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WHOLE GRAIN PROCESSING AND EFFECTS ON CARBOHYDRATE DIGESTION AND FERMENTATION

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by

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Whole grains are a major source of dietary fibers in the human diet that provide specific nutrients to the gut microbiota and thereby plays a major role in modulating microbiota composition and increasing diversity of the gut ecosystem. A common approach of consuming whole grains is in the form of ready-to-eat extruded breakfast cereals. Studies reported herein established that extrusion conditions not only affected the physicochemical properties but also in vitro starch digestibility, β-glucan extractability and in vitro fermentation characteristics of whole grain oats. Moderate screw speed (300 rpm) led to higher slowly digestible starch (SDS) with an accompanying decrease in rapidly digestible starch (RDS). Low moisture conditions (15%) resulted in the highest resistant starch (RS) and water-extractable β-glucan (WE-BG). Extrusion moisture significantly affected WE-BG in the extrudates, with samples processed at 15% moisture (lowest) and 21% moisture (highest) having the highest concentration of WE-BG. Extrusion moisture conditions was also found to significantly affect the production of acetate, butyrate and total SCFA by the microbiota during the first 8 h of fermentation. After 24 h, samples processed at 15% moisture supported lower Bifidobacterium counts than those produced at other conditions, but had among the highest Lactobacillus counts. Besides oats,

there are other whole grain cereals and their brans that have unique structural characteristics that may impart distinct effects on fermentation by the gut microbiota with subsequent effects on the host. Since dietary fiber intake has an impact on functionality of the gut microbiota, another study was conducted to establish whether the gut microbiota from individuals consuming high dietary fiber diets (G1) could metabolize the dietary fibers from grains more efficiently and produce higher concentrations of beneficial metabolites compared with donors with lower dietary fiber intakes (G2). Fecal microbiota from G1 subjects showed less decrease in diversity during fermentation and these microbiotas showed higher carbohydrate utilization and butyrate production compared with microbiota from G2 subjects. More carbohydrates were fermented from whole grains than brans. Rye induced high carbohydrate fermentability and butyrate production accompanied by low ammonia production, but only when using fecal microbiota from G1 subjects.

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PREFACE

This dissertation is organized as follows: a literature review (Chapter 1) followed by manuscripts describing three research projects (Chapters 2, 3, 4) and a conclusion (Chapter 5). Chapter 1 provides a current literature review of interaction between whole grains and human gut microbiome. This chapter has been formatted using the guidelines for the Royal Society of Chemistry for publication in the upcoming book entitled Cereal Grain-based Functional Foods, edited by Trust Beta and Mary Ellen Camire. Chapter 2 describes the effects of selected extrusion parameters on physicochemical properties and in vitro starch digestibility and β-glucan extractability of whole grain oats, which has been published in the Journal of Cereal Science (Brahma et al., 2016). Chapter 3 describes how extrusion moisture conditions impact the in vitro fermentation characteristics of whole grain oats. This chapter has been published in the Food Research International (Brahma et al., 2017). Chapter 4 introduced the concept of how long-term dietary pattern of fecal donor impacts the in vitro fermentation properties of different whole grains and brans. This chapter has been published in the Journal of Functional Foods (Brahma et al., 2017). Finally, Chapter 5 provides a conclusion that summarizes the findings provided in this thesis.

Objectives of this research:

Chapter 2: To determine how extrusion conditions affect the physicochemical properties of whole grain oat extrudates, with emphasis on in vitro starch digestibility and changes in water-extractability and molecular weight of β-glucan.

Chapter 3: To investigate the influence of moisture content during extrusion on the in vitro fermentation of whole grain oats by human fecal microbiota.

Chapter 4: To determine changes in microbial metabolite concentrations and fecal microbiota composition during in vitro fermentation of whole grains/brans using stool samples collected from individuals with substantially different diets.

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Chapter 1 . Interactions between grains and the microbiome

1.1. Introduction

The human gastrointestinal tract (GI) is one of the largest interfaces between the host and the environment in the human body. The microbes that colonize the GI tract are termed the gut microbiota, and these microorganisms have evolved with the host to form an intricate and mutually beneficial relationship.[1] The number of microorganisms populating the GI tract has been estimated to be 10^{13} cells, which is equivalent to the number of human body cells.[2] The adult human microbiota typically includes five dominant commensal phyla: Firmicutes, Bacteroidetes, Proteobacteria, Fusobacterium and Actinobacteria, of which Firmicutes, Bacteroidetes are present in the greatest abundance (>90%).[1] The microbiota confers many benefits to the host such as regulating immune function,[3] harvesting energy[4] and maintaining gut integrity.[5] Potential disruption of microbial composition may lead to 'dysbiosis', which can promote development of metabolic diseases.[6-8] Although the composition of the microbiota is relatively stable within an individual, both long-term and short-term perturbations, such as diet changes, have been reported to induce both structural and functional changes to the gut microbiota.[9-11]

Cereal grains are major sources of dietary non-digestible food carbohydrates that are potentially available to be fermented by the gut microbiota in the large intestine. The human genome does not encode for enzymes that break down the complex carbohydrates such as cellulose, arabinoxylan, β-glucan, and fructans, that make up the dietary fibers in whole grains; however, bacteria are able to use these substrates for energy. Bacterial metabolism of these carbohydrates confers health benefits to the host, for example by

producing the beneficial short chain fatty acids (SCFA) such as acetate, propionate and butyrate.[12] Whole grain cereals are also abundant in phytochemicals such as phenolic acids, flavonoids, and anthocyanins, which are considered to evoke significant health impacts in prevention of chronic diseases.

Several studies have been documented pertaining to the impact of whole grain foods and components of whole grains on human metabolic health and the gut microbiota.[7, 8,13-21] However, much is still unknown about how specific components of whole grains interact with the gut microbiota and how they pertain to human health. Furthermore, findings from whole grain intervention studies are not consistent with respect to shifts in the microbiota and corresponding host benefits.[13, 17, 19] Complicating matters further, some intervention trials suggest that gut microbiota composition at enrollment into a study is predictive of host benefits in response to whole grains.[22-24] Finally, not all non-digestible components in whole grains are available for metabolism by the microbiota[25, 26]; therefore, optimizing processing methods and grain types to enhance the quantity of carbohydrates available for gut microbial fermentation is an area ripe for research.[27-33] The purpose of this review is to discuss whole grain-gut microbiota interactions and identify new areas of research that may contribute to a better understanding of the underlying mechanisms linked to human health.

1.2. Grain components that are likely to interact with the microbiome

The most important whole grain components that are likely to interact with the microbiota are dietary fibers and polyphenols. Other non-digestible compounds, such as waxes, saponins, phytates, phytosterols and other lipophilic compounds, and resistant proteins, may also interact with the gut microbiota[34], but much less is known about the

impacts of these compounds on the gut microbiota. It must be emphasized that carbohydrate and polyphenol compositions of whole grains vary among grains (Figure 1.1 and Table 1.1).

1.2.1. Dietary fibers

Dietary fiber concentration in whole grains depends on many factors and that typically ranges from as little as 4% in brown rice to as much as 16% in rye (Figure 1.1). The main dietary fiber components in whole grains are non-starch polysaccharides, which can be classified into poorly fermentable (by the gut microbiota), such as cellulose and water-unextractable arabinoxylans, and readily fermentable, such as mixed-linkage β-glucans and water-extractable arabinoxylans.[40] Compositional and structural descriptions of the major dietary components that escape digestion in the human small intestine are outlined in the following subsections.

1.2.1.1. Arabinoxylans

Arabinoxylans are the major dietary fiber components in grains, comprising roughly 50% of dietary fiber in all whole grains, except for oats and barley, which contain about 30% of dietary fiber as arabinoxylans (Figure 1.1). These polysaccharides are composed of a linear backbone of β-D-xylopyranosyl (Xyl*p*) residues linked through (1-4) glycosidic bonds. The backbone can contain α-L-arabinofuranosyl (Ara*f*) substitutions at the O-3 and/or O-2 positions on the Xyl*p* residues.[41] Some Ara*f* residues contain an ester-linked ferulic acid moiety (see section 1.2.1.6. Phenolics) at O-5, which can form oxidative cross-linkages with other arabinoxylan chains and other components of the cell wall.[42] Oligosaccharide branches consisting of glucose, arabinose and xylose are also common, as are glucuronic acid residues.

Arabinoxylan can be categorized to water-extractable and water-unextractable. Water-extractable arabinoxylans dissolve in aqueous solutions and are present in far lower concentrations than the insoluble, water-unextractable arabinoxylans.[41] The water-extractable arabinoxylans can be considered "precursors" to the water-unextractable arabinoxylans, which act as the "glue" that hold the plant cell wall together through phenolic cross-linkages and non-covalent bonds. Because water-unextractable arabinoxylan is made unextractable in large part by ester-linked phenolic cross-linkages, a large portion of water-unextractable arabinoxylan can be made soluble by treatment with alkali.

The structure of arabinoxylan can vary among grain types. For example, wheat arabinoxylans contain more O-2 and O-2,3 substituted Xyl*p* residues than rye, which contains more O-3 substituted Xyl*p* residues.[42] Rye also contains more unsubstituted Xyl*p* residues that are more uniformly distributed along the xylan backbone, while wheat contains less Xyl*p* residues that tend to cluster in contiguous groups along the backbone.[43]

The structure of arabinoxylan also varies among different anatomical parts of the grain. For instance, when water-extractable arabinoxylan were analyzed from wheat bran and the starchy endosperm the arabinose: xylose ratios (a measure of the degree of branching) as well as the concentrations of arabinoxylans were different in each fraction.[41-43]

Structural features associated with degree of branching, molecular weight, spatial arrangement of arabinoxylans, and ratio of arabinose/xylose in cereals influence their fermentability which in turn could further affect functionality of gut microbiota.[44, 45] For

instance, Rose et al.[44] determined that among the corn bran, rice bran, and wheat bran alkali-extracted fractions, corn arabinoxylans resulted in highest SCFA production compared to fractions from wheat and rice. Rice and corn arabinoxylans were hypothesized to degrade by a debranching mechanism due to their regular branching patterns, whereas wheat arabinoxylans were hypothesized to ferment in two stages due to the irregularity of the branches along the Xyl*p* backbone: the unsubstituted regions first followed by the highly branched regions.[44] In another study, no differences in fermentation rate patterns with respect to molecular mass or arabinose/xylose ratio were reported; however, rice and sorghum arabinoxylans were shown to have a simple branched structure that was associated with rapid fermentation compared to wheat and corn arabinoxylans.[45]

Other recent studies have demonstrated the impact of arabinoxylans on modulation of the gut microbiota by promoting certain probiotic bacteria (e.g., *Lactobacillus*, *Bifidobacterium*) and enhancing the production of SCFA.[29, 46,47] For instance, Damen et al.[46] studied the impact of arabinoxylan fractions isolated from wheat bran in rats. The fractions: water-unextractable (40% purity), water-extractable (80% purity), arabinoxylan oligosaccharides (79% purity), and their combinations, were included in a standardized diet at 5% arabinoxylan for 14 d. The authors observed that the ternary combination of water-extractable, water-unextractable, and arabinoxylan oligosaccharides, increased colonic butyrate production, promoted reduced pH, limited proteolytic metabolites and *Bifidobacterium* growth in the colon compared to diets with only the individual arabinoxylan fractions. Truchado et al.[47] studied the modulatory effects of two doses of water-extractable, long-chain arabinoxylans (3 and 6 g/L), three times per day for three

days on luminal and mucosal microbiota in a human intestinal microbial ecosystem (M-SHIME). The authors concluded that the higher dosage stimulated *Bifidobacterium* and could be potentially beneficial to human host health.

