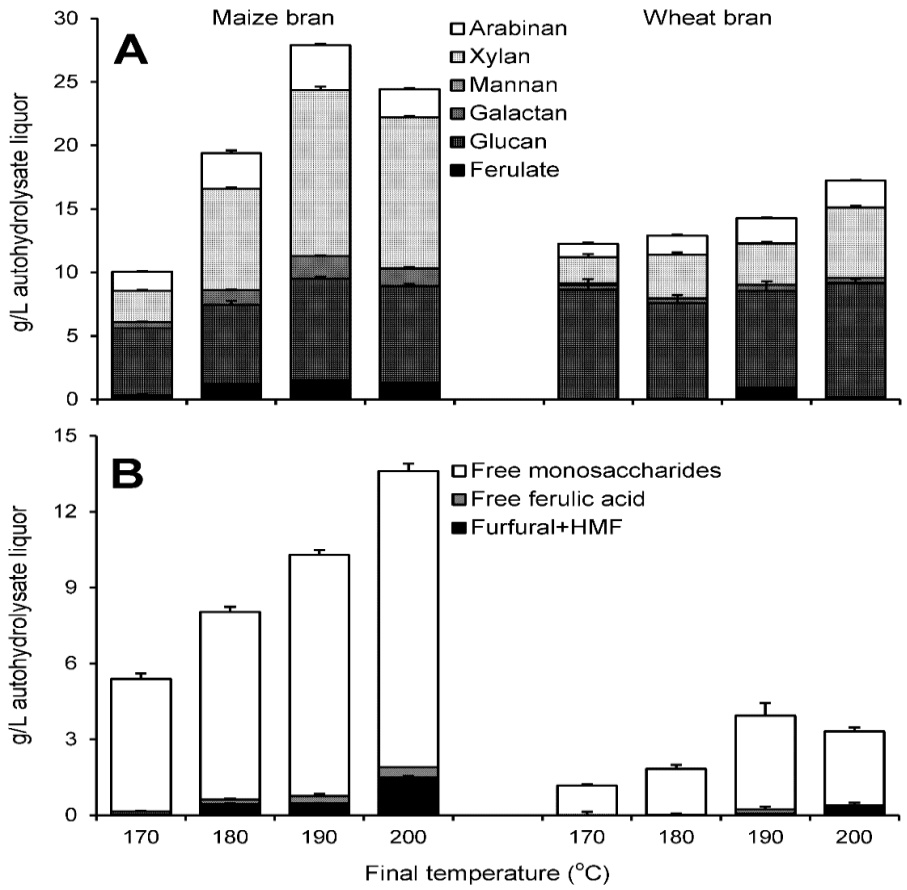

Figure 2. Composition of autohydrolysate liquor upon hydrothermal treatment to selected temperatures: (A) FOPS and (B) non-FOPS material released by autohydrolysis treatment. HMF = hydroxymethylfurfural. Error bars show standard deviation (n = 2). Some error bars were too small to plot.

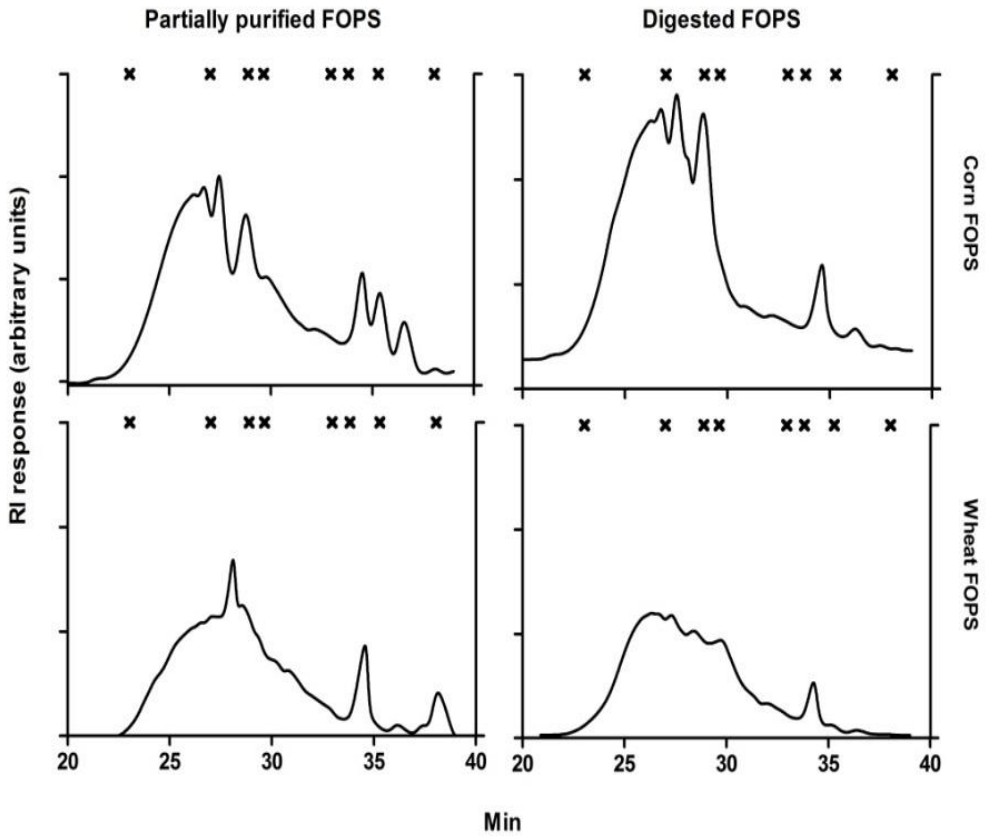

Figure. 3. Size-exclusion chroamtographs of feruloylated oligo- and polysaccharides (FOPS); figures on left are partially purified FOPS; figures on right are digested FOPS.peak retention times of glucan standards of molecular weight 2.00×10^5, 2.11×10^4, 9.60×10^3, 5.9×10^3, 1.15×10^3, 8.29×10^2, 5.04×10^2 and 1.80×10^2 are marked along the top of each subfigure.

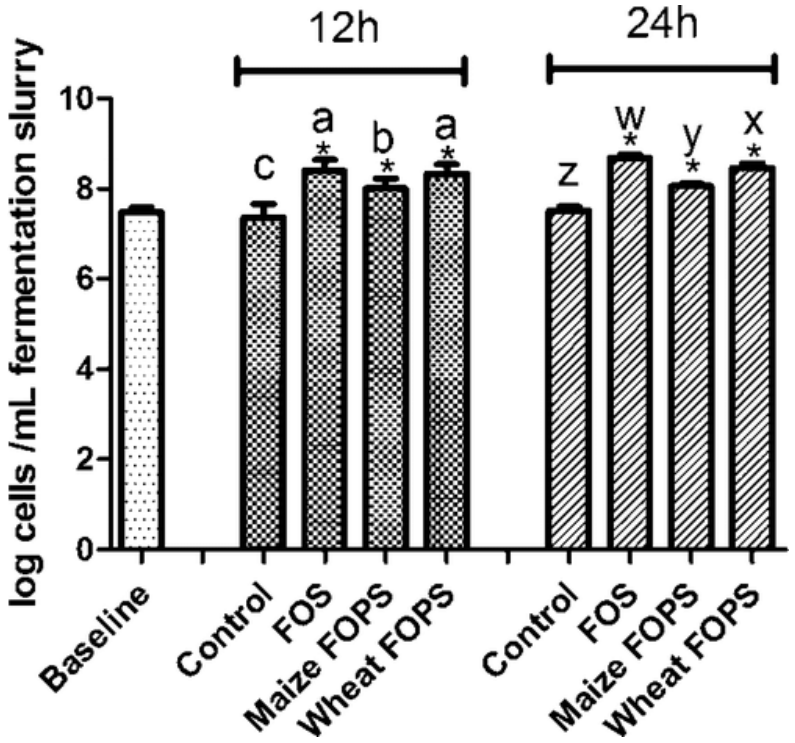

Figure 4. Enumeration of *Bifidobacterium* during *in vitro* fermentation. The baseline represents *Bifidobacterium* in the fecal inoculum before fermentation (0 h). The control represents a sample after fermentation with no added carbohydrate substrate. FOPS = feruloylated oligo- and polysaccharides. FOS = fructooligosaccharides. Bars marked with different letters are significantly different within each time point. (∗) Significantly different from the baseline ($p < 0.05$). n = 2 (baseline) or 3 (fermentation samples). Error bars show standard deviation.

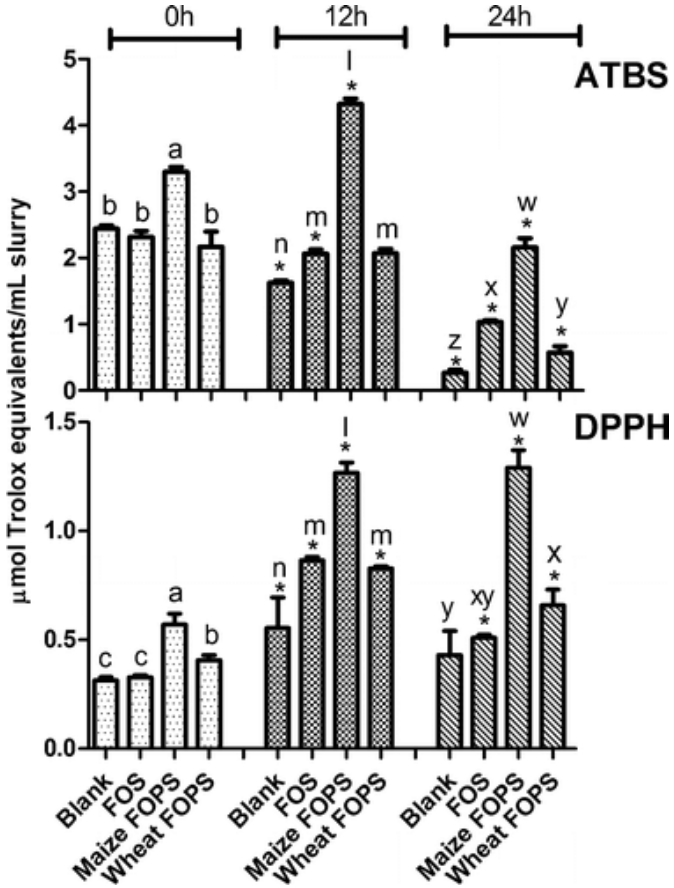

Figure 5. Antioxidant activity of fermentation medium before and after 12 and 24 h *in vitro* fermentation. The blank represents a sample after fermentation with no added carbohydrate substrate. FOPS = feruloylated oligo- and polysaccharides. FOS = fructooligosaccharides. Bars marked with different letters are significantly different within each time point. (∗) Significantly different from the corresponding substrates at the previous time point ($p < 0.05$). n = 3. Error bars show standard deviation.

Chapter 3 . Disparate metabolic responses in mice fed a high-fat diet supplemented with maize-derived non-digestible feruloylated oligo- and polysaccharides are linked to changes in the gut microbiota

1. Abstract

Scope: Recent studies have suggested a positive link between colonic fermentation of dietary fibers and reduced incidence or severity of metabolic disorders. The objective of this study was to determine if consumption of a particular maize-derived dietary fiber—non-digestible feruloylated oligo- and polysaccharides (FOPS)—could counteract the deleterious effects of high-fat (HF) feeding and modulate the gut microbiota in mice.

Methods and results: C57BL/6J mice (n=8/group) were fed a low-fat (LF; 10 kcal% fat), HF (62 kcal% fat), or HF diet supplemented with FOPS (5%, w/w). Four FOPS-fed mice (F-FOPS) showed enlarged ceca with enhanced short chain fatty acid (SCFA) production accompanied by significant improvements in glucose metabolism and plasma resistin compared with HF-fed mice. The remaining four FOPS-fed mice showed normal cecal fermentation with no metabolic improvements. Three F-FOPS mice showed increases in *Blautia* and *Akkermansia* and reduction in plasma leptin accompanied by reductions in body and adipose tissue weights compared with the HF-mice.

Conclusions: FOPS could be an important promoter of SCFA production and shifts in the gut microbiota that could lead to reduction in metabolic disorders. However, more research aimed at identifying the cause of the disparate metabolic responses to dietary FOPS is warranted.

2. Introduction

Obesity is associated with a cluster of metabolic disorders including insulin resistance, type-2 diabetes, and fatty liver disease [1]. These diseases diminish the quality of life of a growing proportion of the world's population, and there is an urgent need for strategies to reduce the prevalence of these diseases. The human gut microbiota exerts substantial effects on its host's metabolic and immune functions; abnormalities in its composition are associated with many diseases, including obesity, diabetes, heart disease, and some types of cancer [2-6]. One of the most promising means of manipulating ratios of species in the microbiota towards putatively beneficial microbial ecologies is through the use of prebiotics, a

special class of dietary fibers that are preferentially fermented by select species of beneficial bacteria in the gut [7]. The selective increase in favorable bacteria as a result of prebiotic intake may benefit the host by precluding growth of detrimental bacteria through competitive inhibition, production of antibacterial compounds, metabolism of harmful substances, strengthening of gut barrier function, and synthesis of short chain fatty acids (SCFA) [7]. SCFA themselves may also benefit the host by improving glucose homeostasis, blood lipid profiles, and reducing body weight and colon cancer risk [8]. Thus the combined effects of prebiotics would likely contribute to long-term prevention of chronic subclinical inflammation that can develop into metabolic syndrome [9-12].

In a previous study, we identified a hydrothermal process to produce non-digestible feruloylated oligo- and polysaccharides (FOPS) [13]. FOPS are composed of hydrolysates of the hemicellulosic component of maize bran, which is principally a complex heteroxylan comprised of a (1, 4)-linked β-D-xylopyranosyl backbone with single or multi-unit branches consisting of α-L-arabinofruanose, β-D-xylopyranose, β-D-glucuronic acid, and a non-carbohydrate antioxidant, ferulic acid [14]. The multi-unit branches in maize heteroxylan are rare among cereal dietary fibers, as are some of the linkages between sugars on these branches [e.g., (1, 2)- and (1, 3)-linkages between β-D-xylopyranose and α-L-arabinofruanose]. Our previous in vitro data using human fecal microbiota has shown that this complex structure is more difficult for the microbiota to ferment than the hemicellulosic components of other cereals and thus may contribute to sustained SCFA production [13, 15]. Dietary fibers that are capable of supporting prolonged saccharolytic bacterial fermentation may help in maintaining beneficial SCFA production in the distal colon, which is low in SCFA and particularly prone to disease [16-18]. Our in vitro studies have also shown enhanced SCFA production of FOPS from maize bran, especially butyrate, compared with fructans and FOPS produced from wheat bran. Additionally, because of the high ferulic acid content, fermented FOPS samples contained high antioxidant activity. Antioxidants in the gut have been shown to reduce the damaging effects of free radicals such as nitric oxide that are released in the colon as a result of inflammation [19, 20]. Because of these promising in vitro results, the objective of this study was to determine if consumption of FOPS could counteract the deleterious effects of high-fat (HF) feeding and modulate the gut microbiota in mice.

3. Materials and methods

3.1. Production and composition of FOPS

FOPS from maize bran was produced as described [13]. In brief, 150 g of finely milled maize bran (Bunge Milling, Crete, NE) was dispersed in 1.35 L of water in a 2 L high-pressure reactor (Model 4848, Parr, Moline, IL). The slurry was heated to 190 °C at the rate of 4 °C/min under constant stirring (400 rpm), and then was cooled to 80 °C using an internal serpentine coil with circulating cold water (ca. 15 min). The slurry was then centrifuged at 10, 000 × g for 10 min and the supernatant was retained. Supernatants from a total of ~25 batches were pooled and loaded into a reverse osmosis system (Model R, GEA Filtration, Hudson, WI, USA) equipped with a membrane (molecular weight cut off: 1000; GE 1207106, GEA Process Engineering Inc., Hudson, WI, USA). The FOPS were circulated with ~75 L of distilled water to separate contaminants (permeate) from FOPS (retentate). Following reverse osmosis, FOPS were freeze-dried (Thermal-Vac Technology Inc., Orange, CA, USA). The freeze-dried material was subsequently analyzed for total carbohydrate, total starch, free monosaccharides, free and esterified ferulic acid, furfural, hydroxymethylfurfural (HMF), and protein as described [13]. FOPS was calculated as the sum of all non-starch polymeric sugars and esterified ferulate [13]. The final FOPS preparation contained 59% FOPS, 16% starch, 3.4% other sugars, 2.9% protein, and 8.8% moisture (Table 1).

3.2. Experimental diets and mouse experiment

The three diets used in the study were prepared by a commercial provider (Research Diets, New Brunswick, NJ USA): low fat [LF; rodent diet with 10 kcal% fat (no sucrose); D12450K), HF (rodent diet with 62% kcal% fat; D12492), and HF supplemented with 5% FOPS (w/w; Table 2). FOPS were incorporated into the HF diet at the expense of cellulose. Because the FOPS preparation contained small quantities of starch and protein, the amounts of these compounds in the HF diet formulation were reduced to the extent necessary to match the macronutrient content of the HF control diet. The LF diet was included to confirm metabolic aberrations induced by the HF diet.

Twenty four 8-week old male C57BL/6J mice were purchased from Jackson Lab (Bar Harbor, Maine USA). The animals were maintained in an environment with a 14 h light/10h dark cycle and controlled temperature and humidity. The mice were randomly assigned to one of the three dietary treatments (n=8 mice/group). Mice were housed in individually ventilated cages in pairs for a total of 4 cages per treatment group and maintained on autoclaved bedding, and fed the same autoclaved water. The

mice were acclimated to the cages for 1 week while consuming a regular autoclaved chow diet before starting the intervention. The dietary intervention continued for 8 weeks. Diet replacement and recording of food intake was performed every 2 weeks and the body weights were recorded every week.

Feces were collected three times during the experiment (weeks 0, 1, and 8) and stored at -80°C until further analysis. After 8 weeks of experimental diet feeding, the mice were euthanized via CO_2 asphyxiation. Blood was harvested by cardiac puncture and plasma was collected by centrifugation at 13,000 × g for 3 min at 4 °C. The liver, adipose tissue [visceral adipose tissue (VAT), epididymal adipose tissue (EAT), and subcutaneous adipose tissue (SAT)], and cecal tissue and contents were carefully removed and weighed. All biological samples were snap frozen in liquid nitrogen and then stored at -80 ˚C until analysis. The Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln approved all procedures involving animals.

3.3. Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was carried out at 7 weeks. Food was removed 6 h prior to the test. Thirty minutes prior to the test, blood was collected from the tail vein to measure fasting glucose concentration using a glucose meter (ACCU-CHEK, Aviva Plus system, Indianapolis, IN, USA). An aliquot of blood was also saved for subsequent insulin analysis by ELISA (Mercodia Insulin ELISA Kit, Uppsala Sweden). At time zero, glucose solution (20 g/100 mL) was injected into the peritoneal cavity (1 g glucose/kg body weight) [21]. Blood glucose was measured 0, 15, 30, 60, 90 and 120 min after glucose injection. The homeostatic model assessment of insulin resistance (HOMA-IR), an indicator of insulin resistance [22], was calculated using the fasting insulin and blood glucose concentration as: HOMA-IR = [glucose (mg/dL)*insulin (μU/mL)]/405 [23].

3.4. Plasma lipids, cytokines, and hormones

Plasma amylin, C-peptide, leptin, monocyte chemoattractant protein-1, interleukin 6, tumor necrosis factor-alpha, glucagon, peptide YY, pancreatic polypeptide, gastric inhibitory polypeptide, and ghrelin were measured by a multiplex immunoassay (Mouse Metabolic Magnetic Bead Panel Kit; Merck Millipore, Billerica, MA, USA). Plasma triacylglycerol and total cholesterol were determined using

enzymatic reaction coupled to spectrophotometry detection (Infinity TG/Cholesterol kit; Thermo Electron, Waltham, MA, USA).

3.5. Cecal short and branch chain fatty acids

SCFA, acetate, propionate, and butyrate, and branch chain fatty acids (BCFA), *iso*-butyrate and *iso*-valerate, were quantified in the cecal contents collected at necropsy by GC [24].Quantification was done by means of internal calibration with 2-ethyl butyric acid.

3.6. Characterization of the fecal microbiota composition

DNA was extracted through mechanical and enzymatic cell lysis as previously described [25]. Microbial composition was assessed at weeks 0, 1, and 8 by 16S rRNA gene sequencing (MiSeq; Illumina; San Diego, CA, USA) [26] targeting the V5-V6 region with primer pair 784F (5'-RGGATTAGATACCC-3') and 1064R (5'-CGACRRCCATGCANCACCT-3'). Initial quality filtering and demultiplexing of the resulting reads was performed with Illumina Software. Next, reads were merged with the merge-Illumina-pairs application, which also removed primers and performed further quality check of the sequences [27]. Subsequently, the UPARSE pipeline [28] was used to process the sequences and perform operational taxonomic unit (OTU) clustering, using a 98% similarity cutoff. Sequences were independently subjected to taxonomic classification for phylum to genus characterization of the fecal microbiome using the RDP MultiClassifier 1.1 from the Ribosomal Database Project [29]. Taxonomic bins were computed as proportions based on the total number of sequences in each sample. α-Diversity were calculated using QIIME [30].

3.7. Statistical analysis

We divided the mice on the FOPS diet into those with enlarged ceca that fermented the FOPS (F-FOPS) and those with ceca of normal size (N-FOPS) (Result 4.1). Results are presented as mean ± SEM or SD, as indicated in figure or table captions, with the exception of results shown in Fig. 6 and Supplementary Fig. 1, where results are shown as individual observations. The impact of dietary treatments on body mass, metabolic markers, and gut microbiota α-diversity were analyzed using one-factor ANOVA (diet). Body weight and blood glucose during the IPGTT were analyzed using two-factor repeated measures ANOVA (diet, time). Significant differences for ANOVA models were assessed using Bonferroni's post

hoc test with comparisons (HF vs. LF; HF vs. N-FOPS; HF vs. F-FOPS; N-FOPS vs. F-FOPS). Differences in individual fecal microbiota OTUs between N-FOPS and F-FOPS groups were assessed using t-tests for each of the three weeks that fecal pellets were collected. Principal components analysis (PCA) of fecal microbiota OTU abundances were also computed. Correlations between bacterial groups and host physiological measurements in the HF- and FOPS-fed mice were calculated using Pearson's coefficients. Microbial taxa <1% in abundance in sum in all mice at all-time points were excluded from the analyses. $P < 0.05$ was used to consider all statistical significance. All data were analyzed using SAS software (version 9.4; SAS Institute, Cary, NC, USA) with the exception of PCA, which was performed using XLSTAT software (Statistical Innovations, Belmont, MA USA).

4. Results

4.1. Allocation of mice in the FOPS group into fermenters and non-fermenters

During necropsy we observed dramatic cecal enlargement in four mice in the FOPS group, while the other four mice and all of the mice in the other treatment groups contained ceca of normal size (Fig. 1). This did not appear to be a cage effect; two of the mice with enlarged ceca were from the same cage, while the other two mice shared cages with mice that had ceca of normal size. No significant differences in cecal SCFA or BCFA pools were evident among LF, HF, and N-FOPS (Fig. 2). However, total SCFA pool was significantly elevated in the mice with enlarged ceca compared with the other groups (Fig. 2A, 2C-E). These mice showed marked increases in acetate and propionate pools. Butyrate production was also elevated in this group, but with wide variation among mice. A significant increase in BCFA production was detected in the mice with enlarged ceca compared with others (Fig. 2B); however, the BCFA/SCFA ratio was significantly lower than other groups (Fig. 2F), suggesting that the elevated BCFA production was a result of greater overall fermentative activity and not a tendency toward putrefactive fermentation as is often the case with elevated BCFA production. Given that higher cecal weight and SCFA pool was an indication of increased fermentation and metabolic activity of the gut microbiota [31], we divided the mice on the FOPS diet into those with enlarged ceca that fermented the FOPS (F-FOPS), and those with ceca of normal size (N-FOPS) for subsequent data analysis and presentation.

4.2. Influence of FOPS on body weight and tissue weights

There was no difference in feed intake between the HF and FOPS groups (Fig. 3); however, there were differences in body weight gain among treatment groups (Fig. 4A). The F-FOPS group gained weight at a slower rate during the 8-week study such that at the end of the study the F-FOPS mice exhibited a significant reduction in body weight gain compared with the HF-fed control mice (Fig. 4B). F-FOPS mice also exhibited a significant reduction in epididymal, subcutaneous, and visceral adipose tissue weights compared with the HF-fed control mice (Fig. 4C-E). Remarkably, within the F-FOPS group three mice (6, 7, and 8) consistently showed adipose tissue weights that were similar to the LF-fed mice, while one mouse (3) consistently showed weights similar to the N-FOPS mice and the HF group. In contrast to F-FOPS mice, body weight gain and adipose tissue weights of the N-FOPS mice during the 8 week study mirrored the HF-fed control mice.

4.3. Influence of FOPS on blood glucose and insulin

HF feeding resulted in increased fasting bood glucose, fasting insulin, and HOMA-IR, accompanied by impaired glucose tolerance compared with LF feeding (Fig. 5), indicating metabolic aberrations related to impaired glucose tolerance in the HF-fed mice. The F-FOPS group showed significant decreases in fasting insulin accompanied by a 79% reduction in HOMA-IR compared with the HF-fed mice. Unlike body weight and adipose tissue data, mouse 3 did not stand out from the other FOPS mice but instead showed similar glycemic and insulinemic profiles to mice 6, 7, and 8 in the F-FOPS group.

4.4. Influence of FOPS on plasma hormones and lipids

HF feeding resulted in increased C-peptide and amylin compared with HF-fed mice (Fig. 6A and 6B), in concordance with the insulin data from the IPGTT. Among the markers analyzed in the multiplex assay, only resistin and leptin showed significant differences between HF and LF and were thus associated with metabolic disorder. There was a significant decrease in leptin in F-FOPS compared with HF and N-FOPS and a significant decrease in resistin in F-FOPS compared with HF (Fig. 6C and 6D). Mouse 3 in the F-FOPS group showed separation from mice 6, 7, and 8 for leptin but not for resistin. HF-fed mice showed significantly elevated plasma cholesterol and triacylglycerol concentrations compared with LF-fed mice; however, FOPS did not result in any improvements in these lipids (Fig. 7).

4.5. Influence of FOPS on fecal microbiota

α-Diversity of the fecal microbiota significantly decreased over time for all treatment groups, and the decrease was especially pronounced in the F-FOPS group (Fig. 8A). PCA based on OTU abundance showed no clustering by treatment group at baseline (Fig. 8B), whereas principle component 1 separated FOPS from HF and LF at week 1 (Fig. 8C), which persisted after 8 weeks (Fig. 8D). F-FOPS mice 6, 7, and 8 clustered separately from the other mice, while F-FOPS mouse 3 clustered with N-FOPS mice.