It is to be noted that fermentation of isolated arabinoxylan fractions is much different from that of arabinoxylan in whole grain due to the extensive cross-linkages present in native arabinoxylans.[44, 48, 49] Cross-linking and other factors limit the availability of native arabinoxylan for microbial fermentation, although the extent of fermentation may be altered by various means such as processing (see section 15.5. Increasing whole grain-gut microbiota interactions).

1.2.1.2. β-Glucans

β-glucans are non-digestible polysaccharides composed of mixed linkage (1, 3) and (1, 4)-β-D glucose units with a molecular mass ranging between 50 and 2,300 kDa that are present in the greatest amounts in oat and barley.[50] The highest content of β-glucan has been reported for barley, 2–20 g, and for oats, 3–8 g (g/100 g dry weight). Other cereals such as corn, wheat, and rye also contain β-glucan but in lower concentrations.[50]

As with arabinoxylan, the structure and molecular features of β-glucan such as the ratio of (1, 3) to (1, 4) linkages, ratios of cellotriosyl/cellotetraosyl units (DP3/DP4), and molecular weight play significant roles in viscosity, solubility, dispersibility, and, consequently, the physiological functions that include cholesterol-lowering and glucose-attenuating effects in the GI tract.[51-53] In a human feeding trial, Wang et al.[53] showed the impact of four β-glucan-based experimental diets for five weeks on the gut microbiota composition of mildly hypercholesterolemic subjects. The experimental diets included a

wheat and rice-based control; 3 g/d low molecular weight (LMW) barley β-glucan (288 kDa); 5 g/d LMW barley β-glucan (292 kDa); and 3 g/d high molecular weight (HMW) barley β-glucan (1,349 kDa). Among the treatment groups, the 3 g/d HMW barley β-glucan increased *Bacteriodetes* and decreased *Firmicutes* compared to the control diet. At the genus level, the HMW barley β-glucan diet increased *Bacteroides*, decreased *Dorea*, and tended to increase *Prevotella.* These genera were correlated with changes in markers for cardiovascular disease. The LMW barley β-glucan treatments did not induce any changes in gut microbiota composition.

Other studies have also indicated the effectiveness of β-glucan in modulating the gut microbiota composition and increasing the production of SCFA by the microbiota.[17, 19, 54] For instance, Dong et al.[17] studied how oat products modulated the gut microbiota and reduced obesity in rats. In this study, the authors fed rats either a normal chow diet, a high fat diet, or a high fat diet supplemented with oatmeal, oat flour, or oat bran for 8 weeks. They reported that diets containing any of the oat products modulated the overall gut microbiota composition by increasing the *Bacteriodetes*/*Firmicutes* ratio. Also, the *Acidobacteria* was detected only in the group following the treatment with oat products, more pronouncedly in the oat bran group. A significant increase in fecal SCFA was also noted in the oat products groups compared to the control. Increases in the abundance of *Bacteroidetes* and the *Bacteriodetes*/*Firmicutes* ratio were also found to be negatively correlated with markers of obesity, dyslipidaemia, and inflammation. The authors attributed these results to the oat products aiding in controlling obesity and related metabolic disorders while regulating the gut microbiota composition in obese rats. In contrast to this study, Martínez et al.[19] reported that rolled whole grain barley (60 g/d)

caused a decrease in *Bacteriodetes*/*Firmicutes* ratio along with an increase in the abundance of the genus *Blautia* in a human feeding trial. The authors mentioned that the bacteria responding explicitly to the whole grain barley treatment encode for β-glucanase genes that assist in utilizing the substrate during fermentation.

1.2.1.3. Cellulose

Cellulose is an essential component of cereal cell walls, consisting of linear chains of (1,4)-linked β-D-glucose units. Due to the linear structure of β-glucan, cellulose is insoluble in water and can form three dimensional microfibrillar aggregates that are resistant to digestion by microbial enzymes.[55] Robert et al.[56] demonstrated that the ability of the microbiota to degrade microcrystalline cellulose was greatest in methanogenic individuals. Methanogenic bacteria from these subjects belonged mostly to the *Ruminococcus* genus together with some *Enterococcus*.

Mouse studies have compared diets containing cellulose with those containing more fermentable fibers and have concluded that diets containing fermentable fibers are more important to gut health than cellulose. [57, 58] Native cellulose in plant cell walls behaves differently in the gut from that of purified cellulose.[57] For instance, Van Soest [58] compared the effects of controlled diets with the addition of cellulose from three sources (cabbage, wheat bran, or purified) on the microbial ecology of the healthy volunteers. The authors reported not only lowest fermentation of purified cellulose, but also determined that purified cellulose failed to induce bacterial fermentation and depressed the breakdown of other cell-wall polysaccharides from the diet. Moreover, fermentation of purified cellulose exhibited a lag of 17-20 h which was much longer than cellulose from natural sources. Although cellulose is present in the diets along with other

carbohydrate polymers, more research needs to be conducted to establish whether native cellulose has unique properties in the GI tract.

1.2.1.4. Fructans

Fructans are naturally occurring plant oligo- and polysaccharides built on the repeated fructosylation of sucrose.[59] Among grains, rye has the highest fructan levels, ranging from 3.3-6.6%, followed by wheat and so on (Figure 1.1). Wheat contains fructans known as graminans, which contain both β-(2,1) and β-(2,6) fructosyl linkages in the same molecule and contain an internal glucose unit instead of a terminal glucose.[59, 60] Unfortunately, structural information on fructans from other grains are not currently available.

While studies have demonstrated the prebiotic potential of fructans [61, 62,] few studies have documented the impact of cereal fructans on gut health.[63, 64] Belobrajdic et al.[63] reported similar SCFA concentrations in the caecum and colon digesta of rats fed diets containing oligofructose, wheat stem fructans, or barley grain fructans at the 5% level. Although the number of bifidobacteria in the caecum increased only for the oligofructose group, a significant decrease occurred in the pH of the colonic digesta in the in the barley grain fructan group. Similar to this study, another group of authors evaluated the impact of chain length of fructans isolated from wheat stem and barley on gut microbiota during an in vitro fermentation and compared the data with that of inulin and oligofructose.[64] The authors determined that the graminan fructans produced comparable levels of total SCFA to oligofructose and inulin, indicating that fructans from such novel sources could have metabolic benefits.

1.2.1.5. Resistant starch

Starch can be divided into three categories: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS).[65] RS is the fraction of starch that is relevant to gut health, as this fraction survives transit to the large intestine. RS can be classified into five categories: RS1 (physically inaccessible), RS2 (granular or native semi-crystalline), RS3 (retrograded or re-crystallized), RS4 (chemically-modified), and RS5 (amylose-lipid complexes).[66]

Studies have demonstrated the health benefits of RS on gut microbiota composition.[67-69] In one study, Upadhyaya et al.[68] fed 20 individuals with signs of metabolic syndrome RS4 (30%, v/v in flour) or a control wheat flour for 12 weeks each in a crossover design. The RS4 group had higher concentrations of fecal SCFA such as propionate and butyrate, together with higher abundance of *Bacteroides*, *Parabacteroides*, *Oscillospira*, *Blautia*, *Ruminococcus*, *Eubacterium*, and *Christensenella*. The authors reported significant correlations between changes in the gut microbiota composition induced by RS4 and increased fecal SCFA. Acetate and butyrate levels were correlated with changes in *Ruminococcus lactaris* and *Oscillospira* species. Total SCFA were correlated with changes in *Methanobrevibacter* species and *Ruminococcus lactaris*, and propionate and iso-butyrate were correlated with *Methanobrevibacter* species, *Eubacterium dolichum, Christensenella minuta*, and *Ruminococcus lactaris.* No significant correlations were noted between changes in the gut microbiota and SCFA production on the control flour intervention. Goldsmith et al.[69] studied the impact of whole grain corn flour with RS on gut microbiota in obese rats for 11 weeks. The study included 4 diet groups: normal corn starch; whole grain control flour

(containing 6.9% RS); isolated RS-rich corn starch (25% RS); and whole grain corn flour (25% RS). The isolated RS-rich corn starch contributed to a higher *Bacteroides*/*Firmicutes* ratio compared to the other diet groups, whereas the high RS whole grain treatment induced higher SCFA production and lower cecal content pH than isolated RS.