At week 0, all mice had similar microbial composition dominated by the *Lachnospiraceae* family (Fig. 9). The most remarkable change in microbiota composition over the course of the study was the substantial increase in OTUs belonging to the *Blautia* and *Akkermansia* genera in F-FOPS mice at week 8. Combined, these 2 OTUs represented approximately 80% of the fecal microbiota in F-FOPS mice 6, 7, and 8. Notably these are the same three mice that clustered together on the PCA plots (Fig. 8B-D), and explain the substantial decrease in α-diversity in these mice. In contrast, mouse 3, which was also in the F-FOPS group as it had an enlarged cecum, did not show elevated *Blautia* and *Akkermansia*. Interestingly, at week 0 all mice had very low abundance of *Blautia* (<1%), and, while abundance of this genus remained low in the LF and HF-fed mice (<0.01%), during the course of the study it increased in all FOPS-fed mice (<0.01% at week 0; 7.5% at week 1; 17.5% at week 8).

Differences in fecal microbiota proportions between N-FOPS and F-FOPS at each time point were calculated. At week 0, the F-FOPS mice had significantly higher proportions of OTUs 8 (*Peptococcaceae*) and 33 (*Acetivibrio*) compared with N-FOPS, while N-FOPS mice had a significantly higher abundance of OTU 34 (a member of *Lachnospiraceae* family) compared with F-FOPS (Fig. 10). At week 1, N-FOPS mice had significantly higher abundances of two OTUs belonging to the *Lachnospiraceae* family (OTU 7 and OTU 13) compared with F-FOPS mice. At week 8, F-FOPS mice had significantly higher proportions of OTU 1 (*Akkermansia*) and OTU 9 (*Blautia*), while N-FOPS mice had significantly higher proportions of two OTUs belonging to the *Lachnospiraceae* family (7 and 13).

4.6. Correlation of microbial composition with host metabolic markers

Body weight gain, leptin, and the weight of EAT and SAT were negatively correlated with *Akkermansia, Blautia* and OTU 89 (a member of the *Ruminococcaceae* family; Fig. 11); plasma cholesterol was also negatively correlated with *Akkermansia*. There were positive correlations between SCFA production and the abundance of *Akkermansia, Blautia*, and OTU 89. OTU 6 and in most cases OTU 20

(both members of the *Lachnospiraceae* family) were positively correlated with negative outcomes: body weight gain, fasting glucose, C-peptide, amylin, plasma cholesterol, leptin, resistin, and the weight of EAT and SAT.

5. Discussion

In this study, we investigated how FOPS affected body and tissue weights, markers of metabolic syndrome, and gut microbiota composition in C57BL/6J mice fed a HF diet. We observed disparate metabolic responses to the FOPS diet intervention. Enhanced cecal fermentation with associated metabolic improvements was evident in some mice (F-FOPS), while other mice showed normal cecal fermentation and no metabolic improvements compared with mice in the HF group (N-FOPS).

The F-FOPS group showed greater insulin sensitivity and lower fasting flood glucose compared with the HF-fed mice. These improvements in glucose homeostasis in the F-FOPS mice might be due to enhanced SCFA production from the FOPS fermentation. The mice in the F-FOPS group displayed substantial increases in cecal propionate production accompanied by decreased plasma resistin. Propionate has been associated with a reduction in the expression of resistin, a pro-inflammatory factor in adipose tissue [32]. Decreased resistin has been shown to improve glucose tolerance and insulin sensitivity in obese mice by enhancing insulin-mediated glucose disposal in muscle and adipose tissue [33]. Taken together, these factors may constitute a possible mechanism of the improvements in glucose metabolism in the F-FOPS mice.

F-FOPS mice also showed significant reduction in body weight gain compared with HF-fed mice. However, the mechanism responsible for weight gain reduction appears to be different from that responsible for improvements in glucose tolerance. The overall fecal microbiota structure of F-FOPS mice 6, 7, and 8, which displayed the greatest improvements in body weight gain, adipose weights, and leptin, clustered together and were dominated by *Akkermansia* and *Blautia*. In contrast, this increase in *Akkermansia* and *Blautia* was not evident in F-FOPS mouse 3, and this mouse also did not show improvements in any of the body or adipose tissue measurements or leptin, suggesting that these two bacterial groups may be involved in these specific improvements. Recent studies have demonstrated an inverse association between *Akkermansia* and *Blautia* and markers of obesity-related metabolic disorders [34-36]. The intake of fructans by obese mice increased the abundance of *Akkermansia* by ~100 fold with

concomitant reduction in fat mass gain and adipose tissue inflammation [37]. *Akkermansia* may affect host metabolism through the enhancement of gut barrier function or production of endocannabinoids that control inflammation [38]. In the case of *Blautia*, diet-induced obesity has been shown to strongly reduce the abundance of this genus in mice [39]. Another study suggested that the prevention of obesity and insulin resistance in HF-fed rats by berberine, a yellow pigment found in some plant tissues, may be partially mediated by *Blautia* [40]. Additionally, given that obesity can be linked to leptin resistance and that leptin is primarily involved in energy expenditure [41], the significant decrease in plasma leptin (especially in mice 6, 7, and 8) in F-FOPS suggested enhanced leptin sensitivity in these mice, which might be responsible for the decreased weight gain [41]. Plasma leptin was also negatively correlated with *Akkermansia* and *Blautia*. These findings suggest the importance of *Akkermansia* and *Blautia* in the metabolic improvements related to weight reduction with FOPS. This is in contrast to other reports showing that the metabolic disorders associated with HF feeding in mice may be modulated by shifting the gut microbiota toward that of lean mice using dietary prebiotics or probiotics [42, 43]. This could be due to differences in the types of dietary interventions introduced among studies.

It is unclear why the mice in the FOPS group responded so differently to the treatment. While the phenomenon of responders versus non-responders to prebiotic interventions has been described in human studies [44, 45], it is less common in inbred mouse studies (although not unheard of [46]). Once confirming that the differences in response to FOPS was not a cage effect, we hypothesized that the four mice in the F-FOPS group may have had different gut microbiota profiles at baseline compared with the N-FOPS mice that enabled them to utilize the FOPS substrate. As mentioned, the FOPS contained unusual linkages, including (1, 2) and (1, 3)-linkages connecting branched chain xylose and arabinose, which may have required the presence of uncommon carbohydrate-active enzymes to initiate fermentation [47]. Bacteria capable of expressing such enzymes may allow for the fermentation of FOPS and subsequent changes in the microbiota by cross feeding. Diversity and PCA plots suggested that the overall community structures between N-FOPS and F-FOPS were similar; however, there were subtle differences that may have been meaningful. For instance, *Acetivibrio cellulolyticus*, which was elevated in the F-FOPS mice, can secrete an α-L-arabinofuranosidase [48], a major structural component of FOPS. Alternatively, functional differences,

not detectable through 16S rRNA community profiling, may have existed between the FOPS-fed mice that exhibited an ability to ferment FOPS and those that did not.

Other environmental factors might have also been involved, for instance the frequency and quantity of chow consumed by individual mice could have affected the fermentation patterns of FOPS, although cage 12, which had two F-FOPS, did not appear to have a marked abnormal feed consumption compared to the other cages. Alternatively, mice parentage may be another factor; one study suggested that mouse siblings share similar microbiotas compared with non-siblings [49]. Perhaps F-FOPS mice were closely related and N-FOPS mice were closely related but different from F-FOPS. Further, differences in regulatory mechanisms, such as developmental epigenetic changes, which can dynamically respond to environmental and nutritional exposure [50], might also be a reason for the differences in response to FOPS.

One interesting consideration is that the study duration may have not been long enough to observe a response to FOPS in all mice; it was only long enough to see it in some mice. We observed that all mice in the FOPS group (both N-FOPS and F-FOPS) had a noticeable abundance of *Blautia* after 1 week of the FOPS treatment, while none of the LF- or HF-fed mice did. This suggests that FOPS may select directly for *Blautia.* The bloom in *Blautia* may then create favorable conditions for the growth of *Akkermansia* by consuming hydrogen [51], a gas that inhibits the fermentation pathways used by *Akkermansia* for production of acetate and propionate [52, 53]. Extrapolating this line of reasoning, it may be that the bloom in *Akkermansia* might simply be delayed in some mice; should the experiment have extended for longer than the 8-week time frame we may have eventually seen a bloom in *Akkermansia* in all FOPS mice. Admittedly, this is speculative and we do not have an explanation as to why mice would differ so greatly in their temporal response to FOPS. However, in our present results, one mouse (6) showed the bloom in *Blautia* and *Akkermansia* very rapidly (after only 1 week), while the other two that showed this phenotype (7 and 8) took longer.

Taken together, we observed heterogeneous metabolic adaptation of mice to a FOPS intervention. Although we cannot provide an explanation for the different responses to FOPS treatment, we conclude that the metabolic improvements related to glucose tolerance induced by FOPS in obese mice might be a consequence of colonic fermentation of FOPS and production of SCFA, while the metabolic improvements related to weight reduction might be linked to blooms in *Blautia* and *Akkermansia*. Our findings provide

new evidence for modulating the gut microbiota through dietary fiber treatment and indicate its contribution to the improvement of host metabolism.

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Table 1.Composition of FOPS [a]

Component	Composition
Total carbohydrate (%)	74.4
FOPS (%)	59.1
Arabinan (%)	10.13±0.19
Xylan (%)	33.55±1.29
Mannan (%)	0.30±0.00
Galactan (%)	4.71±0.21
Glucan (%)	4.44±0.56
Esterified ferulate (%)	4.13±0.03
Uronic acid	1.88±0.08
Free monosaccharides (%)	3.44±0.09
Arabinose (%)	1.28±0.02
Xylose (%)	1.06±0.06
Mannose (%)	ND[b]
Galactose (%)	0.53±0.01
Glucose (%)	0.58±0.03
Starch (%)	15.98±0.51
Free ferulic acid (%)	ND
Protein (%)	2.62±0.05
Furfural (%)	ND
HMF[c] (%)	ND
Moisture content (%)	8.77

[a] Values are reported as mean± standard deviation (n=2);

[b] None detected;

[c] Hydroxymethylfurfural.

Table 2. Nutrient composition of diets fed to male C57BL/6J mice.

Diet	LF[a]	HF[a]	FOPS[a]
Ingredient%			
Casein	19.0	25.8	25.1
L-Cystine	0.284	0.388	0.381
Corn Starch	52.1	0	0
Maltodextrin 10	14.2	16.2	14.4
Sucrose	0	8.89	8.73
Cellulose	4.742	6.46	1.32
Crude FOPS[b]	0	0	8.51
Lard	1.90	31.7	31.1
Soybean Oil	2.37	3.2	3.17
Mineral Mix S10026	0.948	1.29	1.27
Dicalcium Phosphate	1.23	1.69	1.65
Calcium Carbonate	0.521	0.711	0.698
Potassium Citrate, 1 H_2O	1.56	2.13	2.09
Vitamin Mix V10001	0.948	1.292	1.27
Choline Bitartrate	0.190	0.258	0.254
Red Dye #40, FD&C	0.00237	0	0.00635
Blue Dye #1, FD&C	0.00237	0.00646	0
Weight%			
Protein	19.2	23.1	22.7
Carbohydrate	67.3	26.3	25.9
FOPS	0	0	5
Fat	4.30	35.2	34.6
Fiber	4.70	6.50	6.30
Crude FOPS	0	0	8.50
kcal%			
Protein	20	18	18
Carbohydrate	70	20	20
Fat	10	62	62
Total	100	100	100
kcal/gm	3.90	5.10	5.10

[a] LF: low fat; HF: high fat diet; FOPS: high fat diet with FOPS; LF (D12450K) and HF formulation were based on D12492 from Research Diet.

[b] FOPS preparation is the final product obtained after freeze-drying process and contains a portion of FOPS.

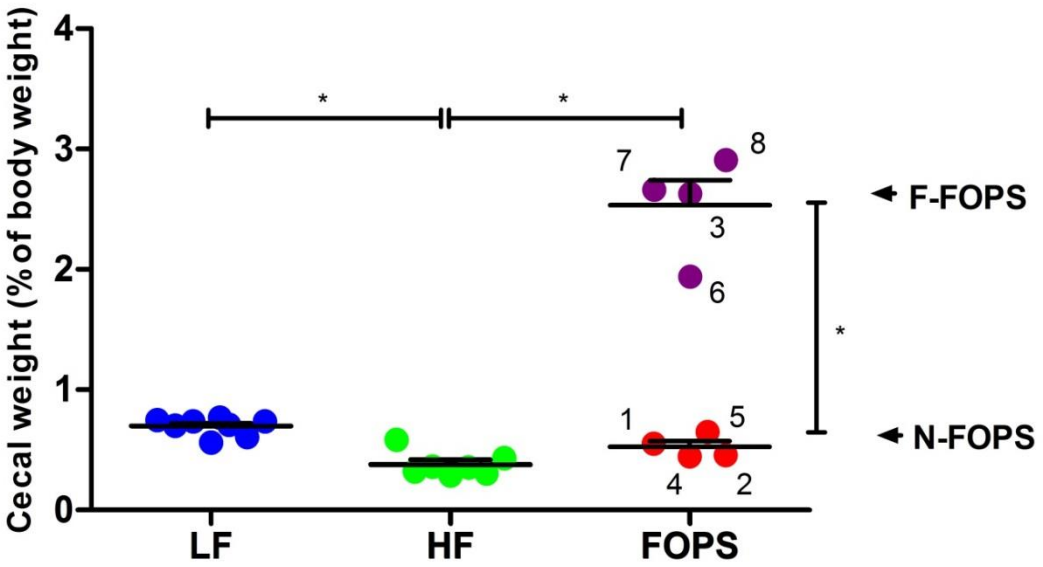

Figure 1. Cecal weights of mice in each treatment group; mean ± SEM; n = 8 mice/group for LF, HF, and FOPS; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. F-FOPS; N-FOPS vs. F-FOPS.

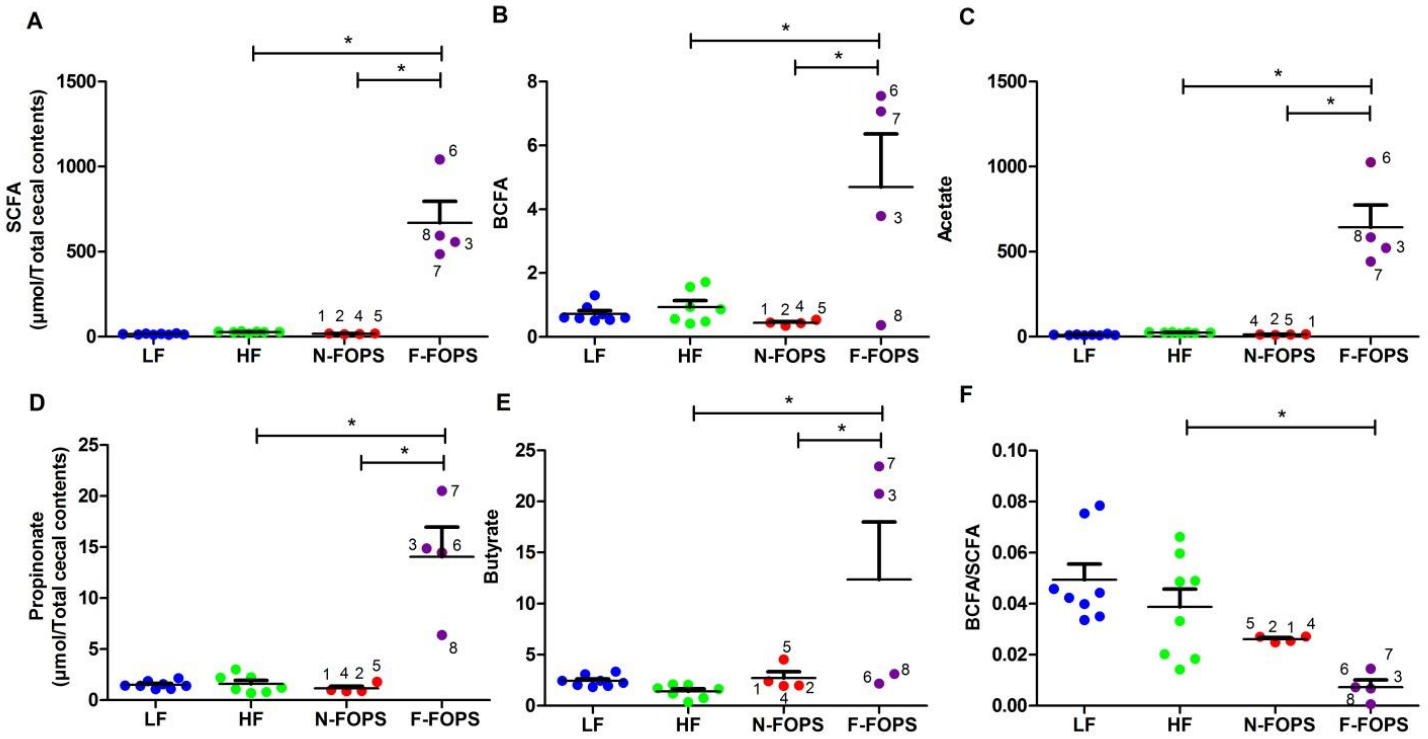

Figure 2. Cecal SCFA (A), BCFA (B), acetate (C), propionate (D), butyrate (E), and BCFA/SCFA ratio (F) of mice in each treatment group; mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. F-FOPS; F-FOPS vs. Y-FOPS.

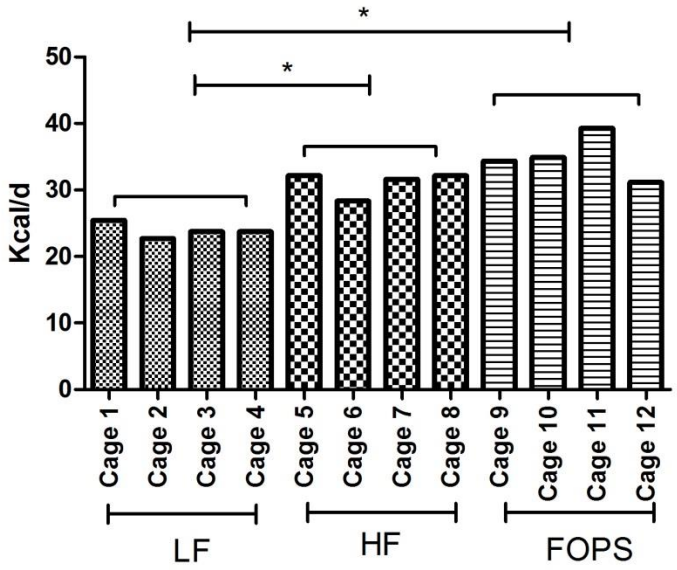

Figure 3. Food consumption of different groups. *p<0.05. Data are shown as individual observations.

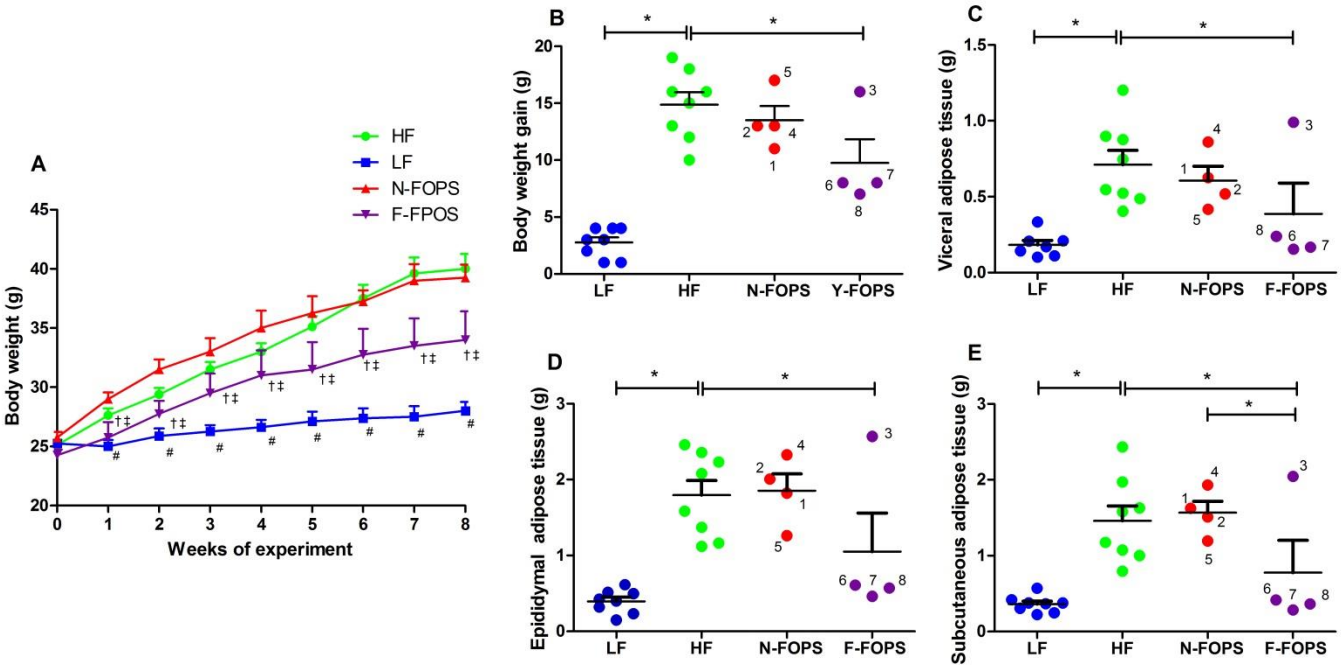

Figure 4. Body weight (A), body weight gain (B), and visceral (C), epididymal (D), and subcutaneous (E) adipose tissue weights at week 8 by treatment group; mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. F-FOPS; N-FOPS vs. F-FOPS; #, $p<0.05$ vs. HF; †, $p<0.05$ vs. HF; ‡, $p<0.05$ vs. N-FOPS.

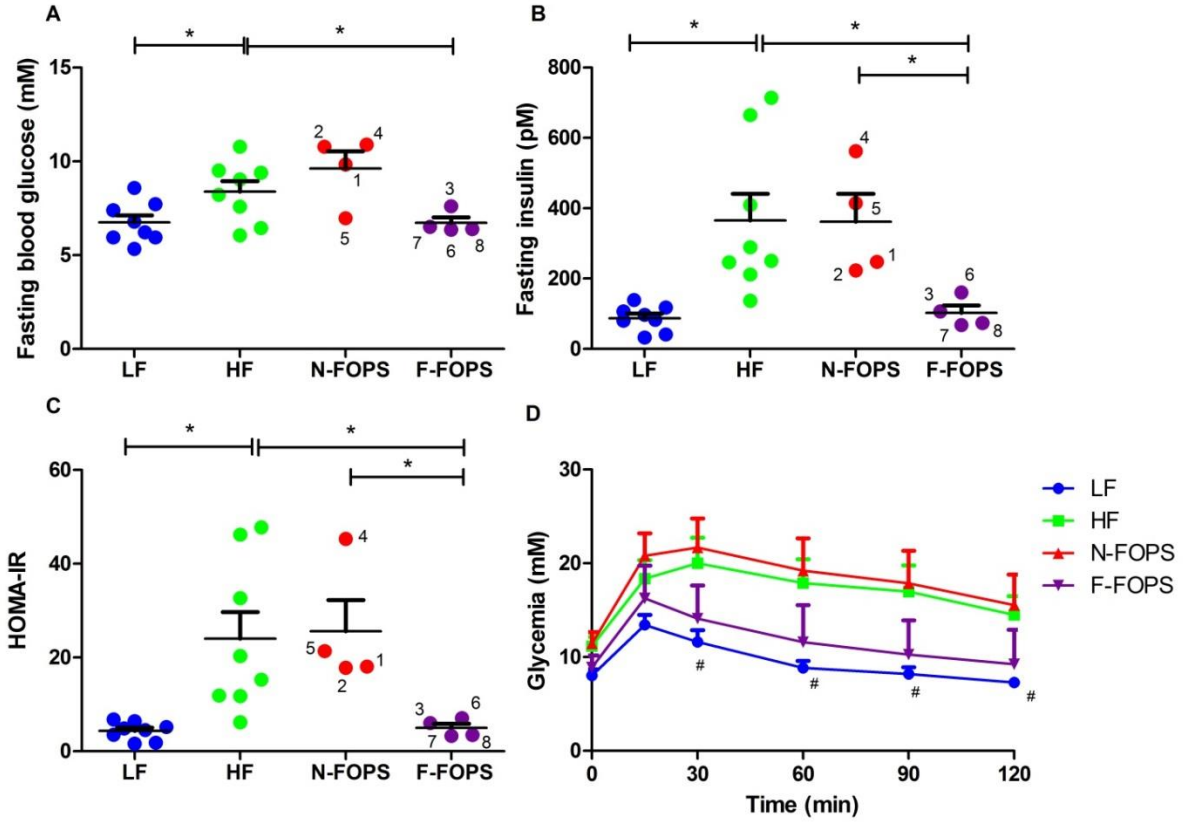

Figure 5. Fasting glycemia (A), fasting insulinemia (B), HOMA-IR (C), and glucose tolerance curve (D) at week 7 by treatment group; mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. F-FOPS; F-FOPS vs. Y-FOPS; #, $p<0.05$ vs. HF.

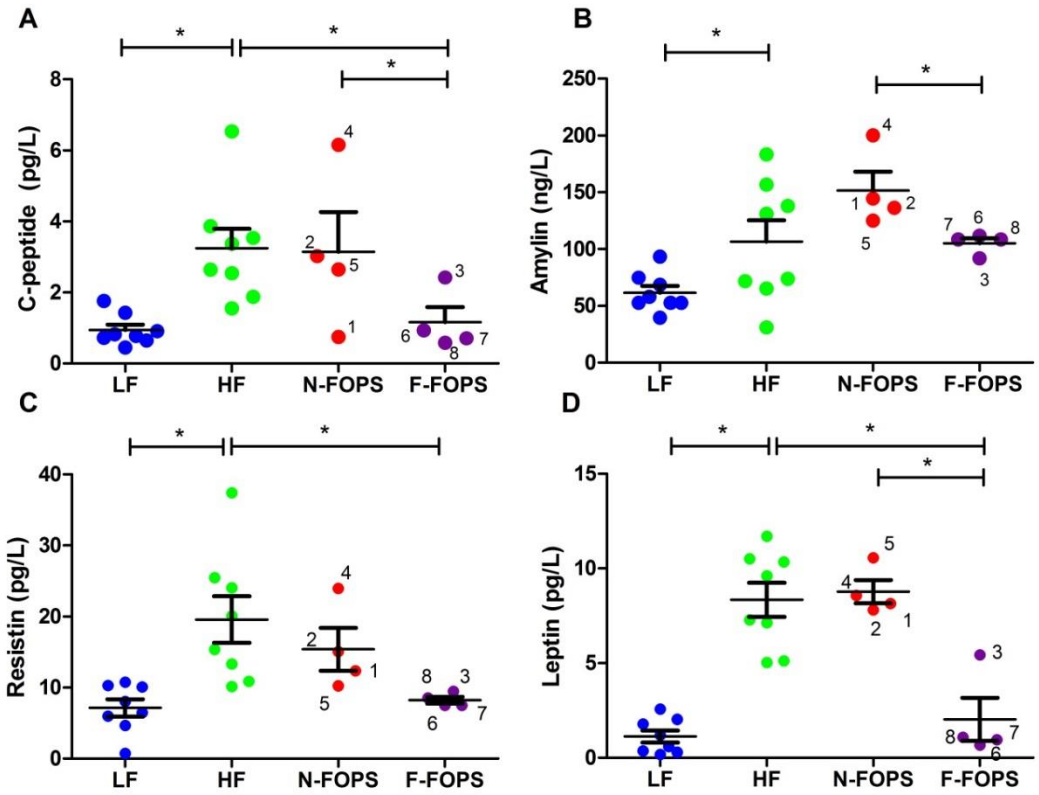

Figure 6. Plasma C-peptide (A), amylin (B), resistin (C), and leptin (D) in different treatment groups; mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. F-FOPS; N-FOPS vs. F-FOPS.

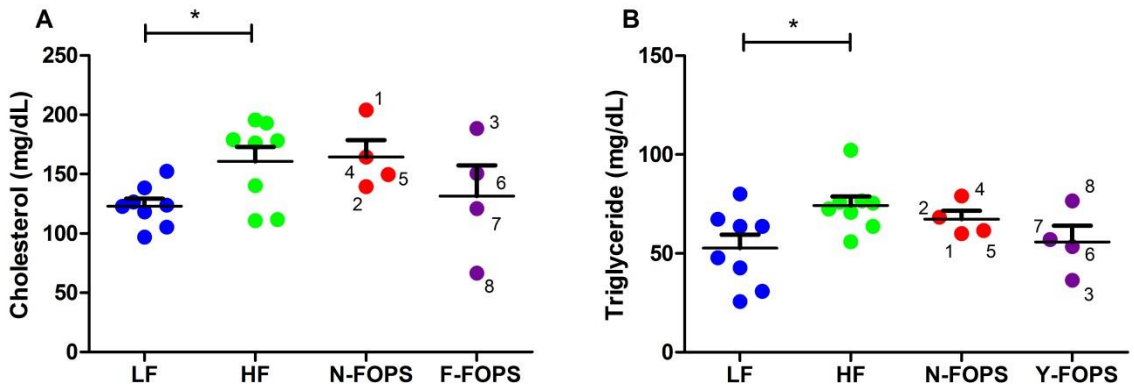

Figure 7. Plasma cholesterol (A) and triglycerides (B) in different treatment groups mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures.*significantly different using Bonferroni's multiple comparison test with four relevant comparisons: LF vs. HF; HF vs. N-FOPS; HF vs. Y-FOPS; N-FOPS vs. Y-FOPS.

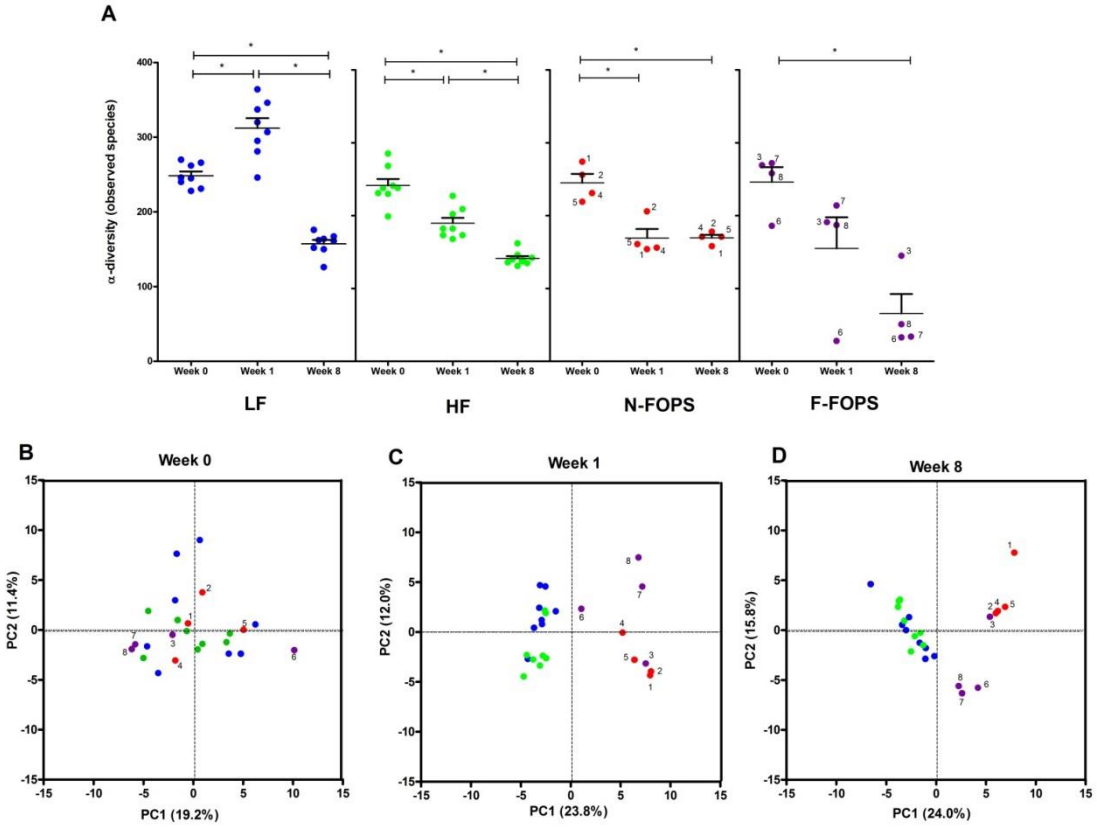

Figure 8. α-Diversity of the fecal bacterial communities (A) and principal components analysis of the abundance of OTU (B-D) at weeks 0, 1, and 8; in (A) mean ± SEM; n = 8 mice/group for HF and LF; n = 4 mice/group for N-FOPS and F-FOPS; in (B-D) blue dots represent LF; the green dots represent HF; the red dots represent N-FOPS; the purple dots represent F-FOPS; mouse numbers are shown for FOPS mice to track individual mice across figures; *p<0.05.

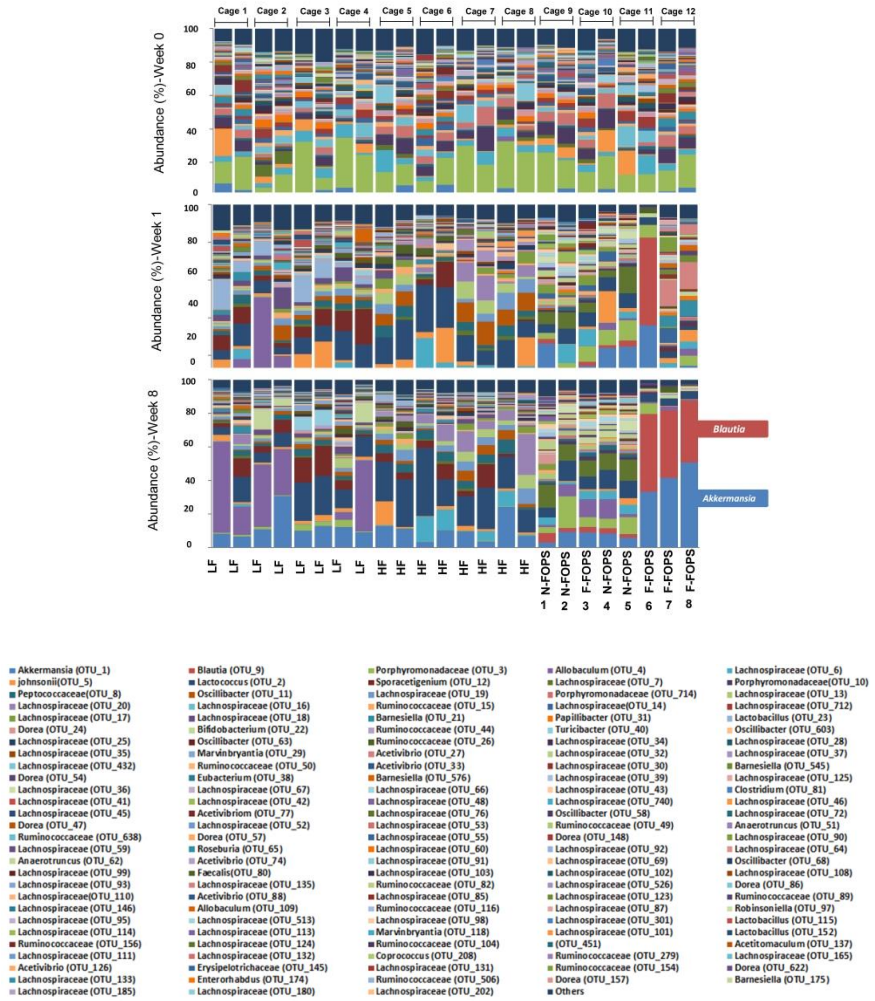

Figure 9. Fecal microbiota composition based on OTU abundance in different mice at weeks 0, 1, and 8; mouse numbers are shown for FOPS mice to track individual mice across figures.

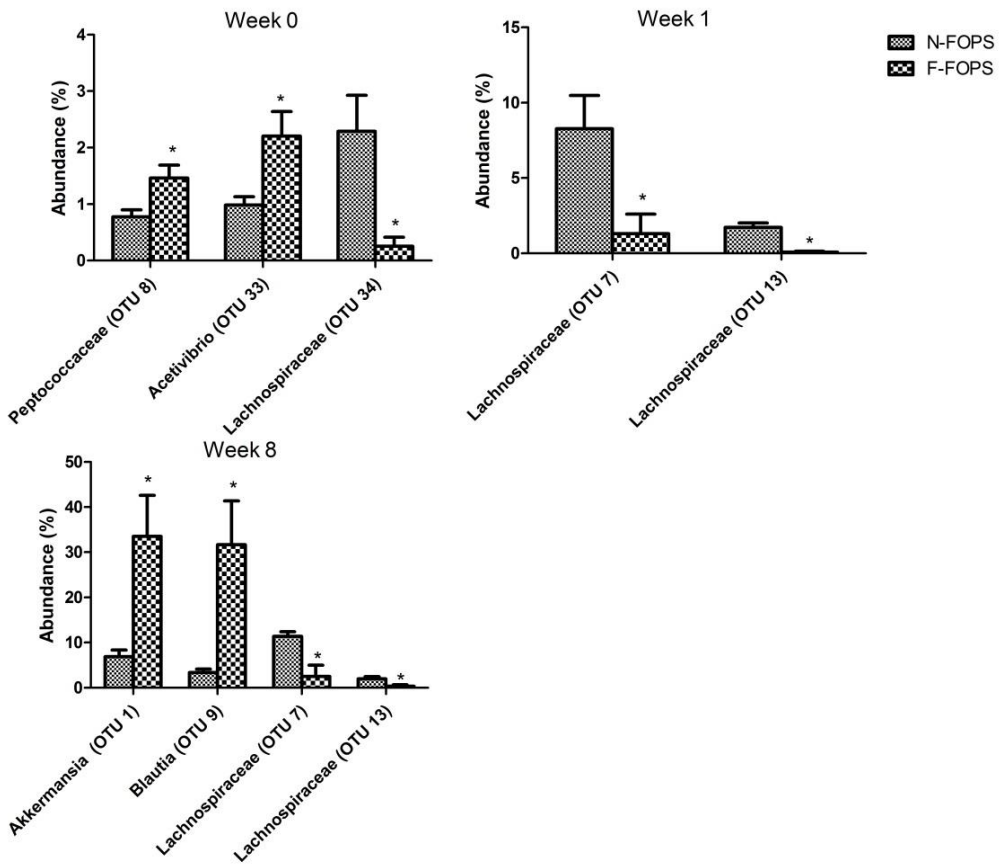

Figure 10. Abundance of bacterial taxa between NFOPS and YFOPS that showed significant differences at week 0, week 1, and week 8. *Significantly different from corresponding NFOPS group ($p<0.05$). Data are means ± SEM; n = 4 mice/group.

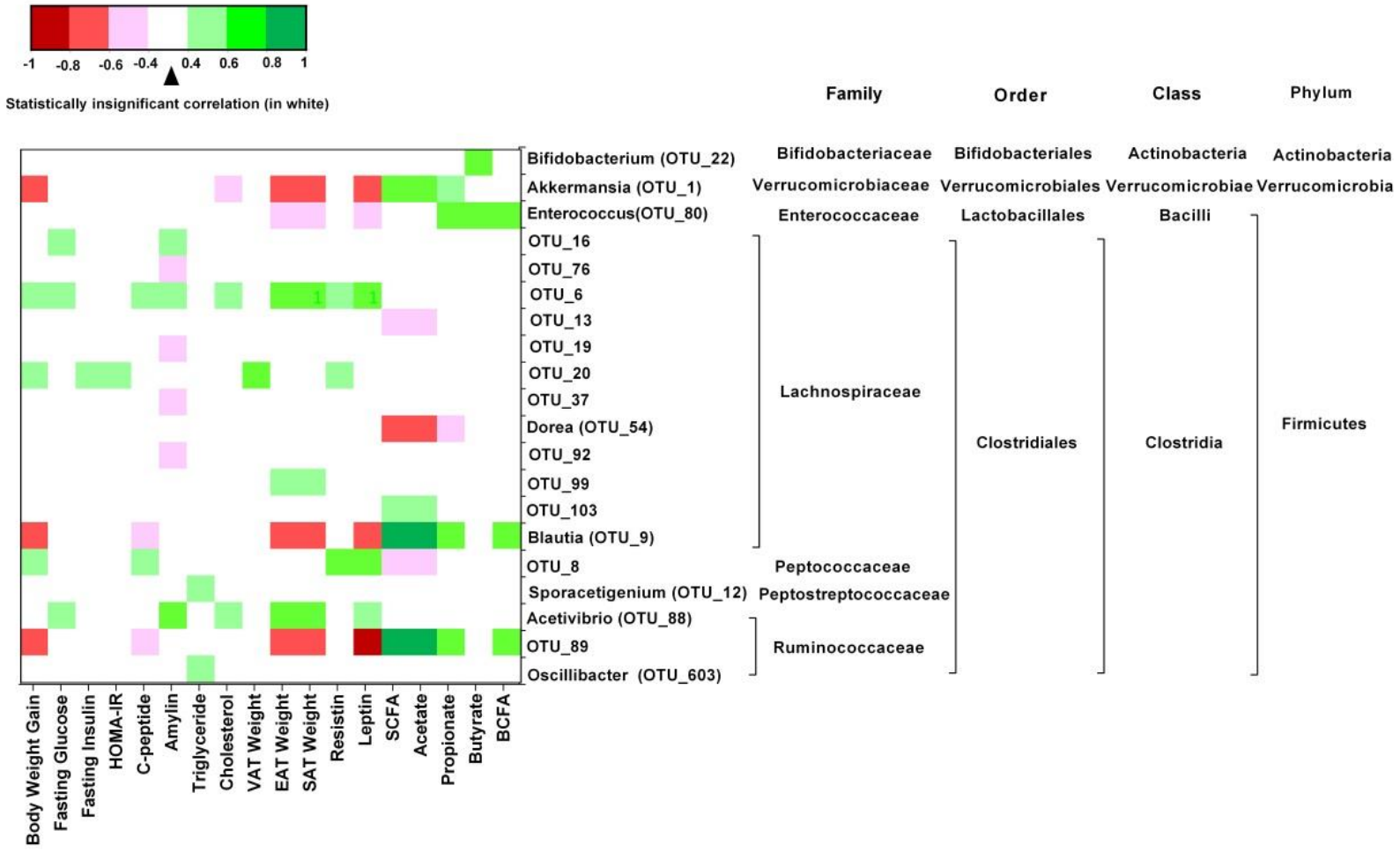

Figure 11. Correlations between relative abundance of OTUs and output variables in different groups; only statistically significant correlation are shaded ($p<0.05$).

Chapter 4 . Long-term dietary pattern of fecal donor correlates with butyrate production and markers of protein fermentation during *in vitro* fecal fermentation

1. Abstract

Diet influences gut microbiota composition. Therefore, we hypothesized that diet would impact the extent of dietary fiber utilization and the types of metabolic end-products produced by the microbiota during *in vitro* fecal fermentation. By obtaining long-term dietary records from fecal donors, we aimed to determine the correlations between dietary intake variables and dietary fiber degradation, short-/branched-chain fatty acid (BCFA) production and ammonia production during *in vitro* fecal fermentation. Eighteen subjects completed 1-year diet history questionnaires and provided fecal samples that were used for in vitro fermentation of a whole wheat substrate. The percentage of dietary fiber fermented was not correlated with nutrient intakes; however, butyrate production was correlated with fecal donor intake of many nutrients of which principal component analysis revealed were mostly contributed by grain-, nut-, and vegetable-based foods. Negative correlations were found for propionate with intake of total carbohydrate, added sugar and sucrose, and for ammonia and BCFA production with intake of unsaturated fats. Thus, our analysis did not support our first hypothesis: the percentage of dietary fiber fermented during in vitro fermentation was not correlated with dietary records. However, production of butyrate; BCFA; ammonia; and, to a lesser extent, propionate was correlated with the diet records of fecal donors, thus supporting our second hypothesis. These results suggest that diets high in plant-based foods and high in unsaturated fats are associated with microbial metabolism that is consistent with host health.

2. Introduction

The human gut is colonized by the gut microbiota, a complex and dynamic microbial community whose collective genome exceeds the size of the human genome by 2 orders of magnitude [1]. The gut microbiota is involved in host energy harvest and storage [2], immune response [3], and development of metabolic syndrome [4].

Previous reports have focused on how diet changes the gut microbiota composition [5], [6], [7] and [8]. One study found that the fecal microbiota from children in Burkina Faso consuming a diet high in dietary fiber contained an enrichment of *Prevotella* and *Xylanibacter*, which contain species that are evolved to efficiently use cellulose and xylans, compared with children in the European Union consuming more refined diets [5]. In another cross-sectional study, long-term intake of protein and animal fat led to enrichment in *Bacteroides* compared with long-term intake of carbohydrates, which is associated with *Prevotella* [8]. Controlled trials have shown that diet can rapidly alter the types of bacteria that appear in the feces. Russell et al [7] showed a decline in *Roseburia*/*Eubacterium rectale* when subjects were on a high-protein, low-carbohydrate diet compared with a maintenance diet. The susceptibility of these butyrate producers to carbohydrate intake was confirmed in subjects consuming diets devoid of carbohydrate [6].

Although knowledge on the compositional changes in the gut microbiota is of interest, the products of gut microbiota metabolism are of at least equivalent importance. For instance, short-chain fatty acids (SCFAs), the major metabolites produced by gut microbiota, have been suggested to be important regulators of energy balance, gut inflammation signaling, and insulin sensitivity [7]. Conversely, ammonia and branched-chain fatty acid (BCFA) productions have been implicated as having undesirable effects on host health [7] and [9].

Past approaches to quantify these metabolites have included analysis of fecal samples for the compounds themselves or for genes involved in their production [7], [10] and [11]. In addition, others have drawn conclusions about metabolite production based on the proportions of bacteria present [12] and [13]. However, given that these metabolites are absorbed and metabolized by the colonic epithelia cells, these approaches either do not reflect the actual production or are only semiquantitative [14] and [15].

When addressing changes in the gut microbiota in response to diet, a wealth of research has concentrated on host benefits of prebiotic oligosaccharide consumption (eg, fructans,

galactooligosaccharides) [16]. These prebiotics are highly fermentable by the gut microbiota and result in desirable shifts in gut microbiota composition. However, intake of these oligosaccharides in most diets (1-4 g/d) [17] is dwarfed by the quantities of complex and poorly fermented dietary fibers (eg, cellulose, cross-linked arabinoxylan, and complex pectic substances) that typically make up the substrates for gut bacteria (11-20 g/d) [18]. Although these dietary fibers are generally poorly fermented by gut bacteria, it could be that adequate and continuous dietary exposure of these materials to the gut microbiota would cause compositional shifts such that the microbiota are more able to use these substrates with subsequent benefits to the host [5].

Unfortunately, quantitative measures of fermentation of specific dietary fibers and resulting SCFA production by the gut microbiota in humans are not practical in vivo. Although conditions in in vitro batch fermentations are very different from conditions in the large intestine (eg, no absorption of metabolites, water, minerals, or other nutrients; no pH control [except buffering]; no mucus layer; etc), batch in vitro fecal fermentation models may be an ideal way to evaluate both the microbial production of certain metabolites, as well as utilization of complex substrates, because compounds are not absorbed and can easily be quantified [19]. Furthermore, in our previous study, we found that changes in the gut microbiota during 12 hours of *in vitro* fermentation were similar to changes reported in the literature for human trials [20].

We hypothesized that diet of the fecal donor would impact the extent of dietary fiber utilization and the types of metabolic end-products produced by the microbiota during in vitro fecal fermentation. To test our hypothesis, we assessed long-term dietary patterns of subjects using a food frequency questionnaire (FFQ) and then used stool samples collected from these individuals as a source of bacteria for in vitro fermentation using predigested whole wheat flour as a substrate. We then quantified carbohydrate fermented, SCFA, BCFA, and ammonia produced during fermentation and correlated the results with intake of nutrients and food groups of the stool donors. This research may allow us to identify dietary strategies to alter the production of metabolites by the gut microbiota in a manner that is consistent with human health.

3. Materials and methods

3.1. Subjects, dietary records, and stool sample collection

Individuals who were 19 years or older, considered themselves to be in generally good health with no digestive diseases or dietary restrictions (excluding voluntary dietary restrictions), and had not taken antibiotics in the last 6 months were recruited using electronic and paper advertisements on campus at University of Nebraska-Lincoln. Individuals who met these criteria were invited to participate in this study. Subjects completed the past-year (long-term) food intake diet history questionnaire (DHQ) II online [21]. The DHQ II is a validated FFQ consisting of 134 food items and 8 dietary supplement questions [22]. The survey included questions about seasonal differences in food intakes. Each subject received a unique login and password and completed the DHQ II after brief instructions on how to properly complete the questionnaire. Subjects' responses were analyzed using Diet*Calc software (Bethesda, MD, USA) [23]. This software used food frequency responses from subjects to estimate daily food intakes and serving sizes based on combined results from the National Health and Nutrition Examination Surveys (NHANES) conducted in 2001 to 2002, 2003 to 2004, and 2005 to 2006. Daily nutrient and food group intakes were generated using the DHQ Database [24], which computed nutrient estimates using data from the USDA Nutrient Database for Standard Reference [25] and the Nutrient Data System for Research [26]. The My Pyramid Equivalents Database [27] was used to calculate daily servings from various food groups.

At the time subjects received their login and password information for the DHQ II, they received stool collection materials and instructions on how to use them. Subjects were instructed to bring a stool sample to the laboratory within 2 hours of defecation. The stool collection materials included a collection container with tight-fitting lid that fit under the toilet seat (Commode Specimen Collection System; Fisher Scientific, Pittsburgh, PA, USA), an insulated cooler with cold packs, and an anaerobic gas-generating tablet (Anaerocult C; BD GasPak, Franklin Lakes, NJ, USA), which subjects placed inside the collection container immediately after defecation and before securing the lid and packing in the cooler. Immediately upon receiving stool samples from subjects, study personnel transferred the sample to an anaerobic hood (Bactron X; Sheldon Manufacturing, Cornelius, OR, USA) where it was packaged into a specimen bag (Fisher). The packaged fecal sample was then stored at −80°C until analysis.

All protocols involving human subjects were approved by the University of Nebraska-Lincoln's Institutional Review Board before initiation of the study (no. 20120512624EP). Subjects provided informed

consent and were modestly compensated for their involvement in this study. Subjects received compensation for completing each of the 2 tasks separately (completion of DHQ II and stool sample donation); they were not coerced into completing both tasks. Thirty-one individuals completed the DHQ II, and of those 31 individuals, 24 also provided fecal samples. Four subjects were excluded from the study due to extreme reported energy intakes (<1300 kcal/d or >3500 kcal/d), and 2 subjects were excluded due to insufficient fecal sample for analysis. In total, 18 samples were used for in vitro fecal fermentation.

3.2. Whole wheat substrate

Hard red winter wheat (*Triticum aestivum* "McGill") was obtained from Husker Genetics, the University of Nebraska-Lincoln's Foundation Seed Division. Wheat was milled with a cyclone sample mill equipped with a 1-mm screen (UDY Corporation, Fort Collins, CO, USA).

The flour thus obtained was subjected to an *in vitro* digestion procedure as described [28]. Briefly, wheat flour (25 g) was mixed with 300 mL of water and boiled for 20 minutes with constant stirring. Upon cooling to room temperature, the pH was adjusted to 2.5 with 1 M HCl, and then 10 mL of 10% (wt/vol) pepsin (P-700; Sigma, St Louis, MO, USA) in 50 mM HCl was added. The mixture was placed on an orbital shaker (150 rpm) at 37°C for 30 minutes, whereupon 50 mL of 0.1 M sodium maleate buffer (pH 6, containing 1 mM $CaCl_2$) was added and the pH was adjusted to 6.9 with 1 M $NaHCO_3$. Fifty milliliters of 12.5% (wt/vol) pancreatin (P-7545; Sigma) in sodium maleate buffer and 2 mL of amyloglucosidase (3260 U/mL; Megazyme, Bray, Ireland) were then added, and samples were kept in a shaking water bath at 37°C for 6 hours.

After digestion, the slurry was transferred to dialysis tubing (molecular weight cutoff, 12 000-14 000) and dialyzed against distilled water for 4 days with a water change every 12 hours. The retentate was then freeze dried. The resulting material contained 2.16% ± 0.08% starch (mean ± SD, n = 3) and 63.1% ± 0.8% total dietary fiber, as measured by a total starch kit (K-TSTA; Megazyme) and by approved method 32-25 [29] with the following modifications: 5 μL of 2-octanol was added before the reduction step to minimize loss of ammonium hydroxide, and reduction time was increased from 60 to 90 minutes.