1.2.1.6. Phenolics

Whole grains are good sources of phenolic compounds that may act as antioxidants and have anti-inflammatory, anti-microbial, and anti-carcinogenic effects against degenerative diseases such as heart disease and cancer.[70] Phenolics are secondary metabolites of plants that are involved in defense mechanisms against ultraviolet radiation or to protect the plant from pathogens.[70] The total phenolic content in grains ranges from 0.04% in oats up to 0.4% in foxtail millets (Table 1.1). Brans have higher percentage of phenolics compared to their corresponding whole grains, ranging between 0.42-0.45%.[71]

All phenolic compounds have a phenolic ring and can be classified into different categories as a function of number of phenol rings they contain and the structural elements that attach these rings to one another.[72] Examples of the most common categories of phenolics are phenolic acids, flavonoids, condensed tannins, and alkyl resorcinols.[72] Phenolic acids are derivatives of benzoic and cinnamic acids and are usually represented by two types: hydrobenzoic acids such as gallic, vanillic, syringic acids, and hydrocinnamic acids with C6-C3 structures such as coumaric, caffeic, ferulic, and sinapic acids.[71] Flavonoids have a typical C6-C3-C6 structure, consisting of two aromatic rings attached by a three-carbon linkage that include flavonols, flavones,

isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins), found mostly in sorghum, millets, barley, maize, rye, rice and wheat.[73] Condensed tannins are polymerized flavanol units that can bind to proteins, carbohydrates and minerals, are mostly located in grains such as sorghum, barley, and red finger millets.[73] Lignans are phytoestrogens and the two most common plant lignans are secoisolariciresinol and matairesinol, which are present predominantly in cool seasonal cereal grains such as barley, oat, rye, triticale, and wheat.[74] Alkylresorcinols are mostly present in the brans of wheat, rye, triticale and barley, but not in maize, oats, millets, rice or sorghum. They are1,3-dihydroxybenze derivatives with an odd-numbered n-alkyl side-chain at C-5 on the benzene ring.[75]

Phenolic acids are the most abundant antioxidants in whole grains and can be present in free and bound forms. The bound phenolics are mostly linked to arabinoxylan chains as explained (section 15.2.1.1. Arabinoxylans). Grains have higher bound phenolics and lesser free ones: about 85, 75, and 62% of the total phenolics present in corn, wheat, and rice, respectively, are in the insoluble bound forms.[76] Some varieties of barley may contain bound phenolics ranging between 54 and 90%.[77]

The release and absorption of free phenolics from the food matrix occurs either by direct solubilization in the intestinal fluids under GI conditions and/or by the action of digestive enzymes that hydrolyze macronutrients and favor the release of phenolics from the food matrix.[78] Once absorbed, phenolic compounds may be subjected to biotransformation in the enterocytes and hepatocytes, generating water-soluble conjugate metabolites such as methyl, glucuronide, and sulfate derivatives that are distributed to host tissues and ultimately excreted in the urine.[78]

In contrast, the release of bound phenols from the food matrix occurs only to a limited extent. Kroon et al.[79] reported that gastric and small intestinal enzymatic treatment released 0.41 and 2.46 nmol of free ferulic acid, respectively, and 6.91 and 4.70 nmol of esterified ferulic acid, which in total accounted for only 2.6% of total feruloyl groups in the wheat bran fiber. The majority of bound phenolics traverse the small intestine intact along with dietary fiber and reach the colon, where they serve as substrates for gut bacteria.[80] Andreasen et al.[81] compared the release of free diferulic acids (8-5- diferulic acid, 5-5- diferulic acid, 8-*O*-4-diferulic acid and 8-5-benzofuran diferulic acid) from wheat and rye bran by human fecal microbiota. The microbiota released 36% of 8-5-diferulic acid, 4% of 5-5-diferulic acid, 4% of 8-O-4-diferulic acid, and 7% of 8-5-benzofuran diferulic acid during fermentation of the wheat bran matrix. In rye bran, human fecal microbiota was unable to release any of 8-5-diferulic acid or 5-5-diferulic acid and only small amounts of 8-O-4-diferulic acid and 8-5-benzofuran diferulic acid (6% and 3%, respectively). However, the extent of bound phenolics released from the matrix can be altered (usually increased) by non-thermal and thermal processing techniques such as fermentation processes in food, germination, roasting, extrusion cooking and boiling (see section 15.5. Increasing whole grain-gut microbiota interactions).

Once released by gut bacteria, phenolic compounds are rapidly metabolized through hydrogenation, demethylation, dehydroxylation, and decarboxylation. The first step during fermentation of methyl ferulate by the human colonic microbiota is the process of demethylation into ferulic acid, followed by several reactions that ultimately yield phenylpropionic acid.[82] Only a few bacterial genera such as *Escherichia*,

Bifidobacterium, *Lactobacillus*, *Bacteroides*, and *Eubacterium* have been documented to be able to metabolize phenolics.[78]

Covalently attached phenolics in grains or grain fractions can impact gut health.[83, 84] For instance, Duncan et al.[83] reported that wheat bran promoted the enrichment of five key species of bacteria *Butyrivibrio fibrisolvens*, *Eubacterium rectale*, *Roseburia faecis* and *Roseburia intestinalis*, *Eubacterium siraeum* that were not only known butyrate producers, but also were responsible for the release of ferulic acid thereby playing pivotal roles in fermenting wheat bran. Yang et al.[84] fed a low-fat diet, a high-fat diet, and a high-fat diet supplemented with maize-derived non-digestible feruloylated oligo- and polysaccharides to mice for 8 weeks. The authors observed blooms in the gut microbial genera *Blautia* and *Akkermansia* in three of the mice fed with feruloylated oligo- and polysaccharides. These shifts were attributed to decreased body and adipose tissue weights compared with the mice fed with the control high-fat diet, thus indicating the changes could depend on the ability of the microbiota of an individual to ferment feruloylated oligo- and polysaccharides. However, the underlying interactions between whole grain dietary fibers and associated phenolics remain elusive. In addition, the specific effects of dietary phenols on the modulation of gut ecology remains vague and needs further investigation.

1.2.1.7. Other compounds

Whole grains also contain other combinations of minerals and phytochemicals depending on the type of cereals. Besides phenolic compounds, other examples of phytochemicals are phytosterols and tocols (terpenes and terpenoids), betaine, folate, α- and β-carotene, lutein, β-cryptoxanthin and zeaxanthin and phytates.[85] Phytosterols are

steroid compounds present in plants and can be classified into sterols and stanols depending on the number of carbon side chains and presence or absence of double bonds. Sterols are unsaturated compounds with a double bond in the ring, whereas stanols are saturated compounds. Stanols represent only 10% of total dietary phytosterols, sitosterols with campesterols being the most abundant sterols present in plants and human diet.[86] Phytic acid, also known as inositol hexaphosphate, is the storage form of phosphorous in grains and cereals. The concentration of phytate in grains varies among cereals, ranging from 0.5-2.0%.[87]

Several studies have revealed the impact of phytosterols and phytic acid on the gut microbiota.[88, 89] Markiewicz et al.[88] studied how diet shaped the ability of the microbiota to degrade phytate in in vitro using fecal samples from adults on conventional and vegetarian diets and breast-fed infants. The authors reported that regardless of the diet group, the gram-positive anaerobes and lactobacilli had the lowest ability to degrade phytate, whereas coliforms and proteobacteria-bacteroides cultures showed the highest potential to degrade phytate to intermediate myo-inositol phosphates. The authors concluded that a well-balanced cooperation of aerobic and anaerobic bacteria is essential to degrade phytate, and a diet rich in phytate could enhance the potential of microbiota to degrade phytate. Another study by Rasmussen et al.[89] exhibited how plant sterol esters made with fatty acids from soybean oil, beef tallow or purified stearic acid could impact the cholesterol absorption when fed to male hamsters for 4 weeks. A control group was also included where the hamsters were fed a diet devoid of sterol-esters. The authors noticed that hamsters fed with purified stearic acid and plant sterol esters showed significant lower cholesterol absorption and reduced concentrations of plasma non-HDL

cholesterol and liver cholesterol, thus suggesting that cardioprotective benefits can be achieved by increasing consumption of stearate-enriched plant sterol esters.

1.3. Whole grain intervention studies

Intervention studies have been conducted to understand the impact of whole grain consumption on markers of cardiovascular and metabolic health (Table 1.2). These studies have shown that consumption of whole grains and their components have been associated with lower body mass index (BMI), adipose tissue, obesity, cardiovascular diseases, and type 2 diabetes, although findings are not consistent. Moreover, these studies have shown differing effects on gut microbiota composition, which could be due to differences in study design and the types of whole grain foods used. Whole wheat and wheat bran breakfast cereals caused an increase in lactobacilli/enterococci and *Bifidobacterium* spp.[16] Lappi et al.[18] examined the differences in gut microbiota composition after intake of high fiber rye bread and low fiber wheat bread in Finnish adults. They reported a decrease in *Bacteroidetes* and an increase in *Clostridium cluster IV*, *Collinsella*, and *Atopobium* spp. during the 12-week intervention. In another study, human subjects consumed a daily dose of whole grain barley, brown rice, or an equal mixture of both whole grain barely and brown rice for 17 weeks in a randomized cross-over design.[19] The authors observed a decrease in *Bacteroidetes* and increase in *Firmicutes*. In a 6-week randomized trial using healthy human subjects, by Vanegas et al.[20] showed that by replacing whole grains with refined grains only had a modest impact on gut microbiota composition accompanied by an increase in *Lachnospiraceae*, a decrease in *Enterobacteriaceae,* and an increase in fecal acetate and total SCFA.

1.4. Responders/Non-responders to whole grains

The prebiotic literature discusses a phenomenon termed "responder/non-responder" and is based on how the microbiota of a given individual changes in response to dietary prebiotic interventions.[22, 23, 24] This division among the individuals originated based on whether the microbiota remains stable (unchanged) during the intervention or whether the expected changes, such as an increase in *Bifidobacterium*, are evident after a prebiotic treatment.[19,22,23,107] Undoubtedly this phenomenon is applied to whole grains. Korpela et al.[22] used data from 3 human feeding trials to generate statistical models based on gut microbiota composition to predict responders (i.e., improve health outcomes) and non-responders to the dietary intervention. The authors noted that baseline microbiota composition (before the onset of the study) had the greatest ability to predict host responsiveness.[24] This has huge implications for human feeding trials, as baseline microbiota composition is not usually a factor that is considered when subjects are enrolled in a study. In yet another study, researchers determined that a subset of individuals responded to a barely kernel bread intervention with an improvement in glucose tolerance. These individuals had higher baseline and end of study abundances of *Prevotella*. To further validate whether *Prevotella* could mediate glucose response, a one week study using gnotobiotic mice was conducted that resulted in an improved glucose tolerance with *P. copri*. The conclusion of this study was that this strain alone could improve glucose tolerance in humans without any change in their normal diet.[23]

Many factors may play roles in the responder/non-responder phenomenon. Davis et al.[108] suggested that the specific strain capable of fermenting the test food might not be present in the non-responder population. For example, Martínez et al.[19] showed that a

mixture of whole grain barley and brown rice caused a reduction in IL-6 in subjects during a four-week intervention period. Importantly, the magnitude of reduction in IL-6 was associated with higher baseline abundance of *Dialister* and lower abundance of Coriobacteriaceae. Using data from an in vitro study, a strong relationship between *Dialister* and the ability of the microbiota to metabolize arabinoxylan from whole grains was evident (Figure 1.2)[109] Thus, the higher abundance of this genus may have enabled the responders to respond to the whole grain barely treatment through the metabolism of the arabinoxylan[19]. Other host factors may also dictate the responders/non-responder status of individuals.