3.3. *In vitro* fermentation

In vitro fermentation of freeze-dried substrate with the fecal inocula was carried out following the method described by Yang et al [28] with the modification of sample size. In short, 15 mg of digested,

freeze-dried material was suspended in 1-mL sterile fermentation medium consisting of (per liter) peptone (2 g, BP1420-100; Fisher Scientific), yeast extract (2 g, CAS8013-01-2; Alfa Aesar, Ward Hill, MA, USA), bile salts (0.5 g, LP0055; Oxoid, Hampshire, UK), $NaHCO_3$ (2 g), NaCl (0.1 g), K_2HPO_4 (0.08 g), $MgSO_4.7H_2O$ (0.01 g), $CaCl_2.6H_2O$ (0.01 g), l-cysteine hydrochloride (0.5 g; Sigma), hemin (50 mg; Sigma), Tween 80 (2 mL), vitamin K (10 μL; Sigma), and 0.025% (wt/vol) resazurin solution (4 mL) and then hydrated overnight on ice. Tubes were then inoculated with 0.1 mL of fecal slurry, capped, and incubated at 37°C with shaking (140 rpm) for 12 hours. The fecal slurry was prepared by blending the fecal sample with sterile phosphate-buffered saline using a hand blender for 1 minute and then filtering through 4 layers of cheesecloth. All steps for fermentation were conducted in an anaerobic hood (Bactron IV; Sheldon Manufacturing) containing 5% H_2, 5% CO_2, and 90% N_2. Separate sample tubes were prepared for analysis of carbohydrates, ammonia, and SCFA/BCFA at time 0 and 12 hours of fermentation, and duplicate tubes were prepared for each analysis due to the insoluble nature of a portion of the whole wheat substrate. Thus, for each fecal sample, 12 sample tubes were fermented. At each designated time point, microbial metabolism was stopped according to different methods based on the analysis to be performed. In tubes designated for carbohydrates, microbial metabolism was terminated by adding 0.2 mL of 2 M H_2SO_4 containing 10 mg *myo*-inositol per milliliter as an internal standard. In tubes designated for SCFA/BCFA, microbial metabolism was terminated by adding 0.2 mL of 2 M KOH containing 7 mM 2-ethyl-butyrate as an internal standard (the KOH also had the added benefit of preventing volatilization of the SCFA/BCFA). In tubes designated for ammonia, microbial metabolism was terminated by adding 0.2 mL 5.2 mM $CuSO_4$.

3.4. Fermentation analyses

Samples for carbohydrate analysis were freeze dried, and the residue was subjected to the hydrolysis and derivatization steps using approved method 32-25 [29] with the modifications described above, as well as proportionate scaling down of reagents to match the smaller sample size. Total neutral polysaccharides were the sum of all sugar residues analyzed (arabinan, xylan, mannan, galactan, and glucan), arabinoxylan was the sum of arabinan and xylan, and cellulose was the sum of nonstarch glucan.

Samples for SCFA/BCFA were thawed and centrifuged (10000*g*, 5 minutes). The supernatant was then used for analysis of SCFA after Hartzell et al [30]. In short, 0.5 mL of supernatant was mixed with approximately 0.4 g of NaCl and 0.2 mL of 9 M H_2SO_4. Tubes were capped and shaken vigorously, and

then 0.4 mL of diethyl ether was added. Tubes were capped and inverted 20 times, and then 1 μL of the diethyl ether extract was injected onto a gas chromatograph (Clarus 580; PerkinElmer, Waltham, MA, USA) equipped with a capillary column (Elite-FFAP, 15 m × 0.25 mm inner diameter × 0.25 μm film thickness; PerkinElmer) and detected with a flame ionization detector. Short-chain fatty acid/BCFA was quantified by calculating response factors for each SCFA/BCFA relative to 2-ethyl butyric acid using injections of pure standards.

Samples for ammonia analysis were centrifuged (8000*g*, 5 minutes), and the supernatant was assayed for ammonia using the phenol hypochlorite method [31]. In short, supernatants were diluted to less than 75 μM ammonia (1:100-1:300), and 1 mL of the diluted sample was mixed with 40 μL of phenol-alcohol solution (1 mL liquefied phenol diluted to 10 mL with absolute ethanol), 40 μL of 0.2% (wt/vol) sodium nitroprusside, and 0.1 mL of freshly prepared oxidizing solution (prepared by combining 4 parts of 20% trisodium citrate in 1% NaOH and 1 part of 13% sodium hypochlorite). After 1 hour, the absorbance was read at 650 nm. Quantification was accomplished by means of external calibration with solutions of ammonium chloride (0-75 μM).

4. Statistical analysis

All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). Data for carbohydrate fermentation and metabolite production were analyzed using 1-way analysis of variance with subject as the factor using the GLM procedure. For carbohydrate fermentation and metabolite production analysis of variance models with significant F values ($P < .05$), pairwise comparisons were calculated by the least significant difference procedure. Pearson correlations among percent carbohydrate fermented and microbial metabolites produced during fermentation were computed using the CORR procedure. For correlations of dietary intake of each fecal donor with metabolite production or carbohydrate degradation during in vitro fermentation, Spearman correlation coefficients were computed using mean values from each subject using the CORR procedure and correcting for age and sex. Correlations among dietary intake categories that carried significant correlations with butyrate production were also calculated. These data were also used for principal component analysis (PCA) using the PRINCOMP procedure with a rank transformation (generated using PRINQUAL). Correlations of each principal component with butyrate and with food category intakes were calculated.

5. Results and discussion

5.1. Subjects and dietary records

Of the 18 subjects whose fecal samples were used in the in vitro fermentations, 11 were male and 7 were female (Table 1). Subject IDs were used to track the 18 individuals. The age range was from 20 to 37 years with a mean age of 26.7 ± 5.4 years. Subjects ranged greatly in reported intake of nutrients and other compounds. As expected from NHANES data [18], intake of total fat, saturated fat, cholesterol, protein (of which most was animal protein), and sodium was generally above recommendations, whereas intake of total carbohydrate, dietary fiber, vitamin D, magnesium, and potassium was below recommendations. Subject 15 was noted for having low consumption of energy and fat, whereas subject 1 was noted for the highest intake of many of the micronutrients.

Intake of foods from specific categories was also typical of a US population (Table 2). Reported intakes of total and refined grain, dairy, and meat dominated the diets. Other common food categories included total vegetables, fruit, poultry, and nuts.

5.2. Carbohydrate fermentation and metabolite production

Disappearance of the principal dietary fibers in the whole wheat substrate over the 12 hours of *in vitro* fermentation was monitored (Fig. 1). Clearly, there was great variation in the ability of each fecal microbiota to use the substrate. The fecal microbiotas from subjects 13, 16, and 65 were among the least able to ferment the dietary fibers in wheat, whereas the fecal microbiotas from subjects 21, 27, and 98 used the most. The fecal microbiotas from subjects 1 and 27 were the most efficient at fermenting both arabinoxylan and cellulose.

As with the carbohydrate fermentation data, SCFA production varied greatly (Fig. 2). Interestingly, however, SCFA production was not correlated with the amount of carbohydrate fermented in any instance (*P* values for correlations ranged from .08 [for cellulose fermentation vs acetate production] to .82). The fecal microbiota from subject 99 produced the most total SCFA, which was composed of mostly acetate and propionate; this microbiota was among the lowest in butyrate production. The fecal microbiota from subject 11 produced much more butyrate than any other microbiota. Several other fecal microbiotas, including those from subjects 1, 5, 16, 19, 25, 26, and 27, produced butyrate at concentrations ranging from 33% to 57% of that produced by the microbiota from subject 11.

Like the SCFA data, markers of protein fermentation did not correlate with the percentage of carbohydrate fermented (*P* values ranged from .49 to .97) but did show interesting variation among fecal microbiotas (Fig. 3). The fecal microbiota from many subjects, including 1, 4, 5, 7, 11, 12, 24, 25, 65, and 98, produced very low concentrations of BCFA during the fermentation period, whereas the fecal microbiota from other subjects, particularly that of subject 21, produced appreciable concentrations of BCFAs. Production of these metabolites was highly correlated with ammonia production (***P*** = .0011, .0015, and .0009 for ammonia vs ***iso***-butyrate, ***iso***-valerate, and BCFA, respectively). We also performed the correlation analysis by sex, and the results showed most correlations found before were driven by men only (data not shown here). Thus, it would be an interesting area to investigate the difference response of gut microbiota from different sexes in the future.

5.3. Correlation between dietary records and carbohydrate fermentation and metabolite production

No correlations between the amount of carbohydrate fermented and intakes of any of the nutrients or other compounds analyzed were found (Fig. 4). However, correlation analysis for intake of nutrients and other compounds vs metabolite production during fermentation was quite interesting. Many significant positive correlations were discovered for butyrate production during fermentation. When correlations were analyzed by sex, most nutrients remained significant for men but not for women. Among the rest of the SCFA, significant negative correlations were noted for total carbohydrate, added sugar, and sucrose intakes vs propionate production. Branched-chain fatty acid and ammonia data showed significant negative correlations with fat intake, in particular, with unsaturated fat intake.

A few correlations were noted for intake of certain food categories and carbohydrate fermented and metabolite production during fermentation (Fig. 5). Butyrate again carried the most significant correlations, including positive correlations with “total vegetable,” “potatoes,” “other vegetables,” and “fish high in omega-3s,” and a negative correlation with “eggs.” “Total grain,” “whole grain,” and “refined grain” intakes also tended to be correlated with butyrate (Spearman $\boldsymbol{\rho}$ = 0.43, 0.45, and 0.45; ***P*** = .10, .08, and .08, respectively). The relevance of some of these correlations is limited due to low reported intake (eg, potatoes, fish high in $\boldsymbol{\omega}$-3 fatty acids, “soy products,” and “dry beans and peas”; Table S2) or poor range of intakes (eg, whole grain and eggs) in some of these food categories.

The fecal microbiota from subjects that consumed diets high in whole grains did not appear to be particularly specialized in degrading the whole wheat substrate. For instance, subject 1 had the highest whole grain intake and subject 24 the lowest, whereas the percentage of arabinoxylan and neutral polysaccharides fermented between the 2 subjects was not significantly different.

5.4. Principal component analysis of nutrients and other compounds that were correlated with butyrate production

The nutrients and other compounds that were significantly correlated with butyrate were also significantly correlated with each other in most cases (Table S3). Thus, we desired to look at these nutrients as a whole, rather than individually. Therefore, we used PCA to condense these highly correlated nutrients and other compounds into fewer variables. With the exception of cholesterol, intake of all of these nutrients carried similar positive Eigenvectors on principal component 1 (0.65-0.95), which accounted for 70% of the variation in nutrient intake. Because principal component 1 explained so much of the variation in nutrient intake and was the only principal component that was significantly correlated with butyrate production (r = 0.65; P = .003), we used this principal component as a metric to identify foods that contributed to high intakes of these nutrients. Intake of total grain, refined grain, whole grain, "nuts and seeds," total vegetables, and "other vegetables" were the greatest contributors to high intakes of nutrients that were significantly correlated with butyrate production during fermentation (Fig. 6).

6. Discussion

In this study, we hypothesized that (1) the long-term dietary pattern of the fecal donor would impact the extent to which the microbiota could use a particular substrate, which, in turn, would (2) impact SCFA, BCFA, and ammonia production during in vitro fecal fermentation. Our analysis did not support our first hypothesis: the percentage of dietary fiber fermented during in *vitro* fermentation was not correlated with dietary records. However, production of butyrate; BCFA; ammonia; and, to a lesser extent, propionate was correlated with the diet records of fecal donors, thus supporting our second hypothesis. This suggests that diet does not influence the extent to which dietary fiber is fermented, but rather the types of bacteria that ferment it (which led to the differences in metabolite production).

More specifically, our study revealed 2 significant findings. First, butyrate production during in vitro fermentation was correlated with intake of many macronutrients and micronutrients. This was

accompanied by a noticeable dearth of correlations with other SCFAs (except for a limited number with propionate), suggesting that butyrate production by gut bacteria is highly influenced by diet, with production of the other SCFA much less affected. The correlations with butyrate production are important because butyrate possesses anti-inflammatory properties [32]. Butyrate regulates host intestinal barrier function [33] and may prevent passage of lipopolysaccharide, a detrimental microbial metabolite, from the gut lumen into systemic circulation [15].

The nutrients and dietary compounds that were correlated with butyrate production clearly pointed to foods of plant origin. Correlations with all types of dietary fibers (total, soluble, and insoluble) demonstrated the dependence of butyrate producers on adequate dietary fiber consumption, a finding that is supported by clinical trials showing that butyrate producers are highly dependent on adequate dietary carbohydrate substrates [6] and [7]. Many other micronutrients were also correlated with butyrate production. Although a diet high in dietary fiber-containing foods would undoubtedly also be high in these micronutrients, which was noted by significant correlations among intakes of such nutrients, it is nevertheless interesting to consider the dependence of the gut microbiota not only on dietary fiber but also on dietary vitamins and minerals as well. Only a fraction of the vitamins and minerals consumed in the diet are absorbed in the small intestine [34]; therefore, many of these nutrients make their way to the large bowel where they influence the gut microbiota [35], [36], [37] and [38]. Because of the dense microbial community in this region, it is likely that these nutrients would be in high demand and the less adaptable bacteria would be more susceptible to lack of these compounds. Based on literature data, we might speculate that these vulnerable bacteria would be those bacteria that are considered beneficial, including bifidobacteria and the butyrate producers [6], [7] and [35].

Because butyrate was correlated with the intake of so many nutrients and other dietary compounds that were themselves correlated, we used PCA to determine the types of foods that contributed most to intake of these nutrients and other compounds. This approach revealed that intake of grains, nuts, and vegetables contributed most to intake of these compounds and thus might be important in maintaining butyrate production in the gut. This was consistent with our results from correlative analysis of butyrate production directly with reported intake of these food categories, although in the latter case, some of the correlations were not significant.

The second notable finding was the strong negative correlation of ammonia and BCFA production with intake of unsaturated fats. Although dietary triacylglycerols are typically considered completely digestible, the specific fatty acid composition of the diet influences the gut microbiota [39], [40] and [41]. In particular, studies in mice have shown that, compared with unsaturated fats, saturated fats persist into the distal small intestine where they exhibited an antimicrobial effect [39]. This antimicrobial effect can lead to decreased proportions of beneficial bacteria and a decrease in microbial diversity (an undesirable outcome) [39] and [42]. Our study suggests that a diet high in unsaturated fat may lead to a gut microbiota that produces fewer undesirable fermentation products.

Another notable finding was the negative correlation of propionate production with refined grains and added sugars. Typically, propionate is thought of as a beneficial SCFA [43] and [44]. However, our previous study showed that the fecal microbiota from obese individuals produces more propionate during in vitro fermentation of whole grains [28], and Schwiertz et al [10] reported higher propionate concentrations in feces of overweight and obese individuals. This was attributed to enrichment in *Bacteroides*, which are important propionate producers in the gut. Thus, the fecal microbiotas from subjects consuming diets high in refined grains and added sugars may have started out the fermentation with higher proportions of *Bacteroides*, which translated into higher propionate production during fermentation.

It is interesting to consider why there was a lack of an association between the percentage of dietary fiber used during fermentation and intake of dietary fiber in the diet. It may be that the types of bacteria that are favored when dietary fiber intake is low are also able to adapt quickly to this new substrate upon exposure to it. For instance, several studies have shown that diets that are very low or devoid of dietary fiber-containing foods lead to a bloom in *Bacteroides. Bacteroides* possess more genes that encode for dietary fiber-degrading enzymes than other gut bacteria and thus may be quick to adjust to metabolizing the dietary fibers in whole grains even if they rarely saw these substrates in abundance previously. Notably, we did not assess fermentation rate. Thus, it could be that during the early stages of fermentation, there was a lag in the dietary fiber fermentation rate by the fecal microbiotas from subjects consuming low dietary fiber diets, but by the time 12 hours of fermentation had transpired, these microbial communities had adapted to the whole wheat substrate such that the percentages of dietary fibers fermented were similar to the microbiotas from subjects with higher dietary fiber intakes.

It is well known that different types of dietary fiber impart different physiological effects [20]. For this study, we used whole wheat as the substrate. However, another substrate, such as fructans, resistant starch, or even dietary fibers isolated from vegetables or fruit, may produce different correlations with nutrient intake of the fecal donor with different implications than those reported herein.

We recognize that there were several limitations to this study. The major limitation was the use of an FFQ to assess dietary intake. This approach carried with it the limitations inherent in FFQs, such as differences in how accurately subjects answer the questions and how well the database used to calculate nutrient intakes reflects true nutrient intake [45]. Thus, reported dietary data may not quantitatively reflect actual intakes. Other FFQs or other methods of collecting food intake data, such as 24-hour recalls, may yield different intake results. In addition, some subjects may have had dietary practices that were not covered by the questionnaire, such as use of probiotics. Furthermore, overweight subjects have been reported to underestimate food intakes [46], which was not considered in this study. Another limitation was our small sample size. At the outset of the study, calculation of an appropriate sample size was difficult because there were no previous studies on how diet impacts the performance of the fecal microbiota in an in vitro model system. With a samples size of 18 and 2 covariates (age and sex), we were able to detect correlation coefficients of 0.64 with $\alpha = .05$ and $\beta = .2$. We anticipated that this would be an appropriate, if not small, sample size because similar correlations have been reported between dietary records and gut microbial taxa [8] and because correlations smaller than 0.64, although possibly statistically significant, may not be practically significant due to wide variation among individuals or limited physiological impact of the relationship. Finally, our findings are only correlative and do not imply causation. With these limitations acknowledged, we nevertheless felt that our study design was a reasonable approach to assess the possible relationships between human diet and metabolite production by the gut microbiota, which could serve as valuable information in the design of mechanistic studies and clinical trials on improving human health through interactions of diet with the gut microbiome.

Our study has shown that long-term intake of plant-based nutrients, particularly those from grains, nuts, and vegetables, is correlated with butyrate production during in vitro fermentation of a whole wheat substrate, whereas high intakes of unsaturated fats are associated with fewer markers of protein fermentation, including ammonia production. This suggests that differences in diet can influence the

production of metabolites by the gut microbiota even when exposed to identical substrates. Future studies should determine if changes to dietary patterns can alter metabolite production during in vitro fermentation. This could result in new dietary strategies for improving the metabolism of the gut microbiota in a manner that is consistent with host health.

Supplementary data to this article can be found in Table 1, 2, and 3.

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Table 1. Reported daily nutrient and other dietary compounds intakes of fecal donors (n=18).[A]

Subject number	Gender	Age	Energy, kcal	Fat, g	Sat. fat, g	Trans fat, g	MUFA, g	PUFA, g	ω-3 Fatty acids, g	Cholesterol, mg	Carbohydrate, g	Sugars, g	Added sugar, g	Fructose, g	Sucrose, g	Starch, g	Dietary fiber, g	Soluble fiber, g	Insoluble fiber, g
1	male	33	3117	117	34.7	4.34	47.5	26.6	1.92	217	408	157	77.3	15.7	50.6	197	34.1	8.49	25.7
4	male	24	2045	93.9	26.2	3.94	44.1	15.8	2.72	348	219	121	42.4	31.0	39.6	74.2	11.2	4.16	7.04
5	male	37	1966	72.4	27.3	3.92	25.9	13.5	1.45	254	249	141	119	35.7	41.8	83.0	14.0	4.00	10.0
7	female	24	2236	89.7	28.8	3.28	35.0	18.1	1.59	528	230	97.5	65.6	29.5	29.2	97.5	17.7	4.66	13.0
11	female	24	2391	68.1	21.3	4.15	27.3	13.8	1.36	321	328	151	57.7	24.6	40.2	131	29.4	7.37	22.0
12	male	24	1595	57.7	16.0	2.26	21.1	15.9	1.55	228	208	96.6	54.8	21.7	27.3	83.9	17.5	4.76	12.8
13	female	24	1881	82.4	30.7	2.35	36.0	9.88	1.05	404	189	111	36.2	14.2	26.4	53.9	16.8	5.68	11.2
15	male	21	1331	35.9	12.0	1.64	13.4	7.38	0.780	176	191	57.1	16.3	10.7	17.5	103	17.6	4.41	13.2
16	male	21	3152	105	35.4	7.08	39.8	18.6	1.93	324	353	132	102	19.4	51.7	158	24.7	11.7	12.6
19	male	33	1722	74.6	15.3	2.00	29.1	16.3	1.02	212	173	63.6	24.2	13.8	21.5	69.7	37.1	21.0	16.3
21	male	34	1758	69.8	20.8	3.36	29.4	12.7	1.32	540	203	103	60.5	26.9	34.5	66.3	14.2	6.00	8.09
24	male	27	1608	88.5	28.9	3.47	31.4	20.9	3.24	311	135	71.8	26.4	7.06	15.7	50.8	9.66	2.39	7.21
25	female	26	2470	116	31.3	5.29	44.8	28.5	5.72	476	258	92.8	73.4	17.9	50.7	131	20.9	8.84	12.0
26	female	20	1843	62.2	18.6	4.45	24.0	14.4	1.63	166	251	111	49.3	28.0	43.4	99.1	22.7	5.77	16.8
27	male	36	1834	63.2	18.0	4.07	28.3	10.6	0.900	182	238	68.2	33.6	18.9	18.7	136	27.3	6.38	19.9
65	female	25	1705	94.7	26.0	3.09	37.6	23.5	2.22	414	143	74.7	45.5	23.2	19.5	50.1	7.75	2.53	5.12
98	male	24	2076	91.0	30.3	5.06	33.6	19.3	2.13	429	231	111	76.8	20.7	46.3	95.9	15.3	4.44	10.7
99	female	23	1554	58.6	17.4	2.53	25.0	11.5	1.41	249	195	101	50.1	19.2	33.8	70.6	15.2	4.75	10.4
Mean	NA	26.7	2016	80.0	24.4	3.68	31.8	16.5	1.89	321	234	103	56.1	21.0	33.8	97.3	19.6	6.52	13.0
SD	NA	5.40	501	21.5	7.10	1.34	8.87	5.69	1.15	121	70.1	29.5	26.7	7.42	12.3	39.9	8.18	4.28	5.40
Median	NA	24.0	1862	78.5	26.1	3.70	30.4	15.9	1.57	316	225	102	52.5	20.1	34.1	89.9	17.5	5.22	12.3
25th Percentile	NA	24.0	1709	64.4	18.1	2.67	26.2	12.9	1.33	220	192	79.2	37.8	16.2	22.7	69.9	14.4	4.42	10.1
50th Percentile	NA	24.0	1862	78.5	26.1	3.70	30.4	15.9	1.57	316	225	102	52.5	20.1	34.1	89.9	17.5	5.22	12.3
75th Percentile	NA	31.5	2196	93.2	30.0	4.29	37.2	19.1	2.08	412	251	119	71.4	26.3	43.0	124	24.2	7.12	15.5
Low subject	NA	20.0	1331	35.9	12.0	1.64	13.4	7.38	0.780	166	135	57.1	16.3	7.06	15.7	50.1	7.75	2.39	5.12
Low subject	NA	26	15	15	15	15	15	15	15	26	24	15	15	24	24	65	65	24	65
High	NA	37.0	3152	117	35.4	7.08	47.5	28.5	5.72	540	408	157	119	35.7	51.7	197	37.1	21.0	25.7
High subject	NA	5	16	1	16	16	1	25	25	21	1	1	5	5	16	1	19	19	1

Table 1 (continued). Reported daily nutrient and other dietary compounds intakes of fecal donors (n=18).[A]

Subject number	Protein, g	Animal protein, g	Plant protein, g	Vitamin A, IU	Vitamin D, IU	Vitamin E, IU	Vitamin K, µg	Thiamin, mg	Riboflavin, mg	Niacin, mg	Pantothenic acid, mg	Vitamin B-6, mg	Folate, µg	Vitamin B-12, µg	Vitamin C, mg	Ca, mg	Cu, mg	Fe, mg	K, mg
1	125	61.8	62.7	8725	586	23.3	80.9	3.72	5.38	47.8	9.26	4.25	1065	12.8	73.4	2241	2.37	44.8	4324
4	82.5	65.5	16.8	4093	168	14.1	62.6	1.26	1.96	17.9	4.89	1.77	349	6.68	177	739	1.25	16.1	3044
5	73.5	53.3	19.9	5904	288	9.82	84.3	1.44	2.34	19.8	4.58	1.86	382	6.51	70.4	1107	1.05	14.5	2460
7	129	103	25.6	9621	329	11.4	166	2.95	2.56	29.0	6.23	2.43	387	6.55	77.2	904	1.55	14.9	3349
11	126	89.4	37.0	12329	518	14.0	164	2.59	4.20	33.4	9.02	3.28	671	11.4	110	1920	1.87	25.8	4521
12	62.3	40.0	22.2	8674	226	15.9	73.8	1.71	2.46	20.9	6.22	2.24	474	6.26	111	1300	1.29	17.4	2518
13	97.5	81.5	16.0	12189	388	12.1	292	1.30	3.09	18.6	7.10	1.90	356	6.72	94.1	1838	1.23	11.8	3499
15	64.2	38.7	25.4	13725	204	7.11	219	1.56	1.46	17.4	4.67	1.68	376	3.43	98.3	744	1.30	9.71	2471
16	93.2	58.5	34.6	6590	295	16.4	75.1	2.51	4.48	43.2	10.4	4.11	674	9.54	110	1264	1.55	26.5	4024
19	90.7	55.6	35.0	6748	212	25.0	173	2.71	4.96	43.5	17.1	3.56	717	7.88	186	1037	1.63	23.6	4793
21	75.3	53.9	21.2	4855	246	12.1	71.6	1.42	2.44	17.4	5.60	1.62	372	5.68	142	1328	1.26	13.2	3236
24	68.2	54.7	13.6	13187	357	13.7	553	1.24	2.33	12.2	5.14	1.29	323	5.60	56.0	1227	1.14	9.26	2506
25	99.4	69.5	29.9	11210	122	17.7	466	1.96	2.51	28.1	7.86	1.99	475	4.46	90.1	612	1.46	16.1	2958
26	64.7	39.6	25.0	8720	150	20.9	101	2.15	2.44	28.2	7.54	3.07	581	6.49	199	1251	1.36	20.5	2720
27	74.2	38.1	36.0	9829	41.6	12.2	80.1	1.37	1.31	22.4	3.41	1.81	379	4.32	183	643	1.54	18.1	2921
65	68.4	55.4	13.0	6765	191	15.4	34.3	1.05	1.92	18.5	4.57	1.63	227	9.34	69.4	766	1.35	10.2	1894
98	79.2	58.8	20.4	8329	207	11.9	131	1.44	2.22	20.3	5.12	1.87	361	5.42	96.3	925	1.26	13.7	2753
99	63.9	45.8	18.1	6700	246	11.1	150	1.23	2.23	19.1	5.11	2.03	382	5.61	66.8	981	1.00	12.4	2494
Mean	85.4	59.1	26.2	8789	265	14.7	165	1.87	2.79	25.4	6.88	2.36	475	6.93	112	1157	1.41	17.7	3138
SD	22.2	17.8	11.9	2859	134	4.64	141	0.745	1.17	10.3	3.18	0.899	200	2.45	45.9	455	0.320	8.52	808
Median	77.3	55.5	23.6	8697	236	13.8	116	1.50	2.44	20.6	5.91	1.95	382	6.50	97.3	1072	1.33	15.5	2940
25th Percentile	68.3	47.6	18.6	6712	195	11.9	76.4	1.32	2.22	18.6	4.95	1.78	364	5.60	74.4	801	1.25	12.6	2509
50th Percentile	77.3	55.5	23.6	8697	236	13.8	116	1.50	2.44	20.6	5.91	1.95	382	6.50	97.3	1072	1.33	15.5	2940
75th Percentile	96.4	64.6	33.4	10864	321	16.2	171	2.42	2.96	28.8	7.78	2.91	555	7.59	134	1291	1.55	19.9	3461
Low subject	62.3	38.1	13.0	4093	41.6	7.11	34.3	1.05	1.31	12.2	3.41	1.29	227	3.43	56.0	612	1.00	9.26	1894
Low subject	12	27	65	4	27	15	65	65	27	24	27	24	65	15	24	25	99	24	65
High	129	103	62.7	13725	586	25.0	553	3.72	5.38	47.8	17.1	4.25	1065	12.8	199	2241	2.37	44.8	4793
High subject	7	7	1	15	1	19	24	1	1	1	19	1	1	1	26	1	1	1	19

Table 1 (continued). Reported daily nutrient and other dietary compounds intakes of fecal donors (n=18).[A]

Subject number	Mg, mg	Mn, mg	Na, mg	P, mg	Se, µg	Zn, mg	Choline, mg	Betaine, mg	Phytic acid, mg	Oxalic acid, mg	Alcohol, g	Caffeine, mg
1	648	8.30	4481	2718	185	25.2	439	360	1797	313	0.030	0.830
4	280	5.23	2382	1156	101	14.7	384	78.7	460	139	1.31	261
5	258	2.16	2896	1374	90.3	11.7	310	133	496	119	8.04	42.0
7	340	3.21	3726	1714	197	15.9	547	178	677	267	0.910	7.75
11	478	5.04	4477	2371	160	19.3	505	332	1012	312	1.52	8.74
12	301	3.64	2586	1204	87.7	12.4	281	140	688	115	2.84	72.7
13	362	3.19	2777	1909	111	12.6	441	175	502	345	2.18	114
15	264	3.00	2490	1063	86.7	7.80	259	91.9	622	213	0.030	1.36
16	437	4.45	4155	1871	124	17.1	495	278	805	202	68.4	544
19	469	4.82	2368	1437	123	18.2	441	131	1000	263	11.9	1436
21	307	3.42	2922	1387	100	10.8	473	106	428	112	7.21	102
24	276	2.28	2010	1269	91.9	9.46	323	238	246	471	2.25	63.2
25	327	3.85	3384	1266	138	14.1	443	228	682	393	3.48	426
26	296	3.45	2762	1148	81.4	15.3	257	206	816	162	10.3	5.46
27	310	3.41	4269	1049	90.4	15.0	279	118	773	144	3.07	11.0
65	192	1.42	2239	1091	99.1	9.79	349	52.5	287	65.8	2.23	0.290
98	258	2.90	3627	1316	114	11.8	381	163	481	168	4.23	84.8
99	254	2.63	2188	1172	79.3	9.62	296	128	464	193	4.02	99.7
Mean	336	3.69	3097	1473	114	13.9	383	174	680	222	7.44	182
SD	109	1.53	832	470	34.8	4.26	92.7	85.3	353	110	15.6	348
Median	304	3.42	2836	1292	101	13.4	383	151	649	197	2.96	67.9
25th Percentile	267	2.93	2409	1160	90.3	11.0	300	121	468	140	1.69	8.00
50th Percentile	304	3.42	2836	1292	101	13.4	383	151	649	197	2.96	67.9
75th Percentile	356	4.30	3701	1645	124	15.8	442	222	797	301	6.47	111
Low subject	192	1.42	2010	1049	79.3	7.80	257	52.5	246	65.8	0.030	0.290
Low subject	65	65	24	27	99	15	26	65	24	65	15	65
High	648	8.30	4481	2718	197	25.2	547	360	1797	471	68.4	1436
High subject	1	1	1	1	7	1	7	1	1	24	16	19

[A]SD=standard deviation; Sat. fat=saturated fat; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids.

Table 2. Reported food category intakes (servings/d) of fecal donors.[A]

Subject number	Gender	Age	Total grain	Whole grain	Refined grain	Total vegetables	Dark green vegetables	Orange and yellow vegetables	Potatoes	Starchy vegetables not potato	Tomatoes	Other vegetables	Total fruit	Citrus, melon, berries	Other fruit	Total dairy	Fluid milk
1	male	33	14.3	5.42	8.91	1.38	0.13	0.10	0.18	0.21	0.31	0.46	0.64	0.19	0.44	5.32	4.83
4	male	24	4.90	1.40	3.49	0.72	0.02	0.07	0.13	0.15	0.10	0.25	2.84	1.27	1.57	1.15	0.93
5	male	37	4.55	0.60	3.95	1.48	0.14	0.09	0.30	0.19	0.30	0.46	0.70	0.27	0.44	2.60	1.95
7	female	24	5.57	0.83	4.73	2.23	0.33	0.23	0.14	0.23	0.30	0.98	1.29	0.17	1.12	1.72	1.29
11	female	24	7.59	2.24	5.36	2.44	0.34	0.27	0.33	0.39	0.47	0.64	1.95	0.47	1.49	4.45	3.63
12	male	24	5.00	1.18	3.82	1.84	0.07	0.23	0.27	0.37	0.29	0.61	1.05	0.43	0.63	2.06	1.61
13	female	24	3.51	1.72	1.79	1.49	0.60	0.19	0.03	0.08	0.18	0.41	1.63	0.53	1.10	4.83	3.03
15	male	21	4.64	0.51	4.13	2.58	0.39	0.37	0.75	0.32	0.18	0.58	1.02	0.32	0.70	1.47	1.27
16	male	21	8.43	1.36	7.06	1.92	0.08	0.07	0.79	0.15	0.39	0.46	0.91	0.45	0.46	2.15	1.26
19	male	33	2.60	0.75	1.84	2.61	0.62	0.05	0.79	0.12	0.39	0.64	1.08	0.46	0.62	1.05	0.88
21	male	34	3.81	0.47	3.33	1.12	0.10	0.06	0.23	0.11	0.26	0.36	1.51	1.21	0.30	2.15	1.51
24	male	27	2.89	0.31	2.58	1.22	0.72	0.01	0.23	0.04	0.05	0.16	0.48	0.22	0.26	3.09	2.95
25	female	26	7.84	0.58	7.26	1.51	0.51	0.02	0.28	0.10	0.20	0.40	1.33	0.41	0.92	0.40	0.18
26	female	20	6.15	0.97	5.17	1.58	0.22	0.20	0.29	0.22	0.21	0.43	2.83	1.30	1.53	1.19	0.69
27	male	36	6.33	0.89	5.44	2.80	0.10	0.31	0.11	0.41	1.11	0.76	1.59	0.42	1.17	0.14	0.01
65	female	25	3.19	0.71	2.48	0.78	0.01	0.04	0.13	0.21	0.13	0.26	0.99	0.50	0.49	1.70	1.35
98	male	24	5.57	0.59	4.98	1.51	0.17	0.12	0.30	0.12	0.40	0.40	1.21	0.46	0.75	1.89	1.25
99	female	23	3.63	0.67	2.96	1.06	0.22	0.07	0.14	0.07	0.20	0.36	1.72	0.20	1.51	2.26	1.65
Mean	NA	26.7	5.59	1.18	4.40	1.68	0.27	0.14	0.30	0.19	0.30	0.48	1.38	0.52	0.86	2.20	1.68
SD	NA	5.40	2.77	1.17	1.94	0.63	0.22	0.11	0.23	0.11	0.23	0.20	0.66	0.36	0.45	1.43	1.22
Median	NA	24.0	4.95	0.79	4.04	1.51	0.20	0.10	0.25	0.17	0.28	0.45	1.25	0.44	0.73	1.98	1.32
25th Percentile	NA	24.0	3.68	0.59	3.05	1.26	0.10	0.06	0.14	0.11	0.19	0.37	1.00	0.28	0.47	1.26	1.01
50th Percentile	NA	24.0	4.95	0.79	4.04	1.51	0.20	0.10	0.25	0.17	0.28	0.45	1.25	0.44	0.73	1.98	1.32
75th Percentile	NA	31.5	6.29	1.32	5.31	2.15	0.38	0.22	0.30	0.23	0.37	0.60	1.62	0.49	1.16	2.52	1.88
Low	NA	20.0	2.60	0.31	1.79	0.72	0.01	0.01	0.03	0.04	0.05	0.16	0.48	0.17	0.26	0.14	0.01
Low subject	NA	26	19	24	13	4	65	24	13	24	24	24	24	7	24	27	27
High	NA	37.0	14.3	5.42	8.91	2.80	0.72	0.37	0.79	0.41	1.11	0.98	2.84	1.30	1.57	5.32	4.83
High subject	NA	5	1	1	1	27	24	15	16,19	27	27	7	4	26	4	1	1

Table 2 (continued). Reported food category intakes (servings/d) of fecal donors.[A]

Subject number	Yogurt	Cheese	Meat, poultry, fish	Beef, pork, veal, lamb, game	Sausages, lunch meat	Poultry	Fish high in omega-3s	Fish low in omega-3s	Eggs	Soy products	Nuts and seeds	Dry beans and peas	Alcoholic beverages
1	0.10	0.38	2.24	0.95	0.02	1.13	0.02	0.10	0.16	0.01	5.66	0.10	0.00
4	0.10	0.12	6.26	5.10	0.43	0.05	0.06	0.61	0.62	0.00	0.31	0.00	0.10
5	0.05	0.60	3.56	1.39	0.65	0.81	0.18	0.51	0.36	0.01	0.39	0.12	0.60
7	0.21	0.22	9.92	7.38	0.42	0.89	0.52	0.70	1.07	0.09	1.41	0.04	0.07
11	0.34	0.47	4.79	1.60	0.62	1.72	0.30	0.56	0.69	0.05	0.71	0.19	0.10
12	0.02	0.43	2.49	0.82	0.85	0.46	0.06	0.30	0.62	0.01	0.35	0.09	0.21
13	0.72	1.08	4.07	0.65	1.11	1.84	0.13	0.34	0.98	0.01	0.14	0.08	0.16
15	0.10	0.09	3.11	1.07	0.10	0.91	0.14	0.89	0.32	0.59	0.71	0.03	0.00
16	0.00	0.88	3.99	2.09	0.69	0.59	0.17	0.45	0.50	0.01	0.48	0.08	5.09
19	0.02	0.13	5.27	2.50	0.17	1.76	0.18	0.66	0.34	0.00	4.20	0.06	0.92
21	0.10	0.53	2.74	1.13	0.85	0.51	0.06	0.19	2.05	0.01	0.59	0.12	0.54
24	0.02	0.11	3.08	2.17	0.15	0.22	0.16	0.38	0.60	0.15	0.19	0.02	0.16
25	0.02	0.19	6.79	4.03	0.18	2.23	0.13	0.21	1.02	0.34	1.04	0.00	0.25
26	0.02	0.47	3.29	1.44	0.49	1.06	0.18	0.12	0.21	0.01	0.33	0.05	0.76
27	0.02	0.11	5.46	4.51	0.53	0.35	0.03	0.04	0.03	0.01	0.17	0.55	0.22
65	0.05	0.30	4.57	2.16	0.97	1.25	0.00	0.00	0.98	0.01	0.80	0.00	0.16
98	0.05	0.58	5.20	1.88	2.54	0.59	0.03	0.15	1.06	0.03	0.08	0.03	0.31
99	0.21	0.39	2.58	0.94	0.24	1.11	0.07	0.21	0.65	0.07	0.60	0.09	0.30
Mean	0.12	0.39	4.41	2.32	0.61	0.97	0.13	0.36	0.68	0.08	1.01	0.09	0.55
SD	0.17	0.28	1.92	1.80	0.58	0.61	0.12	0.25	0.47	0.15	1.49	0.12	1.16
Median	0.05	0.39	4.03	1.74	0.51	0.90	0.13	0.32	0.62	0.01	0.54	0.07	0.22
25th	0.02	0.15	3.09	1.09	0.20	0.53	0.06	0.16	0.35	0.01	0.32	0.03	0.12
50th	0.05	0.39	4.03	1.74	0.51	0.90	0.13	0.32	0.62	0.01	0.54	0.07	0.22
75th	0.10	0.52	5.25	2.42	0.81	1.22	0.18	0.55	0.98	0.07	0.78	0.10	0.48
low	0.00	0.09	2.24	0.65	0.02	0.05	0.00	0.00	0.03	0.00	0.08	0.00	0.00
low subject	16	15	1	13	1	4	65	65	27	4,19	98	4,25,65	1,15
high	0.72	1.08	9.92	7.38	2.54	2.23	0.52	0.89	2.05	0.59	5.66	0.55	5.09
high subject	13	13	7	7	98	25	7	15	21	15	1	27	16

[A]SD=standard deviation; serving sizes: grains, meats, poultry, fish, eggs, soy, nuts, and seeds=1 oz. (28 g) equivalents; vegetables, fruits, and dry beans and peas (cooked)=1/2 cup (120 cm3) equivalents; dairy products=1 cup (236 mL) equivalents; alcoholic beverages= number of drinks

Table 3. Spearman correlations among nutrients that were significantly corrrelated with butyrate production during *in vitro* fermentation.

	Cholesterol	Carbohydrate	Starch	Dietary fiber	Soluble fiber	Insoluble fiber	Plant protein	Betaine	Phytic acid	Thiamin	Riboflavin	Niacin	Pantothenic acid	Vitamin B-6
Carbohydrate	-0.05													
Starch	-0.28	0.86***												
Dietary fiber	-0.42	0.55*	0.72***											
Soluble fiber	-0.12	0.51*	0.55*	0.85***										
Insoluble fiber	-0.55*	0.52*	0.73***	0.93***	0.65**									
Plant protein	-0.32	0.66**	0.82***	0.93***	0.79***	0.88***								
Betaine	0.00	0.58*	0.49*	0.50*	0.43	0.47*	0.45							
Phytic acid	-0.48*	0.64**	0.74***	0.95***	0.78***	0.93***	0.89***	0.55*						
Thiamin	-0.16	0.61**	0.66**	0.81***	0.64**	0.77***	0.83***	0.60**	0.84***					
Riboflavin	0.09	0.38	0.25	0.59**	0.68**	0.44	0.50*	0.71***	0.63**	0.73***				
Niacin	-0.19	0.67**	0.64**	0.85***	0.76***	0.74***	0.80***	0.59**	0.88***	0.83***	0.71***			
Pantothenic acid	0.02	0.37	0.31	0.67**	0.75***	0.49*	0.54*	0.72***	0.66**	0.74***	0.93***	0.71**		
Vitamin B-6	-0.25	0.59**	0.56*	0.79***	0.70**	0.70**	0.68**	0.63**	0.85***	0.79***	0.78***	0.92***	0.79***	
Folate	-0.36	0.64**	0.66**	0.86***	0.79***	0.77***	0.83***	0.58*	0.89***	0.86***	0.73***	0.89***	0.75***	0.91***
Cu	-0.14	0.50*	0.64**	0.83***	0.69**	0.76***	0.85***	0.42	0.80***	0.79***	0.53*	0.80***	0.59*	0.65**
Fe	-0.29	0.75***	0.68**	0.79***	0.75***	0.68**	0.79***	0.47*	0.86***	0.73***	0.59**	0.89***	0.61**	0.81***
Mg	-0.01	0.45	0.48*	0.80***	0.81***	0.67**	0.75***	0.61**	0.75***	0.73***	0.82***	0.71***	0.80***	0.67**
Zn	-0.11	0.64**	0.57*	0.80***	0.72***	0.68**	0.74***	0.54*	0.83***	0.76***	0.70**	0.89***	0.70**	0.81***

Table 3 (continued). Spearman correlations among nutrients that were significantly corrrelated with butyrate production during *in vitro* fermentation.

	Folate	Cu	Fe	Mg
Carbohydrate				
Starch				
Dietary fiber				
Soluble fiber				
Insoluble fiber				
Plant protein				
Betaine				
Phytic acid				
Thiamin				
Riboflavin				
Niacin				
Pantothenic acid				
Vitamin B-6				
Folate				
Cu	0.70**			
Fe	0.83***	0.73***		
Mg	0.67**	0.74***	0.70**	
Zn	0.74***	0.80***	0.91***	0.84***

*p<0.05; **p<0.01; ***p<0.001.

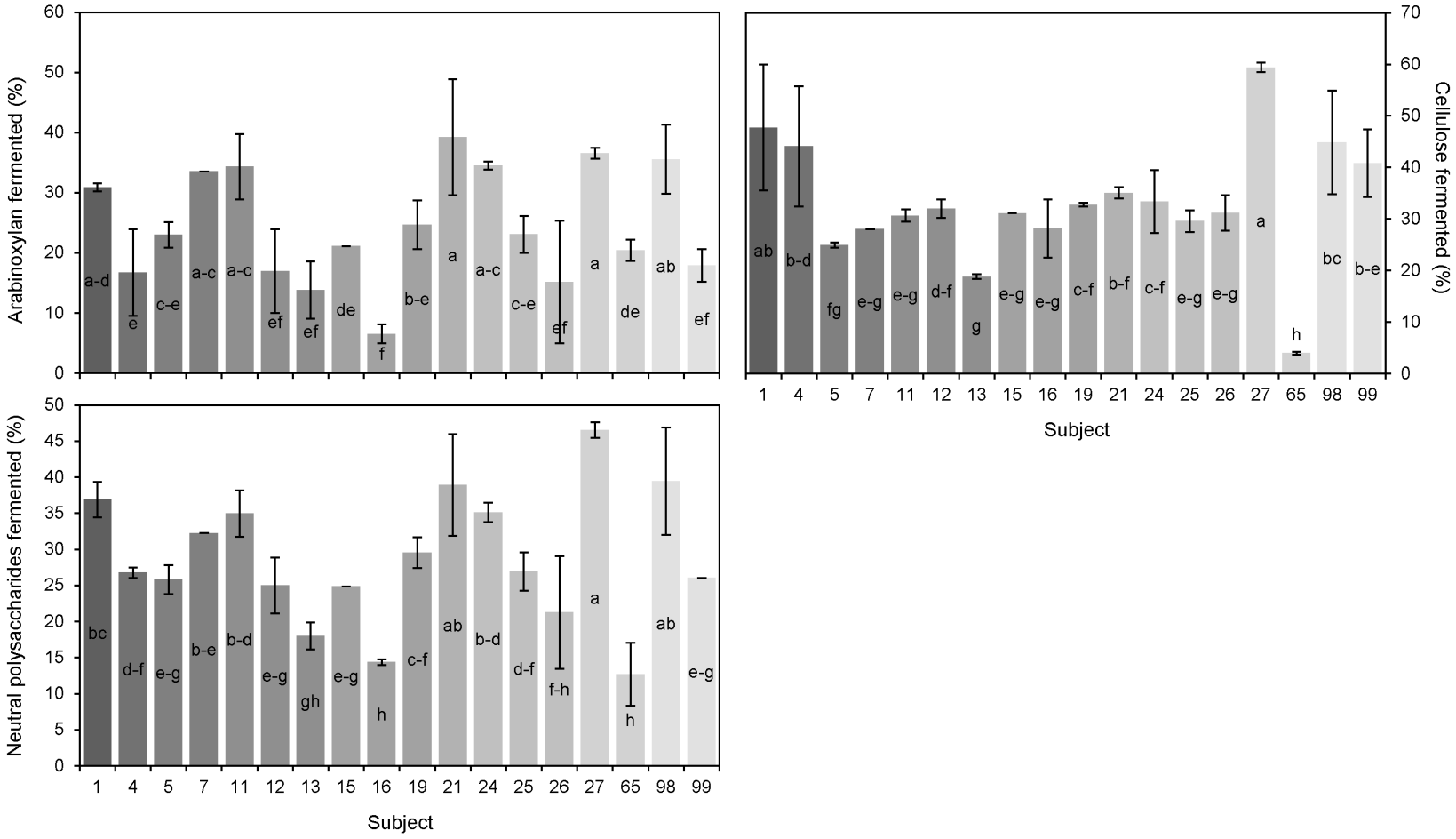

Figure 1. Carbohydrate fermentation during 12 h of *in vitro* fecal fermentation; error bars show standard deviation; bars marked with different letters are significantly different; n=2; note the different scales on the y-axes.

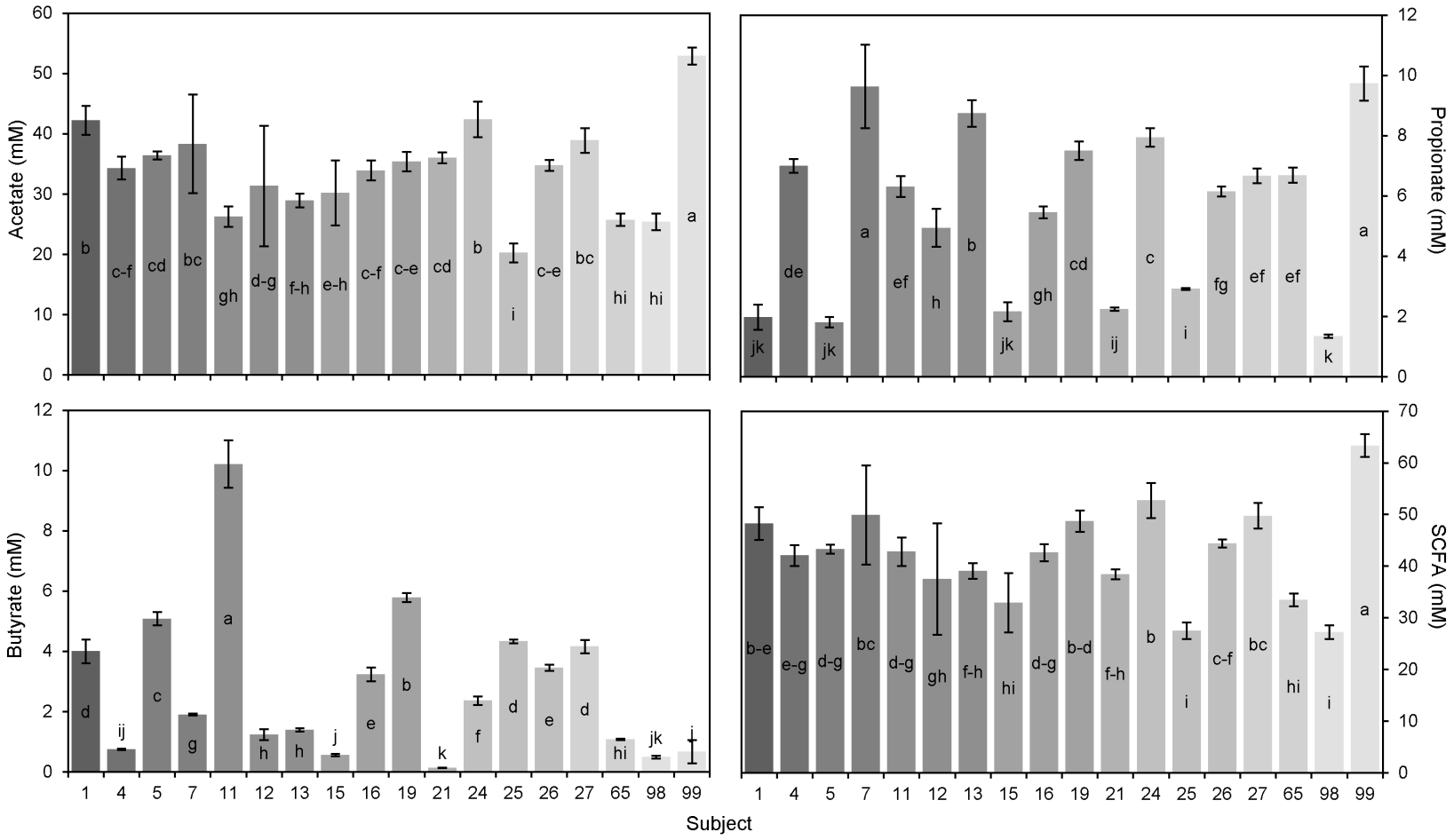

Figure 2. Short chain fatty acid (SCFA) production during 12 h of *in vitro* fecal fermentation; error bars show standard deviation; bars marked with different letters are significantly different; n=2; note the different scales on the y-axes.

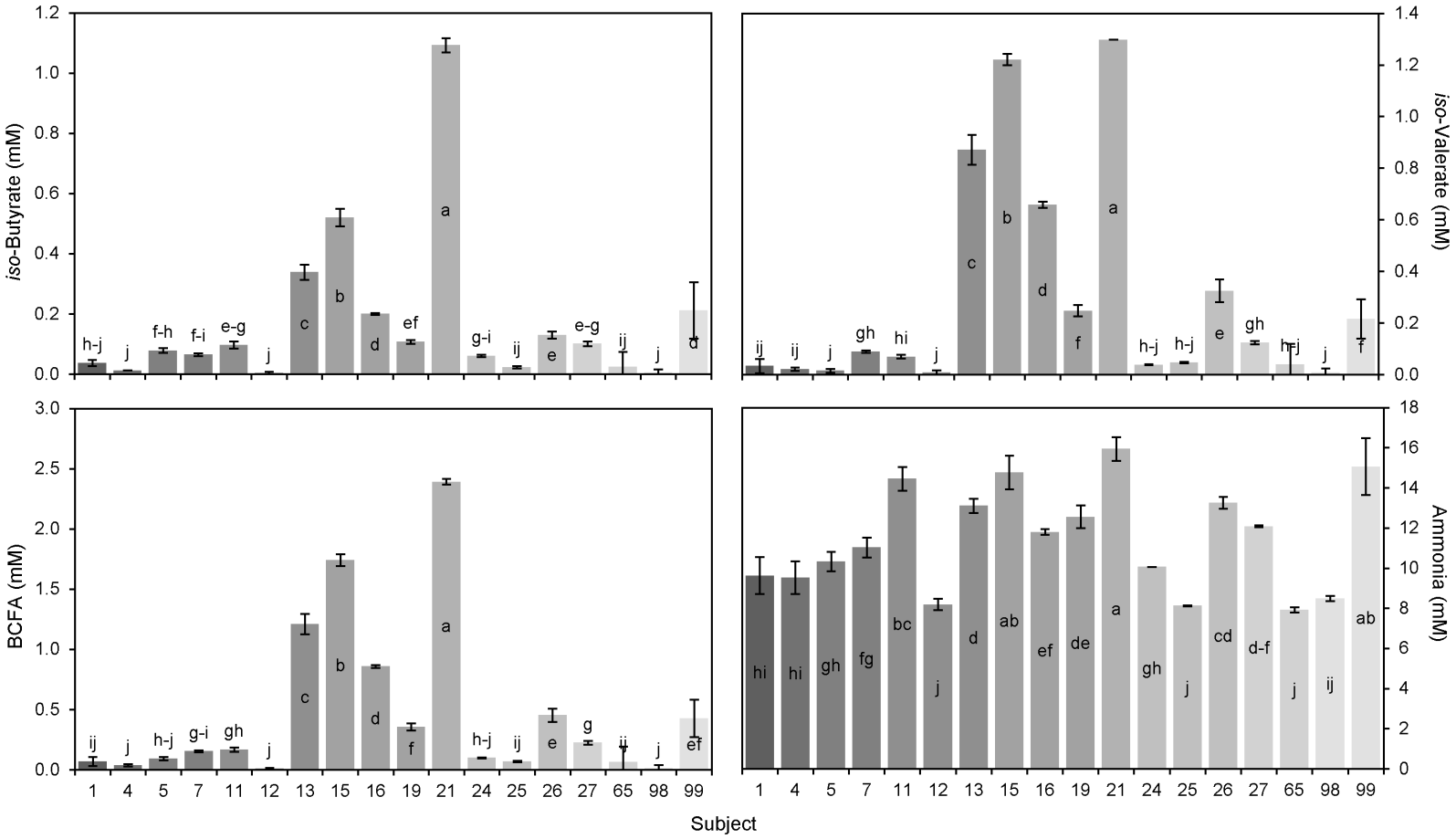

Figure 3. Branched chain fatty acid (BCFA) and ammonia production during 12 h of *in vitro* fecal fermentation; error bars show standard deviation; bars marked with different letters are significantly different; n=2; note the different scales on the y-axes.