1.5. Increasing whole grain-gut microbiota interactions

The interactive effects of whole grains with the gut microbiota must be emphasized along with identifying the metabolic benefits of whole grains which are proposed to be mediated through their interactions with the gut microbiota.[12] However, it is notable that not all non-digestible carbohydrates or phenolics in whole grains are available for fermentation by the microbiota: up to two-thirds of the potentially available non-digestible carbohydrates may pass through the GI tract without modification.[26] Increasing the proportion of non-digestible carbohydrates or phenolics that are available for metabolism by the microbiota may make a positive impact on human health by harnessing more of the potential benefits of whole grains.[25,26]

Many factors may increase the availability of whole grain components to interact with the microbiome. For instance, food processing conditions are known to have significant impacts on structural characteristics of cell wall polysaccharides and these changes have been shown to increase the fermentability of non-digestible carbohydrates

from grains by the human fecal microbiota.[27-29] Low moisture (15%) coupled with low screw speed extrusion conditions (120 rpm) led to the highest availability of non-starch polysaccharides for fermentation, increasing fermentation from 110 g/kg in unextruded wheat bran to 200 g/kg.[27]

Processing and bioprocessing techniques may release the bound phenolics from the insoluble fiber matrix and improve the in vitro bioaccessibility and colonic metabolism of phenolic compounds.[30-33] One such study by Anson et al.[30] investigated the impact of yeast fermentation and enzyme treatment of wheat bran on plasma phenolic concentrations of volunteers. Ferulic acid increased in the plasma to a maximum level of 2.5 μmol/L, which was considerably higher than baseline levels of 5 to 30 nmol. However, the authors were uncertain whether these observed changes would exert any biological effects. Chandrasekara and Shahidi[31], used five dehulled, cooked millets (kodo, finger, proso, foxtail and pearl) and subjected to in vitro digestion and fermentation to assess the bioaccessibility of their phenolic compounds. The authors reported a release of phenolics from all the five millet grains during GI digestion and colonic fermentation that may exert potential health benefits locally and systemically upon absorption.

1.6. Conclusions

Whole grains are rich sources of non-digestible carbohydrates and associated phytochemicals in the human diet. The complex matrix of whole grains and the structural diversity of cell wall polysaccharides make the whole grain carbohydrates an important substrate for the human intestinal microbiota. The phytochemicals associated with whole grains may also influence the health-promoting properties of whole grains through fermentation by the intestinal microbiota. However, to achieve the maximum health

benefits imparted by whole grains, it is important to increase microbiota accessible to whole grain carbohydrates by optimizing processing methods. This would enhance the whole-grain gut microbiota interactions and thereby establishment of the relationship of the specific interactions pertaining to human health.

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Table 1:1. Major bioactive components of selected whole grains (% dry matter).[37-39]

Component	Wheat	Rye	Oats	Barley	Rice
Phytic acid	0.04-0.14	0.05-0.15	0.04-0.12	0.04-0.11	0.45-0.8
Tocols	0.003-0.01	0.004-0.01	0.002-0.004	0.005-0.01	0.4-0.9
Phenolic acids	0.03-0.12	0.05-0.11	0.04-0.09	0.03-0.07	Not reported
Phytosterols	0.07-0.09	0.11-0.14	0.06-0.07	0.09-0.12	Not reported
Alkylresorcinols	0.02-0.07	0.08-0.12	Not present	0.003-0.01	Not present
Avenantramides	Not present	Not present	0.004-0.01	Not present	Not present

Table 1:2. Summary of whole grain intervention studies on host health and gut microbiota

Author	Subject Characteristics	Feeding Trial	WG treatment	Study Design	Non- significant Results	Significant Results	Ref.
Cooper et al., 2017	46 healthy adults; BMI 20 to 28; low whole grain consumers (<1 serving/d)	WG vs. RG; WG based on estimated energy needs (e.g., 13.7 g fiber/d from WG for a 2000 kcal/d diet)	Bread, rice, pasta, snacks, breakfast cereals, tortilla, baking mixes	6 wk parallel-arm	BMI, HDL, triglycerides, GI symptoms, no changes in fecal microbiota composition	↓LDL, non-HDL cholesterol, fasting blood glucose; ↑bowel movement	90
Philip Karl et al., 2017	81 adults; 40–65 y; BMI <35	WG (207 g/d WG) vs. RG	Western-style diet consisting mostly of wheat but oats and brown rice were also included	6 wk parallel-arm	Glycaemia	↑ Plasma alkylresorcinols, resting metabolic rate, stool weight, fecal *Lachnospira* and *Roseburia*; ↓ Enterobacteriaceae	91
Vanegas et al., 2017	Same as Philip Karl et al., 2017	Same as Philip Karl et al., 2017	Same as Philip Karl et al., 2017	Same as Philip Karl et al., 2017	Stool propionate, butyrate; α and β-diversity, IgA, DTH, IFN, IL-17, TNF-α, IL-6, TGF-β, white blood cells, lymphocytes, monocytes, eosinophils, basophils, neutrophils	↑Stool frequency, percentage of terminal effector memory T-cells; LPS-stimulated production of TNF-α, fecal acetate, total SCFA	20

Stefoska-Needham et al., 2017	60 adults	WG 45 g/d) vs. RG	WG sorghum cereal	12 wk parallel-arm	Weight loss, plasma glucose, glycosylated hemoglobin, insulin, cholesterol, triacylglycerides, IL-1b, IL-6, IL-8, TNF-α, hs-CRP, and total antioxidant capacity		92
Vetrani et al., 2016	54 overweight/obese adults with metabolic syndrome	WG (45 g/d) vs. RG	Cereal products	12 wk parallel-arm	Glucose, BMI, HOMA, TAG, cholesterol, HDL, hs-CRP, IL-1 ra, IL-6, and TNF-α, and SCFAs	↓Postprandial insulin; ↑Fasting plasma propionate	93
Ampatzoglou et al., 2015	33 adults; 40–65 y, BMI 20–35; low WG consumers (<24 g/d)	High WG (>80 g/d) vs low WG (16 g/d)	Bread, rice, pasta, snacks, breakfast cereals	6 wk crossover	IL-1, IL-6, IL-8, TNFα, CD8+, ghrelin, GIP, GLP-1, glucagon, leptin	↓IL-10, CRP, insulin, CD4+ T cells, C-peptide, PAI-1	94
De Angelis et al., 2015	26 healthy adults	WG vs. RG	Pasta containing WG durum wheat and barley (3 g/d barley β-glucan)	2 month parallel-arm		↑Clostridiaceae, Roseburia, Ruminococcus, lactobacilli, fecal acetate, propionate, butyrate, 2-methyl-propanoate; ↓Fusobacteria, Enterobacteriaceae, total coliforms, Bacteroides, Porphyromonas, Prevotella, Pseudomonas, Alcaligenes, Aeromonas ↑	13

Vitaglione et al., 2015	80 healthy overweight/ obese subjects; low fruit and vegetable consumers; sedentary lifestyle; BMI: 25–35	WG (70 g/d) vs. RG	Biscuits	8 wk placebo-controlled, parallel-arm	IL-6	↑Serum, fecal, and urinary ferulic acid; ↓TNF-α ↑interleukin (IL)-10, fecal *Prevotella*; ↓Fecal *Dialister, Bifidobacterium, Blautia, Colinsella*	95
Hajihashemi et al., 2014	44 overweight/ obese female adolescents	WG vs. RG	"Dark" breads, brown rice, barley bread, cornflakes, bulgur, popcorn, wheat germ, whole meal biscuits	6 wk crossover	Weight, BMI	↑hs-CRP, soluble intercellular adhesion molecule-1, serum amyloid, leptin	96
Lappi et al., 2013	51 adults with metabolic syndrome; 40–65 y; BMI 26–39	WG (75 g/d) vs. RG	Rye bread	12 wk crossover		No changes in microbiota composition; Subset with highest plasma alkylresourcinols: ↑*Collinsella*, Clostridium clusters IV and XI, ↓*Bacteroides, Prevotella*	18
Martínez et al., 2013	28 healthy adults; 25.9±5.5 y	WG barley, WG barley+brown rice, brown rice (60 g/d)	WG barely (high β-glucan), brown rice	4 wk crossover	Cholesterol, HDL, non-HDL, hs-CRP, LBP	↑Microbial diversity, Firmicutes, Blautia; ↑ (Barley+brown rice only) IL-6, postprandial glucose peak; ↑(Barley only) Fecal *Roseburia, Bifidobacterium, Dialister, Eubacterium*	19
Kristensen et al., 2012	79 overweight/ obese postmenopausal women	WG vs. RG	Bread, pasta	14 wk parallel-arm	Body weight, cholesterol	↓Percentage fat mass	97

de Mello et al., 2011	131 adults with impaired glucose metabolism; 40–70 y; BMI 26–39	Whole-grain-enriched diet (50% WG), healthy diet, control	Sourdough whole wheat bread; white rye bread, whole grain pasta, fatty fish 3 times/wk, bilberries	12 wk parallel-arm		↓Plasma hsCRP	98
Ross et al., 2011	17 healthy adults; 20-50 y; BMI 19-28; low WG consumers	WG vs. RG	WG (64% wheat, 13% oats, 9% rice, 14% barley+rye); RG (66% wheat, 27% rice, 8% corn)	2 wk crossover	HDL-cholesterol, fasting glucose, CRP, homocysteine	↓Cholesterol, LDL-cholesterol, fecal water; ↑stool frequency, *Clostridium leptum*	99
Brownlee et al., 2010	316 adults; low WG consumers (<30 g/d)	WG (60 g/d), WG (60g/d then 120 g/d each 8 wk) vs RG	Bread, breakfast cereal, oatmeal, brown rice, pasta, oat bar, chips	16 wk parallel-arm	Total cholesterol, HDL, LDL, TAG, glucose, insulin, NEFA, QUICKI, R-QUICKI, sialic acid, CRP, IL-6, fibrinogen, PAI-1, ICAM-1, VCAM-1, E-selectin, systolic BP, diastolic BP, weight, waist, body fat percentage		10
Carvalho-Wells et al., 2010	32 healthy adults; mean BMI 23.3	WG (48 g/d) vs RG	Corn cereal	3 wk crossover	Serum lipids, glucose, faecal output	↑Bifidobacteria	15
Giacco et al., 2010	15 healthy adults (12 M/3 F); mean age 54.5 y; mean BMI 27.4	WG (23.1 g DF/d) vs RG (9.8 g DF/d)		3 wk crossover	Fasting blood glucose, postprandial glucose response	↓ Total and LDL cholesterol	101
Landberg et al., 2010	24 men with prostate cancer	WG vs. RG with added cellulose	Bread, crisp bread, muesli, porridge from rye and wheat	6 wk crossover		↑Apoptosis; ↓Plasma total PSA concentrations, fasting plasma insulin, 24-h urinary C peptide excretion	102

Tighe et al., 2010	233 adults; age 40–65 y; BMI 18.5-35	WG vs. RG	Cereals and breads with wheat and oat	16 wk parallel-arm	Cholesterol, Apo A-I, Apo B, Insulin, fasting blood glucose, revised QUICKI, hs-CRP, IL-6	↓Systolic blood pressure	103
Costabile et al., 2008	32 healthy adults; 20–42 y; BMI: 20-30	WG (48 g/d) vs. wheat bran (48 g/d)	Breakfast cereal	6 wk crossover	Fecal SCFA, fasting blood glucose, insulin, total cholesterol, TAG, HDL, stool habits	↑Ferulic acid (both treatments); ↑Bifidobacteria (WG only); ↑ Lactobacilli/enterococci (both WG and bran)	16
Katcher et al., 2008	50 obese adults with metabolic syndrome; 20–65 y	WG vs RG; also received weight loss advice	In addition to WG, 5 servings of fruit and vegetables, 3 servings of low-fat dairy products, and 2 servings of lean meat, fish, or poultry	12 wk parallel-arm		↓ Body weight; ↓Waist circumference, percentage body fat, CRP, and total, LDL, and HDL cholesterol	104
Andersson et al., 2007	30 adults; mean BMI 28	wg (50%) vs RG	3 bread slices, 2 crisp bread slices, 1 portion muesli, and 1 portion pasta	6 wk crossover	Peripheral insulin sensitivity; 8-iso-$PGF_{2\alpha}$, IL-6, CRP, serum lipid concentrations		105
McIntosh et al., 2003	28 overweight healthy men; 40–65 y	High fiber rye, high fiber wheat, low fiber foods	Bread, crisp bread, cereal	4 wk crossover	Body weight, fecal phenolic and bile acid concentrations	↑Fecal output (both wheat and rye); ↑Plasma enterolactone, fecal butyrate (rye only); ↓Fecal β-glucuronidase activity, postprandial plasma insulin, postprandial plasma glucose (wheat and rye)	106

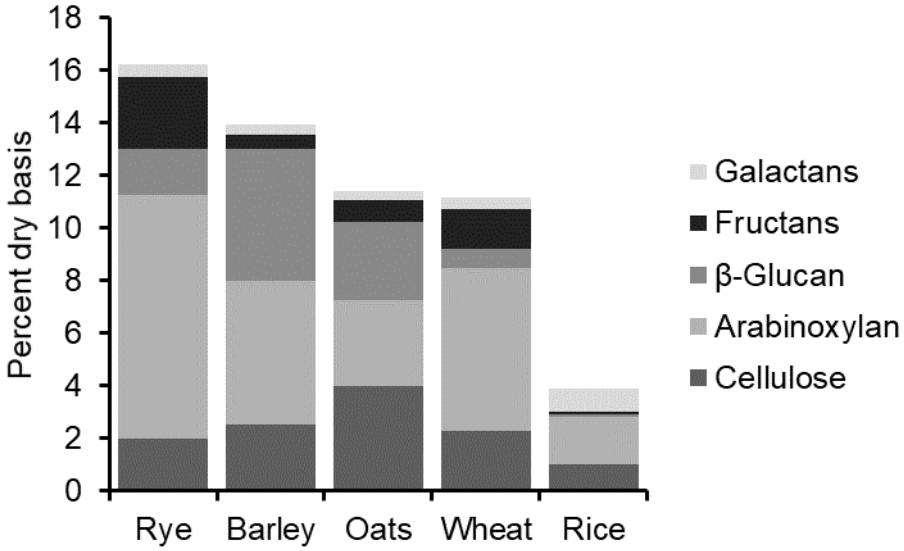

Figure 1-1: Typical non-digestible carbohydrate composition in selected whole grains.[35, 36]

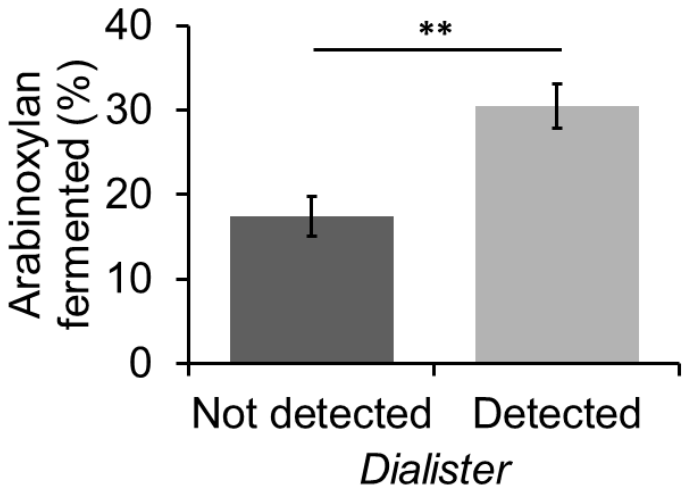

Figure 1-2: Relationship between abundance of *Dialister* in fecal samples and extent of arabinoxylan fermentation in pre-digested whole wheat in vitro.[109]

Chapter 2 . Effects of selected extrusion parameters on physicochemical properties and in vitro starch digestibility and β-glucan extractability of whole grain oats

2.1. Abstract

Whole grain oat flour was extruded under different moisture contents (15%, 18%, 21%), barrel temperatures (100 ˚C, 130 ˚C), and screw speeds (160 rpm, 300 rpm, 450 rpm), and selected physicochemical properties, in vitro starch digestibility, and β-glucan extractability of the extrudates were analyzed. An increase in screw speed resulted in an increase in radial expansion index, water absorption index and water solubility index. Screw speed significantly affected slowly and rapidly digestible starch. Moderate screw speed (300 rpm) led to higher slowly digestible starch with an accompanying decrease in rapidly digestible starch. Low moisture conditions (15%) resulted in the highest resistant starch and water-extractable β-glucan (WE-BG). Under the conditions used in this study, extrusion did not result in changes in WE-BG molecular weight. Thus, extrusion might be beneficial in improving functionality and consumer acceptability by affecting physicochemical properties, in vitro starch digestibility and β-glucan extractability of oat extrudates.

2.2. Introduction

Oats, along with barley, are among the only grains that have received health claims in several countries for reduction in cholesterol (Tiwari and Cummins, 2009). The cholesterol-lowering ability of oats may be due to many components in the grain working synergistically, but the β-glucan fraction seems to play a major role (Wolever et al., 2010). Oat β-glucan is a high molecular weight linear glucan consisting of (1→3) and (1→4) linkages. In the gastrointestinal (GI) tract, β-glucan contributes to viscosity, which slows the rate of cholesterol absorption and decreases enterohepatic recirculation of bile acids, thus reducing blood cholesterol (Queenan and others, 2007; Wolever et al., 2010). In addition to the cholesterol-lowering ability of oats, the viscosity contributed by β-glucan may also provide additional benefits, such as reducing the rate of starch digestion and subsequent glycemic response (Brummer et al., 2012; Kim and White, 2013).

A prevalent way of consuming oats is in the form of extruded ready-to-eat (RTE) breakfast cereals. During extrusion, the grain is subjected to low moisture, high shear and high temperature for a short time, after which the relief of pressure and reduction in temperature causes moisture to flash off and produce an expanded product (Guy, 2001). Previous research has shown that extrusion can affect the solubility and molecular weight distribution of the polysaccharides in grains. For instance, Zhang et al. (2011) extruded oat bran at 10-30% moisture using a twin-screw extruder at 100-160 ˚C and a screw speed of 150 rpm and determined that extrusion increased the yield of soluble dietary fiber (principally β-glucan). In general, the yield of soluble dietary fiber increased as feed moisture decreased, while temperature had less of an influence on the soluble dietary fiber yield. The extracted soluble dietary fiber had a higher solubility and viscosity than that

extracted from untreated oats. The increase in yield may be due to more effective extraction of the higher-molecular weight β-glucan (Zhang et al., 2009a). In contrast to Zhang et al. (2011), Tosh et al. (2010) showed dramatic reduction in β-glucan molecular weight from 1.9×10^6 g/mol to 2.5×10^5 g/mol during extrusion of oat bran, with an accompanying decrease in viscosity. This was likely because extrusion conditions were extremely severe, with moisture content as low as 7% and temperature as high as 237 ˚C.

Starch is also affected by extrusion. As a branched polysaccharide, amylopectin is much more affected than amylose (Li et al., 2014). In fact, moderate depolymerization of amylopectin is important to produce extrudates with acceptable expansion and crispiness (Guy, 2001). The depolymerisation of amylopectin, as discussed with β-glucan above, is dependent on extrusion conditions, with more severe conditions (lower moisture, higher temperature and screw speed) producing higher degradation (Guy, 2001).

Changes in solubility and molecular weight of β-glucan and starch may influence the starch digestion kinetics of extruded oat products. Dust et al. (2004) studied the effects of different conditions of extrusion on in vitro digestibility of selected food ingredients including oat bran. Extrusion under extreme conditions (five reverse lobes at 120-130 °C in a single screw extruder set at 500 rpm and water and steam injection at 15 kg/h and 10 kg/h, respectively) resulted in a 36.5% increase in soluble dietary fiber in oat bran. Under these same conditions, resistant starch also increased (28.8% versus 24.5% in unprocessed oat bran and 25.1% in mildly extruded oat bran). Brummer et al. (2012) showed that under severe extrusion parameter of oat bran cereal, depolymerization of β-glucan depolymerizes as, the cereals possessed diminished the ability to attenuate peak blood glucose response and area under the glycemic response curve. Notably, these cereals also had low

palatability, most likely because β-glucan depolymerization due to the severe processing conditions.

Although different processing conditions affect the properties of β-glucan in oat bran, studies have yet to conduct on the effects of different extrusion conditions of the properties of the whole oat extrudates. Therefore, the purpose of this research was to determine how extrusion conditions affect the physicochemical properties of whole grain oat extrudates, with emphasis on in vitro starch digestibility and changes in water-extractability and molecular weight of β-glucan.