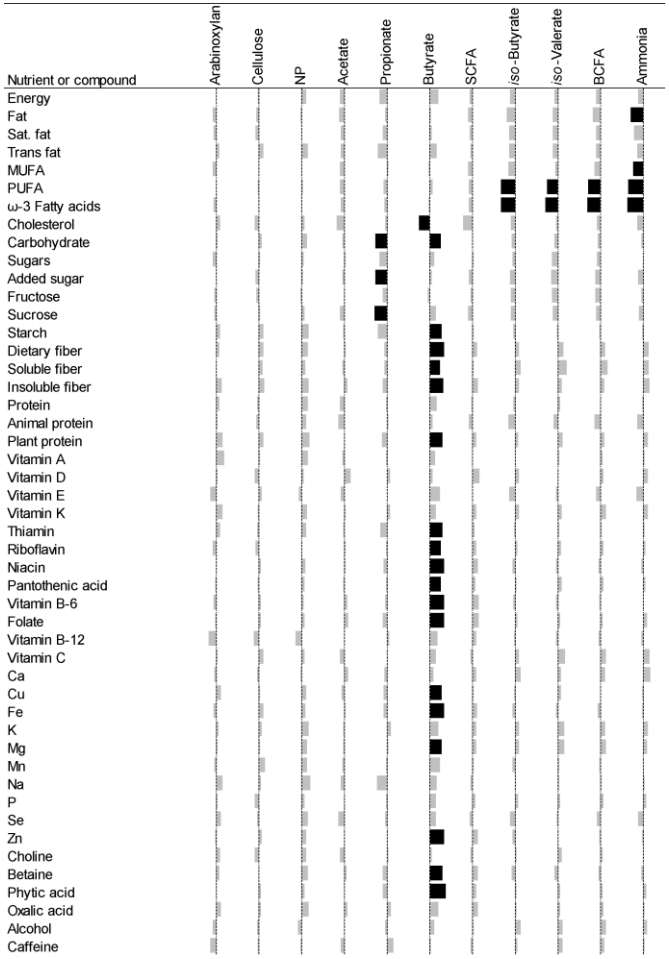

Figure 4. Spearman ρ correlation of dietary nutrient intake of the fecal donor with carbohydrate fermented (%) and metabolite production (mM) during *in vitro* fecal fermentation; bars indicate the direction (left, negative; right, positive) and magnitude of the correlation; gray bars indicate non-significant correlations; black bars are significant correlations; $p<0.05$; n=18.

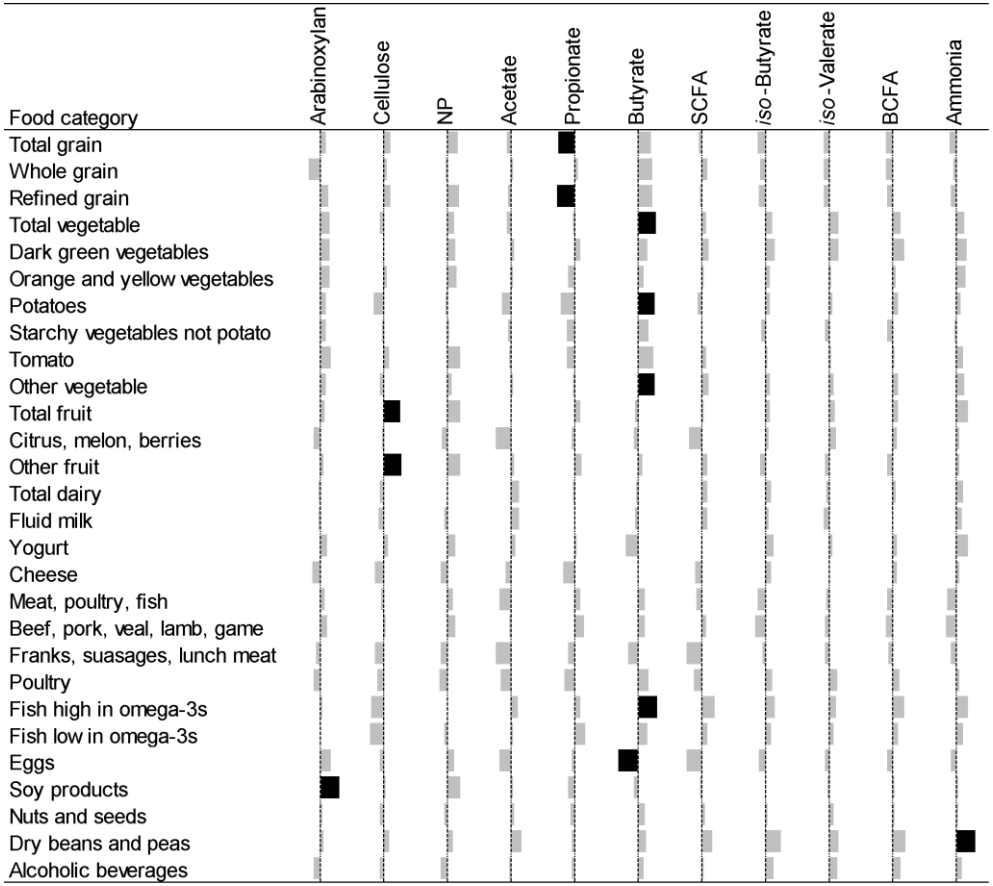

Figure 5. Spearman ρ correlation of food category intake of the fecal donor with carbohydrate fermented (%) and metabolite production (mM) during *in vitro* fecal fermentation; bars indicate the direction (left, negative; right, positive) and magnitude of the correlation; gray bars indicate non-significant correlations; black bars are significant correlations; $p<0.05$; n=18.

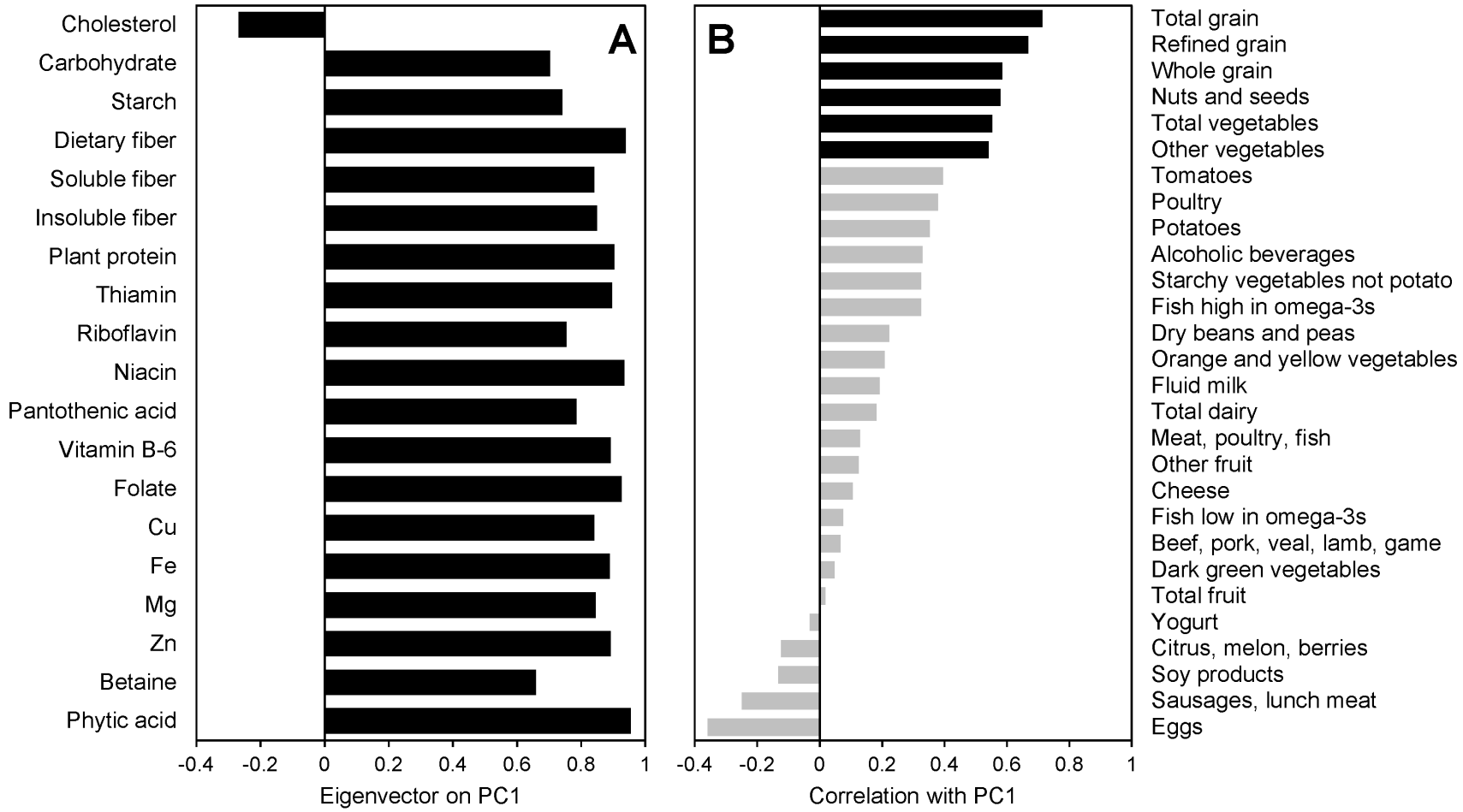

Figure 6. Eigenvectors on principal component 1 (PC1) for each nutrient or other dietary compound that was significantly correlated with butyrate production (A) and correlations of subject loadings on PC1 with reported food category intakes (B); black bars in B are significantly correlated with PC1; $p<0.05$; n=18.

Chapter 5 . Impact of pre-digested whole grain wheat flour on the composition and metabolites of the gut microbiota using *in vitro* fecal fermentation

1. Abstract

Our previous study has identified an approach of *in vitro* fermentation to assess the functionality of the microbiome that is not demonstrated by analyzing fecal SCFA. The study suggested that long-term diets high in plant-based foods and high in unsaturated fat are associated with the production of gut microbiota metabolites that are consistent with host heath after *in vitro* fermentation of predigested whole wheat flour. However, little is known about the influence of long-term dietary pattern and the fermentation of whole grain wheat on the gut microbiota composition. Thus, the objective of the study was to investigate the impacts of long-term dietary pattern on gut microbiota composition and to investigate the change in composition of the gut microbiota during fermentation of predigested whole wheat flour. Eighteen subjects provided fecal samples that were used for *in vitro* fermentation of predigested whole wheat flour. We did not observe significantly positive correlations between specific gut microbiota taxa and the nutrients that have been shown to be associated with the function of the gut microbiota from our previous study (chapter 4). Butyrate production was significantly correlated with the abundance of *Butyricicoccus, Coprococcus, Dorea, Faecalibacterium,* and *Lachnospiracea incertae sedis*. Branched chain fatty acids (BCFA) and ammonia production displayed negative correlations with the abundance of *Roseburia* and *Parasutterella*. *Bifidobacterium*, an indicator for gut health, and *Butyricicoccus*, a butyrate producer, were enhanced by pre-digested whole wheat flour.

2. Introduction

Diet is considered a major driver for changes in the gut microbiota composition. Long-term intake of protein and animal fat resulted in the enrichment in *Bacteroides* compared with long-term intake of carbohydrates, which lead to the increase in *Prevotella* [1]. Diets high in dietary fiber lead to an enrichment of *Prevotella* and *Xylanibacter*, compared with a more refined diet [2].

Other than the composition, the function of the gut microbiota is of equal importance. The gut microbiota can affect the host by either producing the harmful metabolites associated with human diseases or beneficial components that protect against diseases, depending on the dietary intake. The gut microbiota can produce harmful ammonia and amines through proteolytic fermentation [3], while production of short chain fatty acids (SCFA) from carbohydrate fermentation may have health benefits [4, 5]. Particularly, butyrate, which is a preferred energy source of gut epithelial cells, has been shown to promote energy expenditure and improve insulin sensitivity [6]. The investigation of the function of the gut microbiota is difficult to perform *in vivo*. For instance, quantitative measures of fermentation of specific dietary fibers and resulting SCFA production by the gut microbiota in humans are not practical *in vivo*. *In vitro* fecal fermentation models may be an ideal way to evaluate the function of the gut microbiota, such as microbial production of certain metabolites as well as utilization of complex substrates because compounds are not absorbed and can easily be quantified. Our previous study has suggested that long-term dietary pattern affected the function of the gut microbitoa using the *in vitro* model [7].

To date, a vast amount of research has focused on impacts of prebiotics, particularly inulin-type fructans on the gut microbiota (3). However, even if one consumes a very healthy diet, intake of traditional prebiotics would still be low. In contrast, whole grain wheat is the major dietary fiber source in the US diet and accounts for a large portion of the fiber that reaches the colonic site. Whole grain wheat flours are rich sources of dietary fiber and phytochemicals including phenolics, minerals, carotenoids, vitamin E, lignans, and phytates (4). A diet high in whole grain wheat has been shown to improve metabolic disorders through modulating the gut microbiota (5).

Our previous study identified an approach of *in vitro* fermentation to assess the functionality of the microbiome that is not demonstrated by analyzing fecal SCFA, and suggested that long-term diets high in plant-based foods and high in unsaturated fat were associated with the production of gut microbitoa

metabolites that are consistent with host health after *in vitro* fermentation of predigested whole wheat flour [7]. However, little is known about the influence of long-term dietary pattern and the fermentation of whole grain wheat on the gut microbiota composition. Thus, the first objective of the study was to investigate the impacts of long-term dietary pattern on gut microbiota composition. The second objective was to investigate the change in composition of the gut microbiota during fermentation of predigested whole wheat flour.

3. Methods

3.1. Subjects, dietary records, and stool sample collection

Eighteen subjects of 19 years of age or older with no history of digestive diseases and no antibiotics consumption in the last 6 months were recruited to participate in this study. The long-term dietary records of each subject were collected by completing the past-year (long-term) food intake diet history questionnaire II (DHQ II) online described by Yang et al. [7]

Subjects were instructed to bring a stool sample to the laboratory within 2 h of defecation. The stool collection materials included a collection container (Commode Specimen Collection System; Fisher Scientific, Pittsburgh, PA, USA), an insulated cooler with cold packs, and an anaerobic gas-generating tablet (Anaerocult C; BD GasPak, Franklin Lakes, NJ, USA), which subjects placed inside the collection container immediately after defecation and before securing the lid and packing in the cooler. Immediately after receiving stool samples, study personnel transferred the sample to an anaerobic hood (Bactron X; Sheldon Manufacturing, Cornelius, OR, USA) where the stool sample was packaged into a specimen bag (Fisher). The packaged fecal sample was then stored at -80 °C for future analysis. All protocols were approved by the University of Nebraska-Lincoln's Institutional Review Board before initiation of the study (no. 20120512624EP).

3.2. *In vitro* digestion and fermentation

Hard red winter wheat (*Triticum aestivum* "McGill") was obtained from Husker Genetics, the University of Nebraska-Lincoln's Foundation Seed Division. Wheat was milled through a cyclone sample mill equipped with a 1 mm screen (UDY Corporation, Fort Collins, CO, USA). The whole grain wheat flour was subjected to *in vitro* digestion following Yang et al. [7]. Following the digestion, the material was

freeze-dried (Labconco, Kansas City, MO, USA). The freeze-dried material were then subjected to *in vitro* fermentation using stool samples collected from 18 individuals as described [7].

3.3. Fermentation analyses

Microbial composition was assessed at baseline and after 12 h of *in vitro* fermentation. One milliliter of slurry was thawed and the cells were recovered by centrifugation (10,000 g for 5 min at room temperature). DNA was then extracted from the pellet with the phenol/chloroform method as described [8]. Microbial proportions were then assessed using 16S rRNA sequencing (MiSeq; Illumina; San Diego, CA USA) [9]. First, PCR-enrichment of the V5-V6 region of the 16S rRNA gene was done with primer pair 784F (5'-RGGATTAGATACCC-3') and 1064R (5'-CGACRRCCATGCANCACCT-3'). This was followed by PCR tailing to attach adaptors and barcodes. Next, samples were individually quantified and their concentrations normalized. The samples were pooled and the PCR products size selected with AMPureXP beads (Beckman Coulter). The final pool was again quantified and normalized to 2 nM for input into Illumina MiSeq (v3 Kit) to produce 2x300 bp sequencing products. Clustering was done at 10 pM with a 5% spike of PhiX. Initial quality filtering of the reads was performed with the Illumina Software. Next, reads were merged with the merge-Illumina-pairs application, which removed primers and performed further quality check of the sequences [10]. Subsequently, the UPARSE pipeline [11] was used to process the sequences and perform operational taxonomic unit (OTU) clustering. Sequences were independently subjected to taxonomic classification for phylum to genus characterization of the fecal microbiome using the RDP MultiClassifier 1.1 from the Ribosomal Database Project [12]. Phylotypes were computed as proportions based on the total number of sequences in each sample. α-Diversity (observed species) and β-diversity were calculated using tools implemented in QIIME [13] .

Carbohydrate content of the pre-digested whole wheat flour before and after *in vitro* fermentation, as well as SCFA, branched chain fatty acids, and ammonia generation during *in vitro* fermentation of these samples were reported previously [7].

3.4. Statistical analysis

Principal component analysis (PCA) based on OTU abundance was computed using XLSTAT (Statistical Innovations, Belmont, MA USA). Differences in abundance of individual bacterial taxa before

and after *in vitro* fermentation were analyzed using paired t-test. Pearson's correlations between fecal gut microbiota composition and dietary nutrients were computed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). Pearson's correlations between gut microbiota composition and their metabolites after *in vitro* fermentation were computed using SAS software. Only microbial taxa at the genus level were included and microbial taxa with abundance <1% were excluded from the analyses. $P < 0.05$ was used to consider all statistical significance.

4. Results

4.1. Influence of long-term dietary pattern on the fecal gut microbiota composition

Significant correlations between stool donor nutrient intakes and gut microbiota compositions are shown in Fig. 1. *Butyricicoccus* exhibited significantly positive correlation with PUFA, Vitamin A and E., while exhibited significantly negative correlation with fructose. *Erysipelotrichaceae_incertae_sedis* exhibited significantly positive correlation with trans fat, carbohydrate, sugars, fructose, and starch.

Our previous research has suggested significant positive correlations between several plant-based nutrients (carbohydrate, starch, dietary fiber, soluble fiber, insoluble fiber, plant protein, thiamin, riboflavin, niacin, pantothenic acid, vitamin B-6, folate, Cu, Fe, Mg, Zn, betaine, and phytic acid) and the function of the gut microbiota using an *in vitro* model [7]. However, we did not observe significantly positive correlations between these nutrients and specific gut microbiota taxa.

4.2. Influence of digested whole wheat flour on the composition of gut microbiota

The stool microbial communities and the microbial communities after *in vitro* fermentation of digested whole wheat flour were clearly differentiated on PC1, with the fermented samples showing a negative shift along this axis (Fig. 2A). The shift of the gut microbiota community on the PCA plot was driven by the change in several specific OTUs. The OTUs with significant loadings on this PC were identified (Fig. 2B). The loadings of individual OTUs on PC1 revealed that 4 taxa belonging to *Bifidobacterium*, *Bacteroidetes*, *Anaerovora*, and *Escherichia* were primarily responsible for a negative shift on this axis.

Significant changes in the gut microbiota at the genus level during fermentation are shown in Fig. 3. Fermentation of pre-digested whole wheat flour resulted in increases in *Bacteroidetes*, *Bifidobacterium*,

Butyricicoccus, Collinesella, Dorea, *Escherichia, Parabacteroides,* and *Phascolarctobacterium*, while the abundance of *Balutia* and *Lachnospiracea incertae sedis* were significantly decreased during fermentation.

Carbohydrate fermentation and microbial metabolite production were correlated with bacterial populations after fermentation (Fig. 4). Taxa *from the Lachnospiraceae* family, including *Coprococcus, Dorea, Lachnospiracea incertae sedis*, and *Roseburia*, and the *Ruminococcaceae* family, including *Faecallibacterium* and *Butyricicoccus,* exhibited positive correlations with butyrate production. *Bifidobacterium* were negatively correlated with butyrate production. *Clostridium XI* was positively correlated with *iso*-butyrate, *iso*-valerate, and BCFAs production. *Roseburia* and *Parasutterella* were negatively correlated with *iso*-butyrate, *iso*-valerate, BCFAs, and ammonia production.

5. Discussion

Our previous study showed that the consumption of plant based nutrients influenced the functionality of the gut microbiota during fermentation (i.e., production of propionate, butyrate, ammonia and BCFA) [7], while we did not observe correlations between the gut microbiota composition and those plant-based nutrients. This suggested that the impacts of long-term diet on the function of the gut microbiota might be more preserved compared with the gut microbiota composition. Consistent with our results, Daniel et al. has shown the impact of diet on the chemical metabolites of microbiota was greater than the impact of gut microbiota composition, suggesting that diet might exert a more pronounced impact on the functional level of gut microbiota [14]. Indeed, Zhang et al. described a core gut microbiota from healthy subjects, which was made up of a small number of genus-level phylotypes. Interestingly, these core phylotypes showed a common function of producing SCFA [15]. Turnbaugh et al and The Human Microbiome Project Consortium also showed a ‘core microbiome’ at the functional level, rather than at the organismal lineage, and the deviations from this core may result in disease states [16, 17]. Core metabolic functions of the gut microbiota is important because even distantly related bacteria share similar key metabolic functions, and thus these key metabolic functions are directly related to health or disease, rather than core microbial community [18]. Closely related lineages are considered to compete against each other because of the overlapping habitats and ecological roles, however, the microbiota with similar functions have been shown to be likely co-existing in the same niche [19]. For instance, *Faecalibacterium* and *Subdoligranulum*, which are butyrate producers, might be co-existing rather than competing with each

other [15]. Thus, we did not observe significant correlations between dietary nutrients and individual genus; it is likely that the correlations between dietary pattern and the cluster of gut microbiota with similar functions would be observed. Unfortunately, gene expression data that show the function of gut micriota were not obtained in the present study.

Given the importance of gut microbiota function, the metabolites of bacterial taxa were investigated using correlation analysis. It was worth noting that these correlations were obtained in the context of predigested whole wheat fermentation. Thus, the results also showed the types of bacteria that are specifically selected for by whole wheat. Our analyses revealed a positive relationship between the arabinoxylan utilization and *Dialister*. This is supported by recent findings showing that *Dialister* exhibited a gradual increase according to the whole grain dose [20]. High fecal *Dialister* levels were also identified in subjects that showed an anti-inflammatory response to a whole grain diet [21].

SCFAs are produced as a result of carbohydrate fermentation and are beneficial to gut health. In particular, butyrate might improve gut epithelia function and insulin sensitivity [6]. Upon fermentation of pre-digested whole wheat flour, *Faecallibacterium* and *Butyricicoccus* exhibited positive correlations with butyrate production. This finding was in accordance with previous research that showed *Faecallibacterium* and *Butyricicoccus* are the most important butyrate-producing bacteria in the gut [22]. Members of the *Lachnospiraceae* family were also positively correlated with butyrate production. Members of this family have been associated with obesity and protection from colon cancer *in vivo* by enhancing the production of butyric acid [23]. It is interesting to consider why there was a negative correlation of *Bifidobacterium* with butyrate production. Typically, increases in bifidobacteria are accompanied by a butyrogenic effect. While *Bifidobacterium* themselves do not produce butyrate, other commensal microbes can produce butyrate from lactate or polysaccharide breakdown products released by *Bifidobacterium* through cross-feeding [24]. However, other factors may also affect the butyrogenic bacteria. For instance, the competition for carbohydrate substrates with other nonbutyrogenic bacteria, such as *Bacteroides*, may result in a net decrease in butyrate production [25]. This is consistent with the increase of *Bacteroides* observed in our study and may explain the negative correlation of *Bifidobacterium* with butyrate production.

Protein fermentation is recognized to be detrimental to gut health. Protein fermentation results in the production of metabolites such as BCFAs, ammonia, and amines that may be toxic [3]. *Clostridium XI*

were positively correlated with *iso*-butyrate, *iso*-valerate, and BCFAs, suggesting that conditions that favor blooms in *Clostridium XI* might result in more undesirable fermentation products. *Clostridium difficile*, a pathogen that is a member in *Clostridium XI*, can use amino acid fermentation in order to create ATP as a source of energy [26]. *Roseburia* and *Parasutterella* were negatively correlated with *iso*-butyrate, *iso*-valerate, BCFAs, and ammonia, suggesting their potential benefits on gut health. They may compete with some bacterial taxa that ferment protein and thereby reduce fermentation of protein. The increased *Roseburia* have been associated with improved metabolic diabetes and hepatic steatosis [27], whereas a low *Parasutterella* levels were observed in patients with inflammatory bowel disease [28].

The fermentation of pre-digested whole wheat flour resulted in a significant increase in *Bifidobacterium*, which is consistent with human studies [29, 30]. *Bifidobacterium* have been regarded as a marker for intestinal health. For instance, supplementation of *Bifidobacterium* have been reported to reduce the risk of obesity [31]. We also observed a decrease in *Desulfovibrio*. *Desulfovibrio* prefers a slightly alkaline environment to grow, while *Bifidobacterium* may inhibit its growth by decreasing the luminal pH through SCFA production or by producing antimicrobial peptides that targeted *Desulfovibrio* [32]. The decrease in *Desulfovibrio* is potentially important for gut health because *Desulfovibrio* are endotoxin-producing bacteria that are enriched in animals with impaired glucose tolerance [33]. Changes in other microbial genera were also observed, such as the increase in *Butyricicoccus*. Although research on this genus is limited, it has been reported that maize arabinoxylan can increase *Butyricicoccus* and the administration of *Butyricicoccus* has been linked to reduced colitis and improvement in epithelial barrier function by decreasing the TNF-α and IL-12 [34].