2.3. Materials and methods

2.3.1. Oat flour composition

Whole grain oat flour was obtained from General Mills (Minneapolis, MN, USA). The flour was analyzed for moisture content according to AACCI approved method 44-15.02. Protein content was analyzed using a nitrogen analyzer (Leco FP-528, Leco Corporation 3000, St. Joseph, MI, USA) with a nitrogen conversion factor of 5.83 according to AACCI approved method 46-30.01. Lipid content was analyzed according to AACCI approved method 30-25.01. The ash content was determined according to AACCI approved method 08-01.01. Starch concentration was determined following AACCI approved method 76-13.01 using a kit (K-TSTA, Megazyme, Bray, Ireland). Total β-glucan concentration was measured according to the AACCI approved method 32-23.01 using a kit (K-BGLU, Megazyme). Peak molecular weight and concentration of WE-BG were also determined as follows: Two hundred milligrams of flour were digested according to Mkandawire et al. (2013). During in vitro digestion, 4 mL of 3.6% (w/v) of freshly prepared pepsin (P7000, Sigma, St. Louis, MO, USA) in 0.05M HCl was added to

the samples. The tubes were capped, mixed by vortexing and placed horizontally in a water bath at 37 °C with shaking at 250 rpm for 30 min. Next, 2.0 mL of 0.5 M sodium acetate buffer (pH 5.2) was added to each tube with vortex mixing. To initiate starch digestion, at 1 min intervals, 2.05 ml of freshly prepared enzyme solution containing 15% (w/v) pancreatin (P7545; Sigma) and 20 μL of amyloglucosidase (3260 U/mL; Megazyme) per mL in water were added to each tube and digested for 120 min. Following digestion, the slurry was centrifuged at 1000 g for 10 min and then 5 mL of the supernatant was assayed for WE-BG following the European Brewery Convention method 8.11.1 using a kit (K-BGLU, Megazyme). For molecular weight, 0.75 mL of the supernatant was filtered through a 0.45 μm nylon membrane using a centrifugal filter (F2517-4, Thermo Scientific, Nashville, TN, USA), and then 100 μL of the filtrate was analyzed by HPLC as described (Yao et al., 2007). In brief, the HPLC (model 1260, Agilent, Santa Clara, CA, USA) was equipped with a guard column (SB-G, Shodex, Showa Denko, Japan) and three size exclusion columns connected in series (SB-806 HQ, SB-805 HQ, and SB-804 HQ, Shodex). Column temperature was maintained at 35 °C and the mobile phase was 0.02% sodium azide at a flow rate of 0.5 mL/min. Peaks were detected using a refractive index detector (Agilent). Shodex pullulan standards (Showa Denko, Munich, Germany) were used to construct a standard curve and determine the peak molecular weight of the extracted β-glucan.

2.3.2. Extrusion

A 3×3×2 replicated (duplicate) factorial design was used in this study to test the effect of moisture, screw speed and temperature. High (450 rpm), moderate (300 rpm) and low (160 rpm) screw speeds; high (130 ˚C) and low (100 ˚C) temperatures; and high

(21% wb), moderate (18% wb), and low (15% wb) moisture conditions were chosen to generate different processing conditions.

To adjust flour moisture, flour was weighed and blended with a pre-determined amount of water in an upright mixer (H-600-D, Hobart, Troy, Ohio, USA) for 4 min to achieve the desired levels of moisture content. The samples were transferred to closed containers and stored overnight at 4 ˚C for equilibration until extrusion.

The hydrated flours were extruded in a laboratory co-rotating, intermeshing, twin-screw extruder with 3:1 compression ratio, 3 mm die diameter and 20:1 L/D ratio (TSE 20; CW Brabender Instruments Inc., South Hackensack, NJ, USA). The flour was fed into the extruder barrel using a volumetric feeder (FW 40 Plus, C. W. Brabender) set at a constant delivery rate of 76 g/min. Feed rate was calculated by recording the weight of extruded product exiting the die per min. The extruder was operated by a direct current drive unit (Intelli-Torque, Pastic Corder Lab-station, C.W. Brabender) with a 7.5 hp motor. The exit die internal diameter was 3 mm. The experimental extruder variables were adjusted using computer software (Measurement and extrusion program for control systems, version 3.0.2, C.W. Brabender).

Following extrusion, the extrudates were dried in a gas fired belt conveyor dryer (Model 41357-011, Wenger Manufacturing, Inc., Sabetha, KS, USA). The single zone, two pass dryer temperature was 100 °C and the retention time was 3 min for each pass (total dryer retention time of 6 min). Dry extrudates at about 3% moisture (wb) exiting the dryer were conveyed through a 4 m cooling conveyor (ca. 1 min) to cool the cereal to ambient temperature. The extrudates were then packaged in moisture proof zip top bags

and stored at 4˚C until further analysis. All treatment conditions were performed in duplicate.

2.3.3. Physical properties of extrudates

The diameter of the extrudates was measured with a Vernier caliper (Mitutoyo Co., Kawasaki, Japan) with an accuracy of 0.1 mm and the radial expansion index (REI) was calculated as the extrudate diameter divided by the die diameter. Each sample was measured 40 times. Samples were then milled using a cyclone mill (Model 4425, UDY, Fort Collins, CO, USA) equipped with a 1 mm screen for further analysis.

The color of the milled extrudates was measured using a colorimeter (Chroma Meter CR-300, Minolta, Tokyo, Japan). Six measurements were competed per treatment combination and the results were expressed in terms of lightness (L*), redness (a*), and yellowness (b*). Water absorption index (WAI) and water solubility index (WSI) of the extrudates were measured as described by Anderson et al. (1982). The WSI was expressed as the weight of soluble solids recovered after drying the supernatant liquid (103 ˚C, 12 h) divided by the initial wet weight of supernatant liquid. The gel remaining in the tube after centrifugation was weighed, and WAI was expressed as g gel divided by g initial dry extrudate. Four measurements per treatment were recorded.

2.3.4. Chemical composition of extrudates

Extrudates that had been milled using the cyclone mill (see 'Physical properties of extrudates' section) were analyzed for starch and β-glucan as described for the oat flour (see 'Oat flour composition' section). Whole grain oat flour and extrudates were also subjected to in vitro starch digestion as described for the oat flour (see 'Oat flour composition' section). At exactly 20 and 120 min of in vitro starch digestion, an aliquot

of 0.05 mL was removed from each tube and mixed with 0.95 mL of absolute ethanol. The samples were centrifuged at 5000 g for 5 min, and the glucose content was measured in the supernatant by the glucose oxidase-peroxidase method (K-GLUC, Megazyme) and converted to starch multiplying by a correction factor of 0.9. Results were expressed as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). The RDS fraction of starch was converted to glucose in the first 20 min of in vitro starch digestion; SDS was the portion of starch converted to glucose between 20 and 120 min of digestion; RS was the fraction of starch not converted to glucose during the 120 min digestion process.

2.3.5. Data analysis

For all compositional data, the duplicate samples were each measured twice. For physical properties, each duplicate sample was measured 40 (radial expansion), 6 (color), and 4 (WAI and WSI) times. For in vitro starch digestion, duplicate samples were each measured 3 times. All data were reported on a dry weight basis except moisture content, which was on a wet basis.

For comparisons among extrudates, a three factor ANOVA was applied to moisture, temperature and screw speed as the main effects, with all two-way and the three-way interactions included in the ANOVA model. Contributions of each factor to the overall ANOVA model were calculated by dividing each factor's sum of squares by the total sum of squares and multiplying by 100%. This allowed for an effective measure of the magnitude of the contribution by each factor to the response. Only effects with $p<0.05$ were considered significant. Fisher's least significant difference test was used to determine differences among samples with significant effects. Pearson correlations were

calculated using the correlation procedure. All data were analyzed using SAS software (version 9.4, SAS Institute, Cary, NC, USA).

2.4. Results and discussion

2.4.1. Oat flour composition

The composition of the whole grain oat flour is shown in Table 2-1. Values for each component were similar to previous reports (Ajithkumar et al., 2005; Yao et al., 2007). The peak molecular weight of β-glucan was an order of magnitude lower than typically reported (Ajithkumar et al., 2005; Yao et al., 2007), which may be due to endogenous beta-glucanases that were active during in vitro digestion process.

2.4.2. Influence of processing conditions on physical properties of oat extrudates

The REI of the extrudates ranged from 1.12 to 1.64 (Table 2-2). Screw speed and temperature had significant effects on REI (Table 2-3). Screw speed and temperature together contributed a majority (70%) to the ANOVA model for REI. Higher screw speed resulted in higher REI. Screw speed has been previously shown to significantly affect REI (Ozer et al., 2004). Ozer et al. (2004) studied the expansion characteristics of extruded snacks composed of different flours in various proportions, including oat flour, where they showed that the screw speed had the most prominent significant effect on REI, with higher screw speed resulting in higher REI. This response may be caused by high screw speed by introducing more energy to the dough in the barrel, which resulted in faster evaporation of the moisture at the die exit and hence expansion increases. Higher temperature was associated with a decrease in REI. Similar results were reported by Mendonca et al. (2000) who studied extrusion of corn meal containing added corn bran

and concluded that high temperatures leads to excessive breakdown of starch followed by weakening and fragmentation of the extrudate structure.

Lightness/darkness (L*) of the extrudates ranged from 71.9 to 76.6 (Table 2-2). Moisture and screw speed as well as their interaction and the interactions between screw speed and temperature had significant effects on the lightness of the product (Table 2-3). Moisture and screw speed main effects explained 49% of the effect contribution to L*. Redness (a*) of the extrudates ranged from 1.01 to 2.22 (Table 2-2). All main effects had significant, independent effects on the redness of extrudates. Together, they contributed to 77% of the total factor contribution to a*. Yellowness (b*) of the extrudates ranged from 15.6 to 18.1 (Table 2-2). Moisture and screw speed had significant effects on the yellowness of the extrudates (Table 2-3). Similar to L*, the interactions of moisture and screw speed as well as of screw speed and temperature had significant effects on yellowness. Changes in color of the extrudates were most likely a result of differences in development of Maillard-type reaction products during extrusion.

WAI and WSI of the extrudates varied from 5.5 to 6.2 g/g and 10.5 to 28.8 % g/g, respectively (Table 2-2). Screw speed had significant effects on both WAI and WSI and contributed 40% of the effect contribution to WAI and 58% of the effect contribution to WSI. An increase in screw speed led to an increase in both WSI and WAI as compared to low screw speed (Table 2-2). WSI is an indicator of the amount of soluble starch after extrusion and WAI is the measurement of the amount of intact and fully gelatinized starch granules. An increase in WAI with increasing screw speed was demonstrated by Gat and Ananthanarayan (2015). The increase in screw speed during extrusion leads to greater starch breakdown. As a result, the longer starch chains are fragmented to shorter

chains, and shorter chains are more soluble as compared to longer chains (Hagenimana et al. 2006).

2.4.3. Influence of processing conditions on in vitro starch digestibility of oat extrudates

Total starch content of oat extrudates was significantly affected by moisture (Table 2-3). The lowest moisture condition resulted in a decrease in starch concentration compared to the highest moisture condition (Table 2-2). This could be due to the susceptibility of high molecular weight branched amylopectin to shear degradation during extrusion (Li et al., 2014).

The RDS, SDS and RS percentage of extrudates ranged from 62 to 69%, 18 to 28%, and 8 to 15%, respectively (Table 2-2). RS was within the range as reported by Hernot et al. (2008) for extruded whole grain products. Extrusion temperature did not significantly affect starch digestible fractions; however, moisture and screw speed did have a significant effect on starch (Table 2-3).