6. Conclusions

We did not observe significantly positive correlations between specific gut microbiota taxa and the nutrients that have been shown to be associated with the function of the gut microbiota from our previous study. This suggested that the impacts of long-term diet on the function of the gut microbiota might be more preserved compared with the gut microbiota composition. The potential metabolic function of bacterial taxa in the context of whole wheat fermentation was investigated using correlation analysis. Butyrate production was significantly correlated with the abundance of *Butyricicoccus, Coprococcus, Dorea, Faecalibacterium, and Lachnospiracea incertae sedis*. BCFA and ammonia production displayed

negative correlations with the abundance of *Roseburia* and *Parasutterella*. This study has also revealed the changes of gut microbiota with health benefits after *in vitro* fermentation of predigested whole wheat flour. Most notably, *Bifidobacterium,* an indicator for gut health, *and Butyricicoccus,* a butyrate producer, were enhanced by pre-digested whole wheat flour.

7. References

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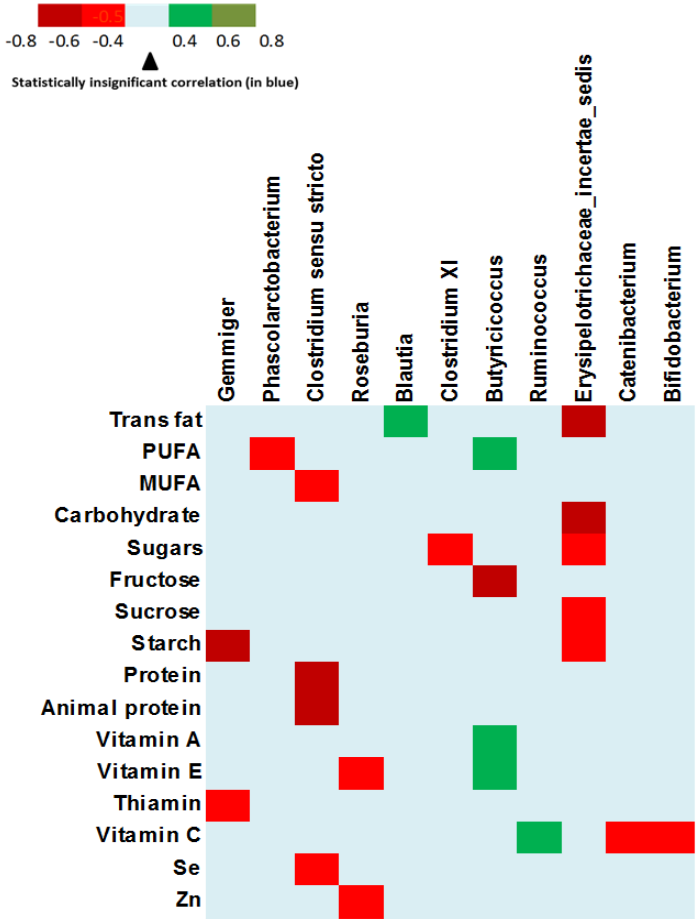

Figure 1. Correlations between the relative abundance of fecal bacterial taxa and dietary nutrients. Only statistically significant correlation are shown ($p<0.05$).

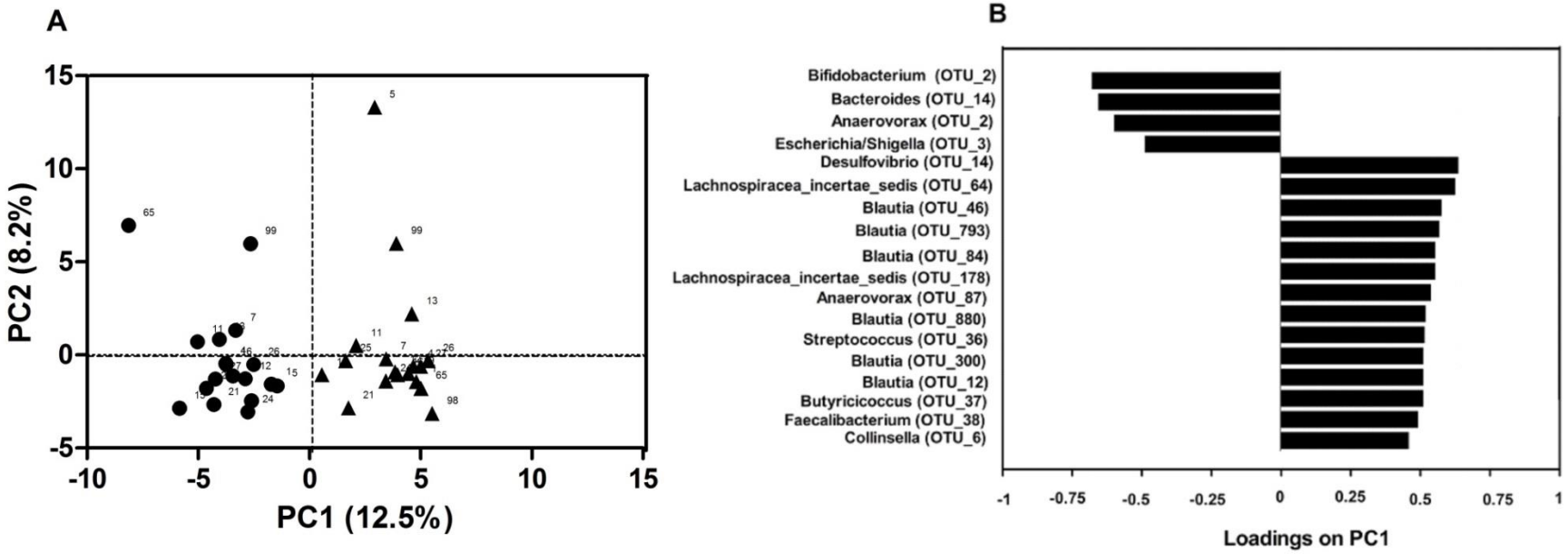

Figure 2. Fecal bacterial communities clustered using PCA analysis of OTU at baseline and after *in vitro* fermentation (A); the loadings on PC1with significant correlations (B). Numbers represent bacterial communities from different fecal donors; the triangle represents bacterial communities at baseline; the circle represents bacterial communities after *in vitro* fermentation.

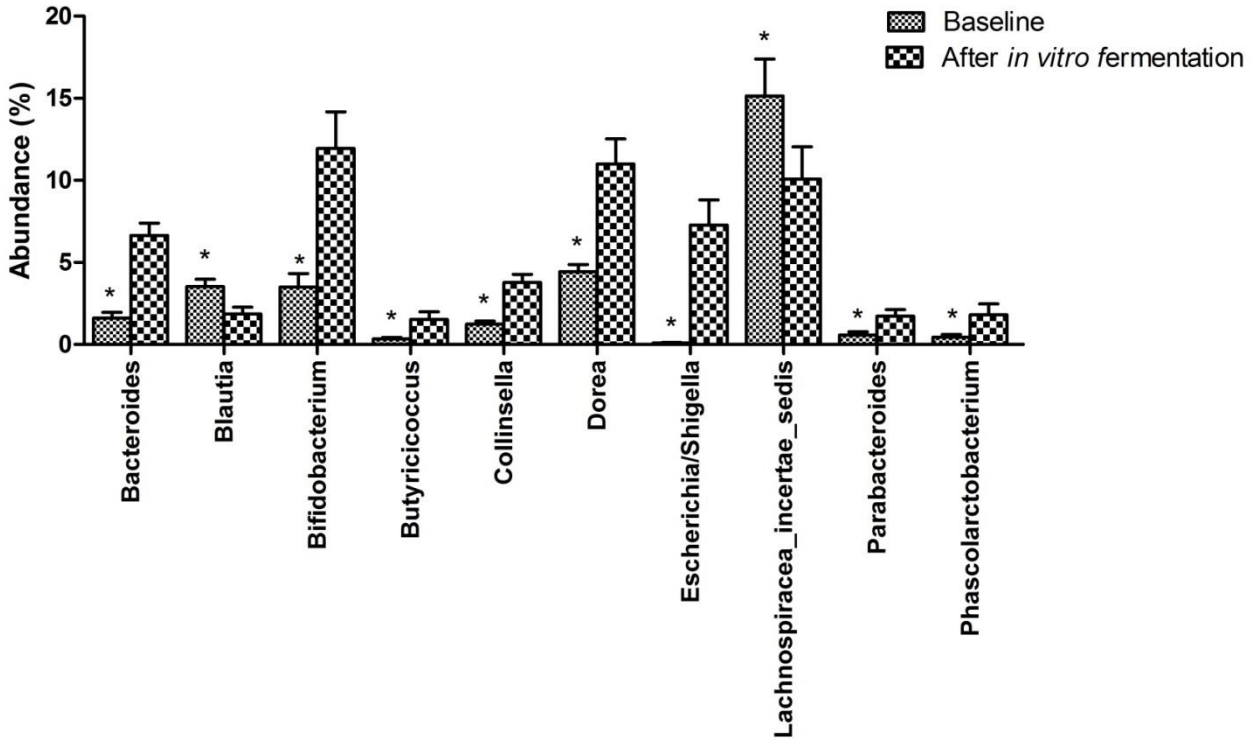

Figure 3. Abundance of bacterial taxa at baseline and after *in vitro* fermentation. *Significantly different from the abundance of bacterial taxa at baseline ($p<0.05$). Data are means ± SEM (n=18).

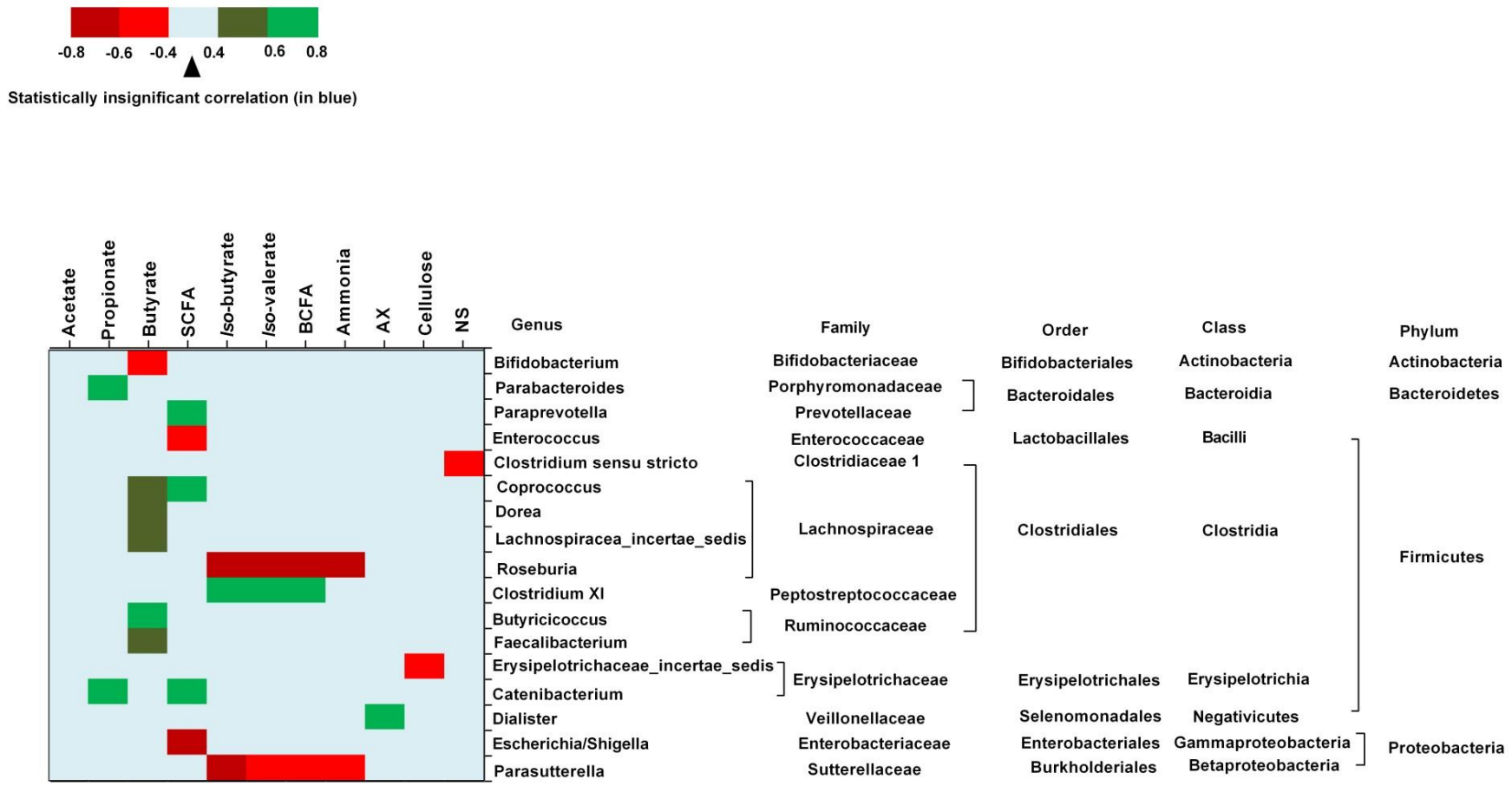

Figure 4. Correlations between the relative abundance of bacterial taxa after *in vitro* fermentation and dietary fiber degradation and short-/branched-chain fatty acid and ammonia production during *in vitro* fermentation. Only statistically significant correlation are shown ($p<0.05$)

General conclusions

For the first study (chapter 2), we hypothesized that FOPS from maize would be more bifidogenic and support bacterial fermentation the longest and produce the highest SCFA compared with FOPS from wheat, thus possibly contributing to a colonic environment that is less susceptible to disease. We determined treatment temperatures for production of FOPS from maize bran and wheat bran and then determined the fermentation properties of partially purified FOPS from maize bran and wheat bran. Although FOPS from wheat bran was more bifidogenic than that from maize, *in vitro* human fecal fermentations indicated that maize FOPS resulted in enhanced production of SCFA, especially butyrate, compared with fructans and FOPS produced from wheat bran. Additionally, maize FOPS showed significantly higher antioxidant activity because of the high ferulic acid content.

For the second study (chapter 3), we hypothesized that unique structural diversity coupled with the presence of antioxidants in FOPS would aid in the improvement of insulin response and lipid profile, alleviating inflammation syndromes, sustaining prolonged bacterial fermentation, and altering the gut microbiota to a healthier state. We selected maize FOPS to test whether the consumption of maize-derived FOPS could counteract deleterious metabolic effects of HF-feeding through modulating the gut microbiota in mice. We observed that mice in the FOPS treatment exhibited disparate metabolic responses to it. Our results suggest that colonic fermentation of FOPS plays an important role in preventing metabolic disorders in HF-fed mice, and that these metabolic improvements depend on specific alterations of the gut microbiota through FOPS fermentation. *Blautia* and *Akkermansia* might be considered potential therapeutic targets for improving body and adipose tissue weights, while we speculated SCFA production seems linked to improvements in glucose metabolism. However, it is unclear why the mice in the FOPS group responded so differently to the treatment. We further hypothesized that functional microbial differences at the strain level are responsible for the disparate responses to dietary FOPS in mice. Future studies should determine the functional microbial differences at the strain level between these FOPS mice.

For the third study (chapter 4), we hypothesized that diet would impact the extent of dietary fiber utilization and the types of metabolic end-products produced by the microbiota during in vitro fecal fermentation. We determined the correlations between dietary intake variables and dietary fiber degradation

and SCFA/BCFA and ammonia production during *in vitro* fecal fermentation. We found that butyrate production was correlated with fecal donor intake of many nutrients of which principal component analysis revealed were mostly contributed by grain-, nut-, and vegetable-based foods. These results suggest that diets high in plant-based foods and high in unsaturated fats are associated with microbial metabolism that is consistent with host health.

For the fourth study (chapter 5), we hypothesized that long-term dietary pattern and the fermentation of whole grain wheat would influence the gut microbiota composition. We then determined the influence of long-term dietary pattern and the fermentation of whole grain wheat on the gut microbiota composition. We found butyrate production was significantly correlated with the abundance of *Butyricicoccus*, *Coprococcus*, *Dorea*, *Faecalibacterium*, and *Lachnospiracea incertae sedis*. *Bifidobacterium* and *Butyricicoccus* were enhanced by pre-digested whole wheat flour. However, we did not observe significantly positive correlations between specific gut microbiota taxa and the nutrients that have been shown to be associated with the function of the gut microbiota from our previous study. This suggested that the impacts of long-term diet on the function of the gut microbiota might be more preserved compared with the composition of the gut microbiota. Thus, we further hypothesize that dietary pattern impacts the function of the gut microbiota. To test this hypothesis, future studies should determine the impacts of long-term diet on the function (based on metabolite profiling and gene expression) and gut microbitoa composition using an *in vivo* model.

Appendices

Appendix 1. Cereal Grain Projects.

Polysaccharide composition of triticale produced over three years in the Great Plains of the USA

Abstract

Triticale, a man made hybrid of durum wheat and rye, is mostly used for animal feed. It merges the yield potential and grain quality of wheat and the environment tolerance of rye. However, the limited market for triticale can discourage many farmers from planting it. Thus, given the beneficial effects of dietary fibers and the potential for starch conversion to ethanol, characterizing the polysaccharide fractions in triticale may help expand the triticale market to food and energy industries. To accomplish this, twelve experimental lines of winter triticale grown over three consecutive years were characterized for starch, β-glucan, and water extractable and water unextractable arabinoxylan (WE-AX and WU-AX). Our results showed starch, β-glucan, WE-AX, and WU-AX ranged from (% dm) 55.5 to 65.2, 0.48 to 0.91, 0.76 to 1.3, and 4.1 to 6.5, respectively. Some genotypes were less variable across production year than others, suggesting that some lines may be selected to breed for certain polysaccharides. The concentration of WE-AX and WU-AX was highly negatively correlated with grain yield. Our data may help growers and plant breeders select genetically stable cultivars with high starch or beneficial bioactive polysaccharides, thereby enhancing the application of triticale in energy and food industries.

Keywords: Triticale; starch; β-glucan, water extractable and water unextractable arabinoxylan

1. Introduction

Triticale (×*Triticosecale* Wittmack), a cross of wheat (*Triticum durum L.*) and rye (*Secale cereale L.*), was developed to harness the yield potential of wheat and the adaptability of rye. However, the utilization of triticale has been limited primarily to animal feed; it is seldom used in bread industry or for bioethanol production (Obuchowski, Banaszak, Makowska, & Łuczak, 2010).

The polysaccharides in triticale consist of starch and dietary fibers [cellulose, arabinoxylan (AX) and β-glucan]. Starch is the major storage polysaccharide of triticale and varies from 59-72% of the weight of the grain (Burešová, Sedláčková, Faměra, & Lipavský, 2010; Rakha, Åman, & Andersson, 2011). Dietary fiber content of triticale (13-16%; Rakha et al., 2011) lies between wheat (10.5-13.9%; Gebrurs et al., 2008) and rye (14.7-20.9%; Nyström et al., 2008). The dietary fibers in triticale include arabinoxylan (AX) as the most abundant component (5.9-7.5%), followed by cellulose (1.9-2.5%), and then a small amount of β-glucan (0.5-1%) (Rakha et al., 2011). AX is composed of a (1, 4)-linked β-d-xylopyranosyl backbone with α-L-arabinofuranosyl moieties substituted at C(O)-2 and/or C(O)-3 (Delcour & Hoseney, 2010). AX can be categorized based on water extractability into water-extractable (WE-AX) or water-unextractable (WU-AX). β-Glucan is a minor component of the starchy endosperm and aleurone cell wall of cereals, and consists of β-D-glucopyranosyl units linked through (1, 4) and (1, 3) glycosidic bonds (Delcour et al., 2010). Because of its limited bioactivity, cellulose was not addressed in this study.

The polysaccharide content in triticale has only been studied in few cases (Dennett, Wilkes, & Trethowan, 2013; Rakh et al., 2011; Saini & Henry, 1989). However, no studies have been carried out using multiple triticale lines produced over several years in the Great Plains, an area of the US covering, Nebraska, South Dakota, North Dakota, and parts of Colorado, Kansas, Montana, New Mexico, Oklahoma, Texas, and Wyoming. The objective of this study was to characterize the major polysaccharides in triticale over three consecutive years, thereby providing plant breeders and producers information on selecting useful cultivars in this region.

2. Materials & methods

2.1. Triticale samples

Ten experimental triticale lines, NT01451, NT10417, NT09423, NT10429, NT05429, NT09404, NT06427, NT07403, NT06422, and NT05421, and two cultivars, NE422T (Baenziger & Vogel, 2003), NT426GT (Baenziger, Jannink, & Gibson, 2005), were obtained from the small grains breeding program at the University of Nebraska-Lincoln (Lincoln, NE USA). Triticale was grown in an experimental field (Lincoln, NE USA) over three

consecutive years (2011, 2012, and 2013). After harvest, the triticale samples were milled with a cyclone sample mill equipped with a 1 mm screen (UDY, Boulder, CA USA).

2.2. Weather information

High/low temperature and total rainfall over the study period was provided by the state climatologist in the School of Natural Resources, University of Nebraska-Lincoln (Lincoln, NE USA). Data were collected from a climatology station located 0.96 km from the field site.

2.3. Polysaccharide analysis

Samples were analyzed for moisture (Approved Method 44-15A, AACC International, 2000) and starch (K-TSTA, Megazyme, Bray, Ireland). β-Glucan was quantified using a mixed-linkage β-glucan kit (K-BGLU, Megazyme, Bray, Ireland). AX was measured as WE-AX and WU-AX by gas chromatography (GC) of alditol acetates obtained after acid hydrolysis. The separation and hydrolysis steps were based on the method described by Frederix et al. (2004) except using 0.4 M H_2SO_4 at 121 ^oC to hydrolyze the samples, while the derivatization step was performed according to Approved Method 32-25 (AACC International, 2000) with some modifications: 1) 5 µl of 2-octanol was added before the reduction step to reduce the loss of ammonium hydroxide, and 2) reduction time was prolonged to 1.5 h. The WE-AX content was calculated according to the following equation: mmol WE-AX = mmol arabinose – 0.7*mmol galactose + mmol xylose to account for arabinose in arabinogalactan peptide (Ingelbrecht, Verwimp, Grobet, & Delcour, 2001). WU-AX was the sum of arabinan and xylan in the insoluble dietary fiber fraction. The arabinose/xylose ratio (A/X) was calculated as the ratio of arabinan to xylan in each AX fraction.

2.4. Data analysis

All analyses were performed in duplicate. All statistical comparisons were performed using SAS software (version 9.4, SAS Institute, Cary, NC). Differences in polysaccharide compositions were analyzed using analysis of variance (ANOVA) in combination with Fisher's protected least significant difference test. No plot replication was included, therefore only the main effects of genotype and year were included in the ANOVA model. Because the year to year variation is usually much larger than plot to plot variation, it is appropriate to assess genotype difference by analyzing over years (Sweley, Rose, & Jackson, 2012). Pearson's correlation coefficients were computed for agronomic and polysaccharide data. $P<0.05$ was used to consider statistical significance.

3. Results and discussion

3.1. Growth and harvest information of different triticale lines

Year 2 was a particularly unusual year with regard to the earlier heading date compared with others (Table 1). Year 1 experienced the highest amount of rainfall followed by year 2 and then year 3 (Fig. 1). Higher temperature fluctuation was also observed in year 1 and year 3 over year 2. The earlier heading date of wheat exhibits a positive correlation with daily minimum temperatures during the spring season (Hu, Weiss, Feng, & Baenziger, 2005), in agreement with the temperature data shown in Fig. 1.

3.2. Starch

Total starch content in different genotypes over three years varied between 55.5 and 65.2% (Table 2), slightly lower than the values reported previously (59.2-72.9%; Rakha et al., 2011; Burešová et al., 2010). The variation may be explained by use of different genetic lines, production practices, production location, and year (Obuchowski et al., 2010).

The starch content in cereal grains has been shown to be strongly influenced by weather conditions. Generally, warm weather and higher rainfall lead to higher kernel starch content (Burešová et al., 2010). The weather may change the starch accumulation in cereals by acting on starch biosynthesis enzymes involved in chain elongation, branching, and granule crystallinity (Beckles & Thitisaksakul, 2014). Considerable year to year variation was observed in the total starch content, with year 3 significantly lower than year 1 and year 2 (Table 2). Less total rainfall in 2013 (Fig. 1) may account for the low starch content (poorer grain fill) observed in this year.

The genotypic response of starch content in triticale over different years varied considerably. Although a decrease in starch content was observed for most lines in year 3 compared with the other years, some stayed unchanged and others even increased (Table 2). NT01451 and NT06422 were the most consistently high in starch over three years (high means coupled with low standard deviation over year). This suggests that these lines may be stable to different environmental conditions and used as genetic stock to develop new cultivars that are high in starch. High starch content would be desirable for triticale designed for animal feed (Theurer, 1986) or bioethanol production (Obuchowski et al., 2010).

3.3. β-Glucan

β-glucan content over three years ranged from 0.48-0.91% (Table 2), in agreement with values reported previously (0.5-1.0%; Rakha et al., 2011). Compared with its parent cereals, the total β-glucan content fell into similar range with wheat (0.5-1.0%; Gebruers et al., 2008), but lower than rye (1.7-2.0%; Nyström et al., 2008).

Harvest year had considerable influence on total β-glucan content, with highest values observed in year 1 and lowest in year 2 (Table 2). The significant impact of weather conditions in different years on β-glucan content has been reported (Fastnaught, Berglund, Holm, & Fox, 1996; Hang, Obert, Gironella, & Burton, 2007; G. Zhang, Chen, Wang, & Ding, 2001). For instance, in barley high temperature leads to increased β-glucan content, which may also apply to triticale (Fastnaught et al., 1996). The high temperature fluctuation in years 1 and 3 over year 2 may partly account for their significantly higher β-glucan content. Hansen et al. (2003) reported lower β-glucan content in rye during rainy years. We did not observe this trend in our study, indicating the association between rainfall and β-glucan content in triticale may be different from rye or that the timing of rain during seed development is important for β-glucan concentration.

Maximum β-glucan content was detected on NT06427, whereas NE422T, NT09423, NT10429, and NT426GT displayed low β-glucan content over the study period (Table 2). For food use, high β-glucan is preferred because of its beneficial health effects on blood cholesterol (Welch, Webster, & Wood, 2011). Although the β-glucan content in triticale is notably lower than oats (~5%) (Welch et al., 2011), β-glucan consumption as low as 20 mg/d were enough to have physiological effects on human health (Samuelsen, Schrezenmeir, & Knutsen, 2014).