Screw speed had significant effects on both RDS and SDS (Table 2-3) that were interchanged relative to one another i.e., an increase in SDS was accompanied by a decrease in RDS (Fig. 2-1A, 2-1B). Moderate screw speed tended to enhance SDS and diminish RDS compared with low and high screw speeds. Enhanced SDS accompanied by diminished RDS is a desirable characteristic due to potentially lowering the glycemic index (Zhang et al., 2009b). This response may be due to long residence time at low screw speed or extreme shear at high screw speed resulting in lower SDS caused by total disruption of the structure of the starch granules. As a result, starch is fully gelatinized and dispersed promoting the loss of its slow digestible property. Thus, breakdown of starch may be important to the distribution of RDS and SDS. However, the effects of

processing variables on the fine structural features of starch and their interactions with other compounds in whole grains, and how this affects starch digestibility is not fully understood. Structural changes in both starch and non-starch components could be responsible for affecting changes in RDS and SDS in oat flour extrudates as a function of processing variables.

Moisture had a significant effect on RS (Table 2-3). Lowering flour moisture content tended to increase RS (Fig. 2-1C). Lower moisture creates harsher conditions in the extruder due to the absence of the lubricating effect of water that leads to increased fragmentation of the starch. Thus, shorter starch polymers generated at lower processing moisture re-associate after cooling due to increased molecular mobility and thereby excluding amylolytic enzymes (Lopez-Rubio et al., 2008). A similar explanation was reported by Htoon et al. (2010) who studied the effect of acid dextrinization on RS content in extruded maize starch.

2.4.4. Influence of processing conditions on β-glucan properties of oat extrudates

Total β-glucan and β-glucan peak molecular weight were not affected by the processing conditions (Table 2-3). While it was expected that total β-glucan would not be affected by extrusion, it was notable that the molecular weight was unaffected, as several authors have reported changes in molecular weight of β-glucan during extrusion of oat bran (Tosh et al., 2010; Yao et al., 2011; Zhang et al., 2011). As discussed, depending on severity of processing conditions, molecular weight profiles of β-glucan have been shown to increase (Yao et al., 2011; Zhang et al., 2011) or decrease (Tosh et al., 2010). Nevertheless, the processing variables used in the current study did not effect β-glucan molecular weight. Being a linear polymer, β-glucan, would probably require very severe

extrusion conditions before major changes in molecular weight occurs as other linear polymers (e.g., amylose) are stable under extrusion conditions (Li et al., 2014).

In contrast to molecular weight, WE-BG was affected by moisture content of the oat flour (Table 2-3). Extractable β-glucan ranged from 0.71 to 1.20 % (Table 2-3). The concentration of β-glucan was significantly higher when oat flour was extruded at 15% moisture compared with 18% (Fig. 2-2). It is not surprising that the lower moisture content resulted in higher extractable β-glucan concentration, as these conditions were harsher than the higher moisture conditions. This could have been a contributing factor to the enhanced RS content in the low moisture extruded samples. Although the extrudates corresponding to 15% moisture extrusion conditions contained numerically higher extractable β-glucan, they were not significantly different from samples that had been extruded at 21% moisture. At the higher moisture conditions, β-glucan extractability could be due to increased hydration during extrusion, although more studies are required to test this hypothesis.

2.4.5. Correlations among response variables

Physical responses that were simple to measure were evaluated to determine if they significantly correlated with starch digestible fractions or β-glucan extractability, which are more time consuming to measure. Unfortunately, there were no meaningful correlations for starch digestible fractions or β-glucan solubility (Supplementary Table 2-7-1).

2.5. Conclusions

Overall, the current study demonstrated the effects of physicochemical properties and digestion profiles of starch in whole grain oats in response to extrusion conditions.

The REI, WAI, and WSI characteristics increased with higher screw speeds. A combinations of extrusion parameters were responsible for the change in color of the extrudates, instead of a single processing variable. Barrel temperature did not significantly affect the starch digestible fractions or extractable β-glucan concentration. However, screw speed significantly affected both SDS and RDS. Moderate screw speed tended to increase SDS and diminish RDS. Lower moisture content significantly affected both RS and extractable β-glucan concentration. Thus, extrusion of whole grain oats under low moisture and moderate screw speed conditions may produce extruded products with the most desirable physicochemical properties in terms of β-glucan extractability and starch digestibility.

2.6. References

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Table 2:1. Composition of oat flour. [a]

Component	Value
Composition	
Moisture (%, wb)	9.57±0.17
Protein (%, db)	13.9±0.0
Lipid (%, db)	7.00±0.40
Ash (%, db)	2.14±0.02
Carbohydrate (%, db, by difference)	77.0
Total starch (%, db)	51.2±0.0
Total β-Glucan (%, db)	3.27±0.30
Water-extractable β-glucan (%, db)	0.86±0.09
β-Glucan peak MW ($\times 10^5$ g/mol)	8.85±0.08
Color	
L*	81.4±0.3
a*	0.76±0.04
b*	13.2±0.1

[a] MW = molecular weight

Table 2:2. Effect of processing variables on physical properties, starch, and β-glucan, in oat extrudates. [a]

Processing variables			Physical responses						Starch responses				β-Glucan responses		
M (%)	SS (rpm)	T (˚C)	REI	L*	a*	b*	WAI (g/g)	WS (% g/g)	Total (%, db)	RDS (%, TS)	SDS (%, TS)	RS (%, TS)	Total (%, db)	Peak MW (×10^5)	WE (% db)
15	160	100	1.19±0.00	74.6±0.3	1.6±0.0	17.6±0.0	5.8±0.0	12.9±0.9	49±0	68±2	18±1	14±2	3.17±0.13	8.8±0.2	1.20±0.11
15	160	130	1.12±0.04	74.5±0.6	1.8±0.1	17.4±0.6	5.7±0.2	12.2±0.3	55±4	69±2	18±1	14±1	3.21±0.03	9.3±0.9	0.74±0.38
15	300	100	1.26±0.13	72.2±0.6	1.8±0.1	17.9±0.5	5.9±0.3	17.7±0.9	51±1	65±5	20±6	15±1	3.29±0.16	8.2±1.0	1.08±0.53
15	300	130	1.19±0.11	73.9±0.8	2.0±0.3	17.3±0.3	5.5±0.3	14.9±1.5	51±1	62±3	25±4	13±1	3.13±0.29	8.9±0.5	1.23±0.25
15	450	100	1.44±0.27	73.3±0.2	1.8±0.2	17.2±0.1	6.1±0.1	19.7±1.7	51±1	65±1	22±6	13±6	3.07±0.21	8.6±1.1	1.07±0.27
15	450	130	1.30±0.10	73.4±0.1	2.2±0.2	17.3±0.5	6.1±0.3	28.8±1.5	50±2	66±4	19±3	15±1	3.12±0.23	8.5±0.5	0.86±0.14
18	160	100	1.15±0.05	73.9±0.3	1.4±0.0	16.9±0.2	5.9±0.0	11.7±0.5	52±1	67±0	21±3	12±3	3.28±0.08	8.6±0.8	0.88±0.06
18	160	130	1.12±0.01	73.0±0.6	1.9±0.0	17.4±0.3	5.5±0.1	11.8±0.3	51±1	67±1	21±3	12±1	3.19±0.03	9.2±0.0	0.77±0.28
18	300	100	1.31±0.05	72.1±0.5	1.8±0.1	17.8±0.0	5.7±0.4	17.3±0.4	52±0	62±1	28±5	10±4	3.19±0.07	8.0±0.2	0.73±0.22
18	300	130	1.17±0.03	71.9±2.2	1.9±0.0	17.3±0.2	5.6±0.4	14.6±0.7	53±3	66±4	21±8	14±4	3.19±0.03	8.8±1.2	0.71±0.27
18	450	100	1.64±0.08	74.1±0.6	1.9±0.2	16.6±0.1	6.2±0.2	20.2±0.1	51±1	68±1	18±4	14±4	3.23±0.04	8.4±1.3	0.98±0.31
18	450	130	1.48±0.16	74.3±0.3	2.0±0.3	16.7±0.6	6.1±0.1	18.2±1.6	52±0	65±1	21±6	14±7	3.19±0.02	8.4±0.1	0.80±0.01
21	160	100	1.15±0.01	76.6±0.1	1.0±0.0	15.6±0.0	6.1±0.1	10.8±0.1	52±2	67±0	24±5	9±4	3.16±0.12	8.9±0.1	0.81±0.18
21	160	130	1.15±0.00	74.7±0.5	1.5±0.3	16.5±1.0	5.7±0.4	10.5±0.7	52±1	66±4	22±3	11±2	2.99±0.09	8.8±0.1	0.80±0.11
21	300	100	1.34±0.03	72.6±1.3	1.4±0.2	18.1±0.7	5.5±0.2	17.1±0.3	53±1	68±1	25±7	8±6	3.20±0.14	9.2±0.2	0.84±0.35
21	300	130	1.29±0.04	74.5±0.7	1.6±0.1	16.9±0.2	5.7±0.1	14.8±0.1	52±1	64±4	27±2	9±2	3.34±0.12	8.6±1.1	0.96±0.52
21	450	100	1.63±0.00	74.1±0.1	1.4±0.0	17.3±0.3	6.0±0.3	21.2±0.3	53±1	68±2	21±3	12±0	3.26±0.05	9.6±0.5	0.90±0.47
21	450	130	1.34±0.09	74.2±0.6	1.8±0.1	17.1±0.3	6.0±0.1	17.2±0.5	58±4	67±5	22±6	11±1	3.19±0.07	8.1±0.0	0.92±0.33

[a] Responses are mean ± standard deviation (n=2); M = moisture (%, wb); SS = screw speed (rpm); T = temperature (˚C); REI = radial expansion index; WAI = water absorption index; WSI = water solubility index; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch; TS = total starch; MW = molecular weight; WE = water-extractable.