3.4. Arabinoxylan

WE-AX concentration varied from 0.76-1.3%, while WU-AX varied from 4.1-6.5%. (Table 2). This WE-AX concentration is slightly higher than those found in the literature (0.55%; (Dennett, Wilkes, & Trethowan, 2013; Rakha et al., 2011; Saini & Henry, 1989); however, consistent with previous reports, the total AX in triticale has been reported by others as 4.4-7.5% (Rakha et al., 2011), which is in consistent with our results. As with the β-glucan data, the AX content of triticale was more similar to wheat (WE-AX: 0.5-0.8%; WU-AX: 3.7-4.6%) (Gebruers et al., 2008) than rye (WE-AX: 1.5-3.0%; WU-AX: 5-8.5%) (Nyström et al., 2008). NT01451 and NT09404 showed the highest WE-AX concentration, while several genotypes displayed low WE-AX concentrations.

WE-AX has been studied for its health-promoting effects. For instance, WE-AX from wheat has been shown to reduce postprandial glucose responses (Lu, Walker, Muir, Mascara, & O'Dea, 2000) and promote beneficial gut microbiota in the colon (Neyrinck et al., 2011). However, in some applications WE-AX is undesirable, for example in brewing WE-AX increases viscosity resulting in filtration difficulties (Li, Lu, Gu, Shi, & Mao, 2005).

NT07403 and NT09404 showed the highest WU-AX concentration, while low concentrations were found in NT05421 and NT10429. Except for its value as a source of dietary fiber, most applications are improved by reducing WU-AX concentration (Dornez, Gebruers, Delcour, & Courtin, 2009).

With regard to year, higher contents of WE-AX and WU-AX were observed in years 2 and 3 compared with year 1 (Table 2). The content of WE-AX in wheat has been negatively associated with temperature and positively with rainfall due to the high endoxylanase activity under cool and wet conditions, resulting in the breakdown of WU-AX into its soluble counterpart (Gebruers et al., 2010). However, in another study the opposite trends have been shown (Zhang et al., 2010). Our results are in line with the latter study, since higher rainfall was recorded in the year with low WE-AX content.

3.5. Correlations of polysaccharide content with yield

A significant correlation was found between grain yield and WE-AX and WU-AX content (Table 3). Dornez et al. (2008) did not find any significant correlation between these parameters in wheat, suggesting differences between AX fractions in triticale versus wheat. This correlation might be used in a breeding program to select desirable polysaccharide compositions.

4. Conclusions

Our study characterized the polysaccharide content and evaluated the environmental and genetic effects on experimental triticale varieties over three years, providing valuable information for both breeders and food manufactures. For instance, the genotypes with stable high starch content may become potential candidates for ethanol production, while the genotypes with stable high β-glucan or WE-AX can be selected as targets to produce functional food with enhanced health benefits. The heredity of these traits can be studied to produce novel cultivars in the future.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Table 1. Heading date and grain yield for triticale over three production years.

	Year 1		Year 2		Year 3	
Genotype	Heading date	Yield (kg/ha)	Heading date	Yield (kg/ha)	Heading date	Yield (kg/ha)
NT01451	26-May	5948	6-May	4829	30-May	2937
NT10417	24-May	6738	3-May	5149	29-May	3840
NT09423	27-May	6643	6-May	5405	30-May	4220
NT10429	28-May	6268	5-May	4652	30-May	3667
NT05429	23-May	6339	26-Apr	4577	28-May	4334
NT09404	28-May	6206	6-May	4854	29-May	3490
NE422T	29-May	5146	8-May	4441	31-May	2937
NT426GT	25-May	6269	3-May	5037	29-May	2780
NT06427	25-May	5561	30-Apr	4660	29-May	3366
NT07403	22-May	6406	22-Apr	5020	27-May	4806
NT06422	22-May	6352	26-Apr	4952	29-May	4688
NT05421	24-May	5618	3-May	4906	30-May	3976

Table 2. Polysaccharide content of triticale lines over three years.[a]

Effect	Starch			β-Glucan			WE-AX			A/X_{WE-AX}			WU-AX			A/X_{WU-AX}		
Level	Mean	SD	$LSD_{0.05}$	Mean	SD	$LSD_{0.05}$	Mean	SD	$LSD_{0.05}$	Mean	SD	$LSD_{0.05}$	Mean	SD	$LSD_{0.05}$	Mean	SD	$LSD_{0.05}$
Genotype																		
NE422T	59.3	2.8	b-d	0.482	0.073	e	0.890	0.166	b-d	0.66	0.01	c	4.64	0.36	d-f	0.77	0.15	bc
NT01451	60.1	2.1	bc	0.692	0.170	bc	1.30	0.07	a	0.78	0.25	ab	5.32	1.09	c	0.74	0.05	bc
NT05421	56.4	1.9	de	0.757	0.058	b	0.879	0.326	b-e	0.66	0.02	c	4.44	1.22	ef	0.75	0.02	bc
NT05429	60.9	5.7	bc	0.683	0.147	b-d	0.916	0.043	b-d	0.82	0.30	a	4.62	1.28	d-f	0.79	0.09	ab
NT06422	60.4	1.9	bc	0.763	0.079	b	0.814	0.284	de	0.64	0.06	c	4.83	1.78	c-e	0.74	0.03	bc
NT06427	55.5	4.0	e	0.906	0.023	a	0.758	0.229	e	0.61	0.04	c	5.45	1.75	bc	0.71	0.03	bc
NT07403	62.5	5.2	ab	0.649	0.138	cd	0.930	0.139	b-d	0.68	0.16	bc	6.51	0.99	a	0.76	0.10	bc
NT09404	58.4	1.4	c-e	0.609	0.056	d	1.22	0.32	a	0.69	0.12	bc	6.03	1.24	ab	0.72	0.01	bc
NT09423	62.6	4.1	ab	0.474	0.095	e	0.947	0.257	b-d	0.63	0.02	c	5.29	1.42	cd	0.67	0.09	c
NT10417	61.2	2.1	bc	0.692	0.157	bc	0.863	0.260	c-e	0.65	0.01	c	5.43	1.11	bc	0.73	0.03	bc
NT10429	61.0	5.0	bc	0.492	0.085	e	1.02	0.07	b	0.67	0.12	bc	4.14	1.66	f	0.83	0.14	ab
NT426GT	65.2	3.0	a	0.477	0.057	e	0.963	0.266	bc	0.68	0.03	bc	5.01	0.86	c-e	0.91	0.28	a
Year																		
1	61.6	2.1	a	0.712	0.139	a	0.753	0.251	b	0.77	0.18	a	3.84	0.85	b	0.74	0.11	b
2	60.9	4.5	a	0.558	0.177	c	1.08	0.16	a	0.63	0.04	b	5.88	0.93	a	0.81	0.16	a
3	58.4	4.5	b	0.649	0.132	b	1.04	0.16	a	0.63	0.03	b	5.71	0.75	a	0.73	0.02	b

[a]Starch, β-glucan, water-extractable arabinoxylan (WE-AX) and water-unextractable arabinoxylan (WU-AX) expressed as % dry basis; A/X_{WE-AX} and A/X_{WU-AX} are molar ratios of arabinose to xylose present in each arabinoxylan type; $LSD_{0.05}$ represents Fisher's least significant difference groupings at α=0.05 (significant differences within column are noted by different letters).

Table 3. Correlations between heading date, yield, and polysaccharide fractions.[a]

Variable	Yield	WE-AX	A/X_{WE-AX}	WU-AX	A/X_{WU-AX}	Starch	β-Glucan
Heading date	-0.111	-0.28[§]	0.203	-0.32[§]	-0.271	-0.184	0.36*
Yield		-0.50**	0.45**	-0.51**	0.045	0.28[§]	0.178
WE-AX			0.068	0.55***	0.124	-0.038	-0.32[§]
A/X_{WE-AX}				-0.44**	0.159	0.214	0.217
WU-AX					-0.165	-0.165	-0.156
A/X_{WU-AX}						0.233	-0.30[§]
Starch							-0.48**

[a]WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; A/X, arabinose/xylose; heading date calculated as day of the year;
[§]significant at $p<0.1$; *significant at $p<0.05$; **significant at $p<0.01$; ***significant at $p<0.001$.

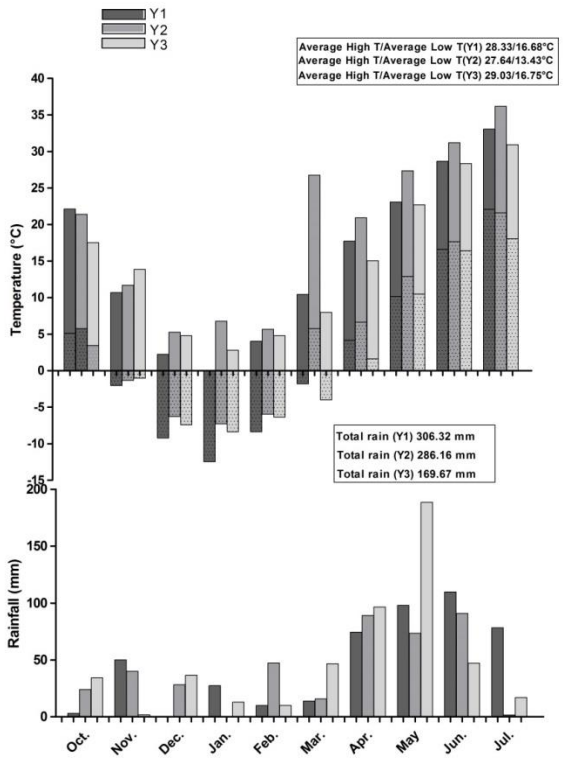

Fig. 1. Average temperature (℃) and total rainfall (mm) in three years of triticale growth; the high temperature is the solid shaded bar, and low temperature is shaded with dots.

Appendix 2. Correlations of long-term dietary pattern of fecal donor with *in vitro* fermentation properties of inulin and comparison with whole wheat substrate

Introduction

Previously, we reported the influence of fecal donor on *in vitro* fermentation properties of whole wheat substrate. Whole wheat was selected as the fermentation substrate because it contains complex and poorly fermented dietary fibers (e.g., cellulose, cross-linked arabinoxylan, and complex pectic substances) that typically make up the substrates for gut bacteria (11-20 g/d) in a typical American diet. We found that butyrate production was correlated with fecal donor intake of many nutrients contributed by grain-, nut-, and vegetable-based foods. The study indicated that diets high in plant-based foods and high in unsaturated fats were associated with microbial metabolism that is consistent with host health.

In contrast to the dietary fibers in wheat, inulin is readily soluble and fermentable by the gut microbiota. In this study, we desired to determine the influence of the long-term diet of fecal donor on *in vitro* fermentation of inulin and compare these results with our previous results using whole wheat as a substrate.

Materials and methods

HP inulin (~100%, average DP $\geq$ 23, Beneo, Germany) was used as the fermentation substrates for this study. Subjects, dietary records, stool sample collection, *in vitro* fermentation, and fermentation analyses were performed as described (chapter 3; Yang and Rose, 2014).

Results and discussion

The amount of inulin fermented over 12 h of *in vitro* fermentation was determined (Fig. 1). There was great variation in the ability of each fecal microbiota to utilize the substrate. The fecal microbiotas from subjects 7, 11, 19, and 24 were among the most able to ferment the inulin, whereas the fecal microbiotas from subjects 15, 21, and 98 used the least. Interestingly, when whole wheat was used as the fermentation substrate, the fecal microbiotas from subjects 21 and 98 were among the most able to ferment the dietary fibers.

In agreement with the percent inulin fermented, the SCFA production varied greatly among fecal microbiotas (Fig. 2). The total SCFA production ($r=0.66$, $p=0.003$), acetate production ($r=0.61$, $p=0.007$), and butyrate production ($r=0.51$, $p=0.03$) were correlated with the amount of inulin fermented. The fecal

microbiota from subject 1 produced the most total SCFA. The fecal microbiota from subject 11 produced much more butyrate than any other microbiota.

Unlike the SCFA data, markers of protein fermentation did not correlate with the percentage of inulin fermented (p-values ranged from 0.23 to 0.93) but did show variation among fecal microbiotas (Fig. 3). The fecal microbiota from most subjects produced relatively low concentrations of BCFA. In contrast, the fecal microbiota from subjects 12 and 21 produced high amounts of BCFAs. Production of these metabolites was highly correlated with ammonia production ($p = 0.0003$, 0.0007, and 0.001 for ammonia vs *iso*-butyrate, *iso*-valerate, and BCFA, respectively).

Many significant positive correlations were discovered for acetate, propionate, butyrate, and the total SCFA production during fermentation (Fig. 4). Interestingly, butyrate showed a significant positive correlation with dietary fiber, starch, plant protein, phytic acid, niacin, folate, Fe, Mg, and Zn, which is consistent with the correlation analysis with whole wheat substrate. There were a few additional significant correlations with inulin that were not observed on whole wheat substrate, including vitamin C and vitamin B-12. In the case of acetate, propionate, and total SCFA, more significant correlations were observed with inulin than whole wheat substrate. *Iso*-valerate showed significant negative correlations with polyunsaturated fat intake, whereas ammonia showed significant negative correlations with energy, carbohydrate, starch, and thiamin intake.

A few correlations were noted between certain food categories and carbohydrate fermented and metabolite production during fermentation (Fig. 5). Butyrate carried the most significant correlations, including positive correlations with “total vegetables,” “potatoes,” “other vegetables,” and “fish high in omega-3s” and a negative correlation with “eggs.” The relevance of some of these correlations was limited due to low reported intake (e.g., “potatoes,” “fish high in omega-3s,” “soy products,” and “dry beans and peas”) or limited range of intakes (e.g., “whole grain” and “eggs”) in some of these food categories.

We performed a correlation analysis for fermentation parameters during fermentation of inulin and wheat substrates (Fig. 6). Interestingly, the only significant correlation was observed between the production of butyrate in wheat and inulin substrate, but not in other SCFA and carbohydrate fermented. This suggests that relative butyrate production is independent of the type of substrate, but highly dependent

on fecal donor. It was worthy nothing that significant correlation was also observed between the production of BCFA in wheat and inulin substrate.

Conclusion

Butyrate showed a significant positive correlation with dietary fiber, starch, plant protein, phytic acid, niacin, folate, Fe, Mg, and Zn, which is consistent with the correlation analysis with whole wheat substrate. In the case of acetate, propionate, and total SCFA, more significant correlations were observed with inulin than whole wheat substrate. We also found that relative butyrate production is independent of the type of substrate, but highly dependent on fecal donor.

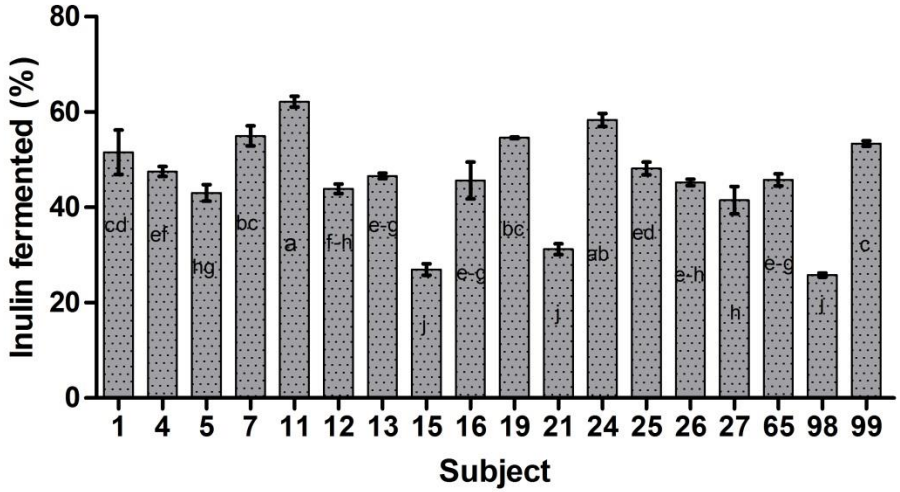

Fig.1. Carbohydrate fermentation during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; n = 2.

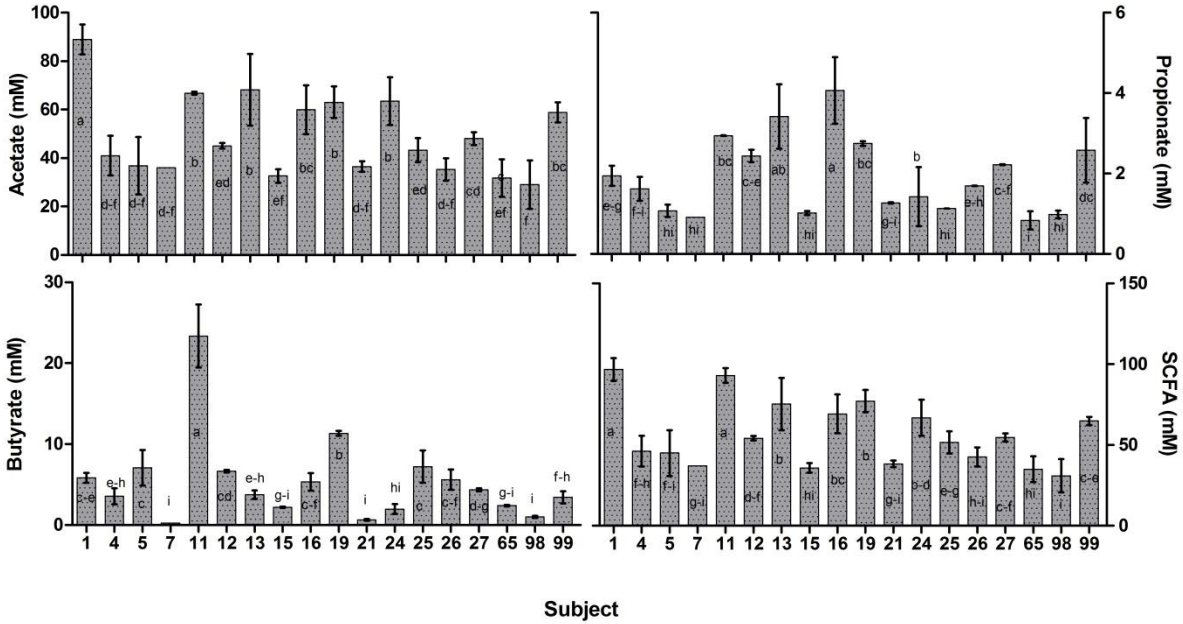

Fig.2 Branched-chain fatty acid and ammonia production during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; n = 2; note the different scales on the y-axes.

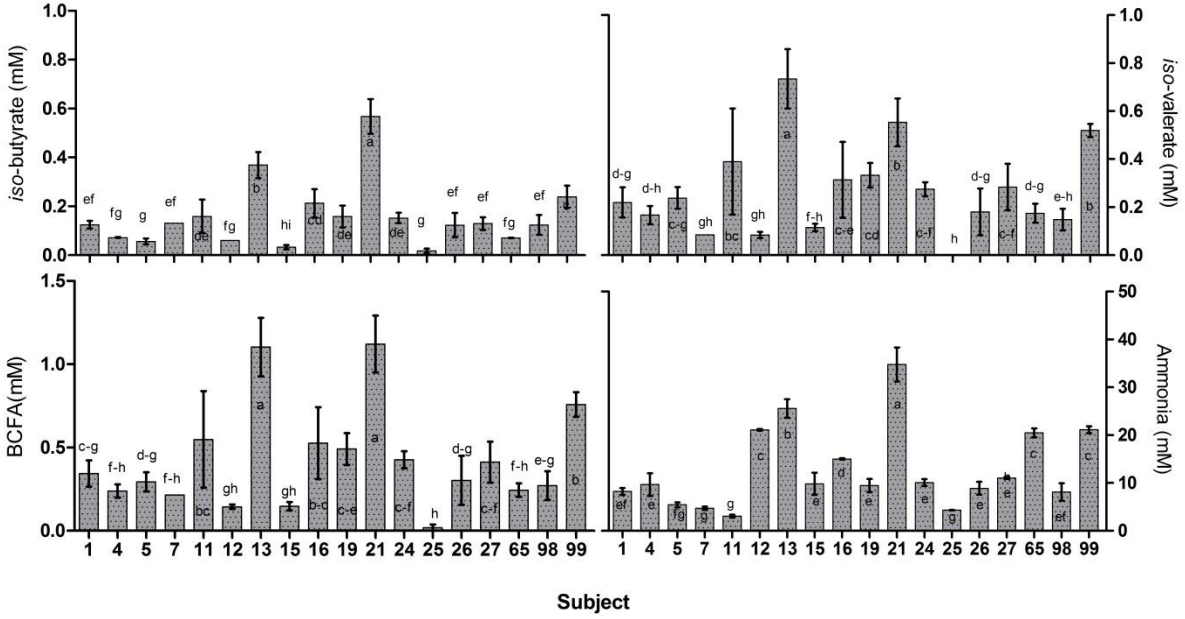

Fig.3. Short-chain fatty acid production during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; n = 2; note the different scales on the y-axes.

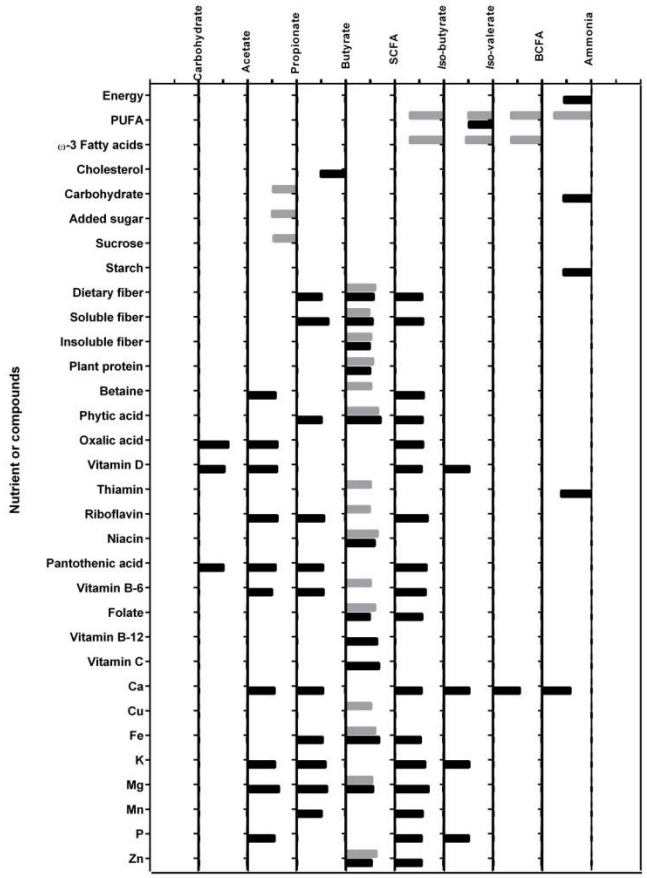

Fig.4. Spearman ρ correlation of dietary nutrient intake of the fecal donor with carbohydrate fermented (%) and metabolite production (mM) during *in vitro* fecal fermentation. Black bars indicate the direction (left, negative; right, positive) and magnitude of significant ($p<0.05$; $n=18$) correlations when using inulin as substrate. Gray bars indicate the direction and magnitude of significant ($p<0.05$; $n=18$) correlations when using whole wheat as substrate. Data for whole wheat substrate were reported in Yang and Rose (2014).

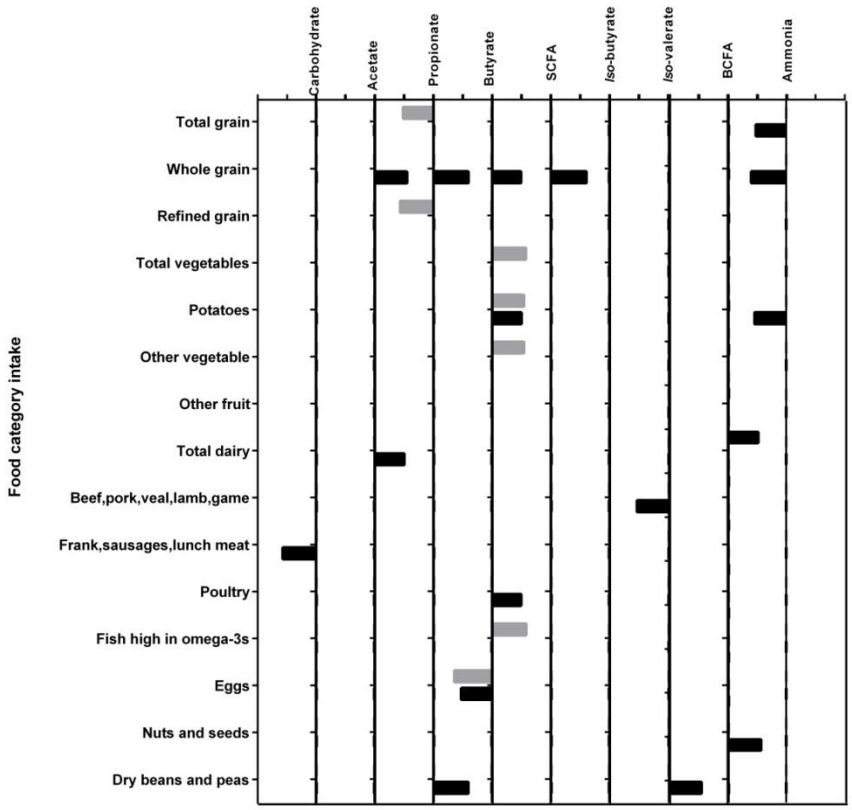

Fig.5. Spearman ρ correlation of food category intake of the fecal donor with carbohydrate fermented (%) and metabolite production (mM) during *in vitro* fecal fermentation. Black bars indicate the direction (left, negative; right, positive) and magnitude of significant ($p<0.05$; n=18) correlations when using inulin as substrate. Gray bars indicate the direction and magnitude of significant ($p<0.05$; n=18) correlations when using whole wheat as substrate. Data for whole wheat substrate were reported in Yang and Rose (2014).

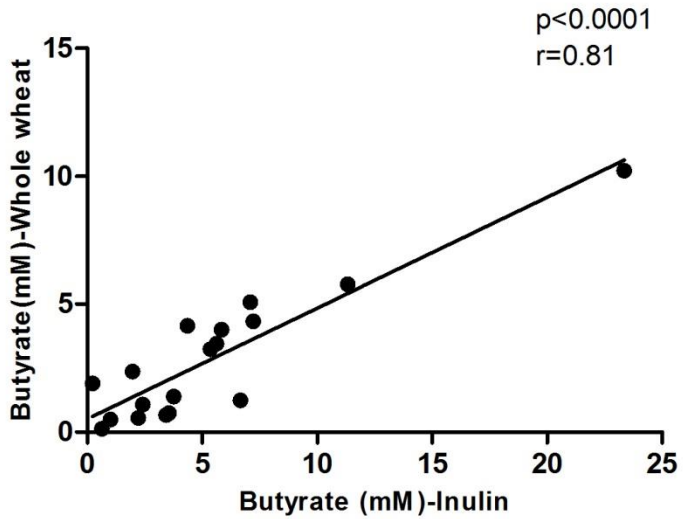

Fig.6. Correlation analysis for butyrate production during fermentation of inulin and whole wheat substrates.