Table 2:3. Factor contributions (%) to ANOVA models for physical properties, starch, and β-glucan, in oat extrudates.[a]

	Physical responses						Starch responses				β-Glucan responses		
Factor(s)	REI	L*	a*	b*	WAI	WSI	Total	RDS	SDS	RS	Total	Peak MW	WE
M	3.46	16.8***	34.9***	12.9*	0.10	4.77	15.34*	0.63	3.45	7.85*	2.66	6.79	9.11*
SS	60.3***	32.2***	17.7***	20.3***	40.2***	58.3***	3.02	6.10*	6.76*	1.87	4.61	7.25	0.72
T	9.65***	0.09	24.6***	0.61	7.72	0.39	3.77	0.32	0.01	0.34	1.83	1.93	1.56
M*SS	5.55	12.3*	0.26	24.9**	4.65	3.83	17.04	2.63	1.57	0.66	18.5	9.64	4.57
M*T	0.08	1.90	1.33	0.61	0.48	3.23	1.58	0.65	0.62	0.35	0.16	14.0	2.08
SS*T	3.84	11.9**	3.38	13.9*	4.78	2.29	1.86	0.17	0.07	0.04	1.11	11.1	3.75
M*SS*T	1.80	5.86	2.79	4.92	7.16	8.16	24.17	3.91	6.43	1.99	14.6	7.77	2.50
Error	15.3	18.9	14.9	21.8	34.9	19.1	33.22	85.6	81.1	86.9	56.6	41.5	75.7

[a] Factor contributions calculated by dividing each factor's sum of squares by the total sum of squares and multiplying by 100%; REI = radial expansion index; WAI = water absorption index; WSI = water solubility index; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch; MW = molecular weight; WE = water-extractable; M = moisture; SS = screw speed; T = temperature; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

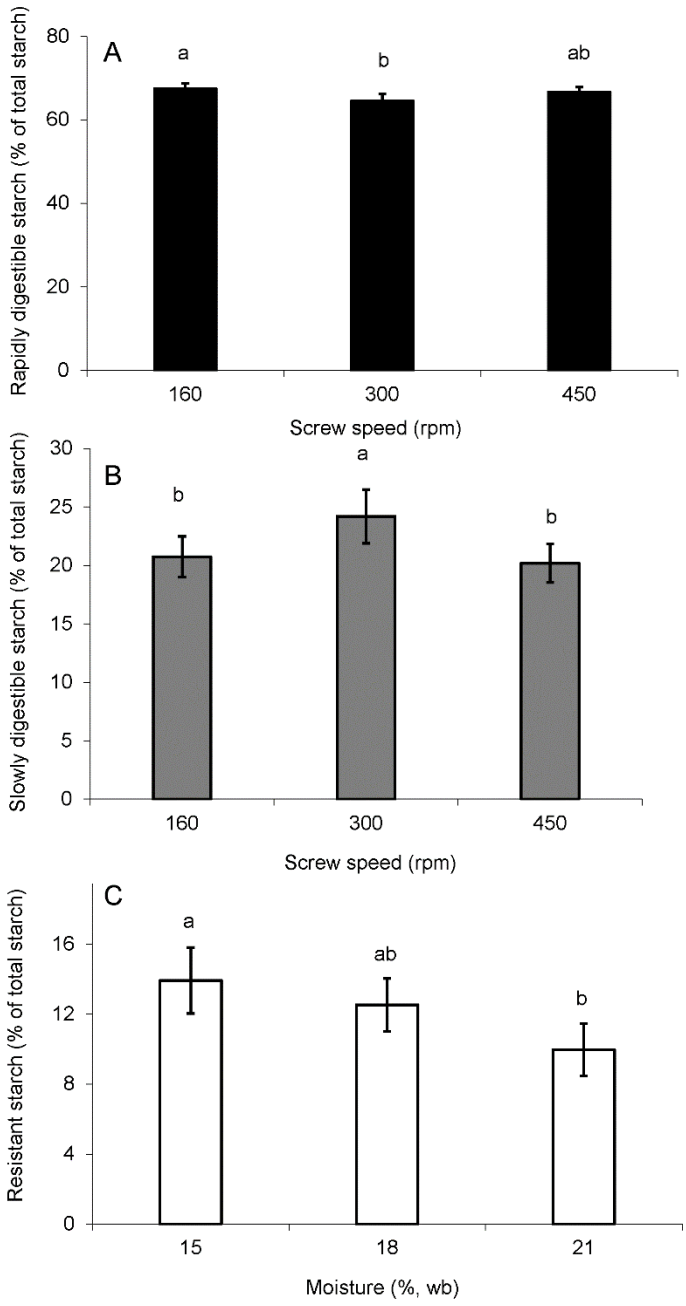

Figure 2-1. Means of significant treatment effects [screw speed (A, B) and moisture (C)] for rapidly digestible starch **(A)**, slowly digestible starch **(B)**, and resistant starch **(C)** in oat flour extrudates; error bars show standard error (n=12); bars marked with different letters are significantly different ($p<0.05$).

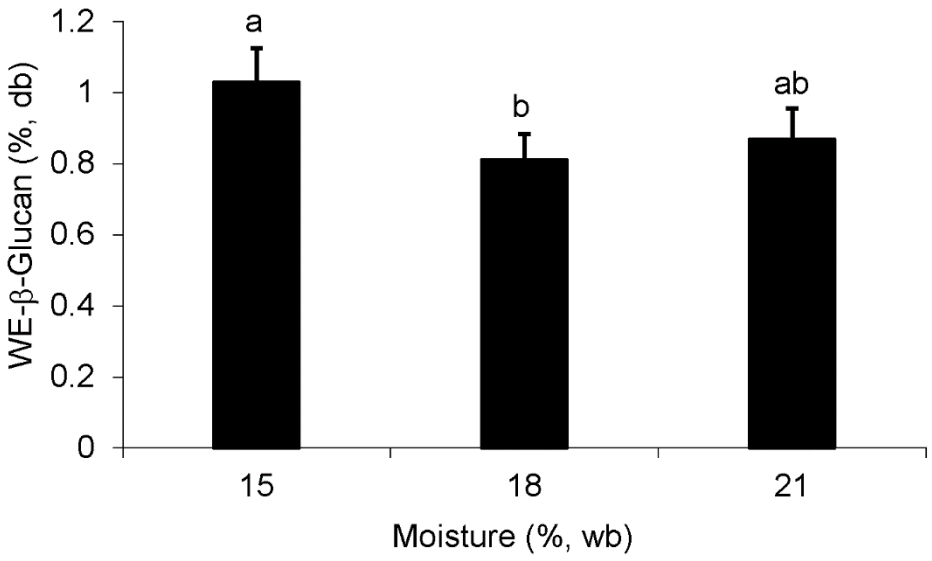

Figure 2-2. Means of moisture treatments for extractable β-glucan in oat flour extrudates; error bars show standard error (n=12); bars marked with different letters are significantly different ($p<0.05$).

2.7. Supplementary Materials

Supplementary Table 2-7-1. Correlations among response variables with at least one significant factor in the ANOVA models. [a]

Variable	SME	REI	L	a	b	WAI	WSI	RDS	SDS	RS
RDS	-0.21	0.09	0.37*	-0.04	-0.29	0.30	0.05			
SDS	-0.09	-0.16	-0.23	-0.12	0.03	-0.23	-0.06	-0.69***		
RS	0.30	0.14	0.00	0.19	0.21	0.06	0.05	0.09	-0.78***	
BG	0.14	0.04	0.05	0.09	-0.06	-0.10	0.06	-0.06	0.03	0.00

[a] SME = specific mechanical energy; REI = radial expansion index; WAI =water absorption index; WSI = water solubility index; RDS= rapidly digestible starch; SDS= slowly digestible starch; RS= resistant starch; BG= Extractable β-glucan; *p<0.05; **p<0.01; ***p<0.001.

Chapter 3 . Moisture content during extrusion of oats impacts the initial fermentation metabolites and probiotic bacteria during extended fermentation by human fecal microbiota

3.1. Abstract

Extrusion exposes flour components to high pressure and shear during processing, which may affect the dietary fiber fermentability by human fecal microbiota. The objective of this study was to determine the effect of flour moisture content during extrusion on in vitro fermentation properties of whole grain oats. Extrudates were processed at three moisture levels (15%, 18%, and 21%) at a fixed screw speed (300 rpm) and temperature (130 ˚C). The extrudates were then subjected to in vitro digestion and fermentation. Extrusion moisture significantly affected water-extractable β-glucan (WE-BG) in the extrudates, with samples processed at 15% moisture (lowest) and 21% moisture (highest) containing the highest concentration of WE-BG. After the first 8 h of fermentation, more WE-BG remained in fermentation media in samples processed at 15% moisture compared with the other conditions. Also, extrusion moisture significantly affected the production of acetate, butyrate and total SCFA by the microbiota during the first 8 h of fermentation. Microbiota grown on extrudates processed at 18% moisture produced the highest levels of acetate and total SCFA, whereas bacteria grown on extrudates processed at 15% and 18% moisture resulted in the highest butyrate production. After 24 h of fermentation, samples processed at 15% moisture supported lower *Bifidobacterium* counts than those produced at other conditions, but had among the highest *Lactobacillus* counts. Thus, moisture content during extrusion significantly affected production of fermentation metabolites by the gut microbiota during the initial

stages of fermentation, while also affecting probiotic bacteria counts during extended fermentation.

3.2. Introduction

Whole grain oats are widely consumed in the form of ready-to-eat (RTE) extruded breakfast cereals. Extrusion exposes flour components to high pressure and shear during processing, which affects the physicochemical properties of the extrudates as supported by several studies (Camire & Flint, 1991; Zhang, Liang, Pei, Gao, & Zhang, 2009; Brahma, Weier, & Rose, 2016). Zhang et al. (2009) showed that fragmentation of water-extractable β-glucan (WE-BG) occurred upon extrusion of oat bran with a concomitant decrease in the (1→3) and (1→4) linkages in oat bran from 1: 2.19 to 1: 0.85 accompanied by a decrease in (1→4) linkages from 72% in unprocessed flour to 48% in extruded samples. Another study reported an 18% increase in the WE-BG in extrudates processed at 15% moisture condition compared to 18% and 21% (Brahma et al., 2016). Camire et al. (1991) reported around 33% increase in insoluble NSP and 14% in total NSP in extruded oatmeal as compared with the raw oatmeal.

Extrusion moisture is the most critical parameter in the extrusion process. Processing moisture content impacts the melting temperature, viscosity and shear stress of materials inside the extruder barrel (Zhang, Bai, & Zhang,2011; Jongsutjarittam & Charoenrein, 2014; Brahma et al., 2016; Sumargo, Gulati, Weier, Clarke, & Rose, 2016). For instance, in our previous study, severe moisture played a critical role in increasing the resistant starch (RS), slowly digestible starch (SDS) fractions as well as WE-BG in extruded whole grain oats (Brahma et al., 2016). Moisture contents ranging from 20-29% in waxy rice flour and 16-25% in rice flour caused structural and physicochemical changes in the extrudates, with the lowest moisture condition causing more damage to the native crystalline structure accompanied by complete gelatinization of the starch granules

(Jongsutjarittam et al., 2014). Another study reported a decrease in rapidly digestible starch (RDS) from 75% to 68% accompanied by an increase in resistant starch (RS) from 1.8 to 12.6% with an increase in moisture from 17.2 to 20.1% in extruded brown rice and pinto bean flours (Sumargo et al., 2016). Zhang et al. (2011) reported an increase in soluble dietary fiber (primarily β-glucan) in oat bran with a decrease in extrusion moisture from with 30-10%. Moreover, extrusion temperatures (100-160 °C) had much lesser influence on the yield of the soluble dietary fiber in this study.

Because extrusion affects the physicochemical properties of the extrudates, it may affect the concentration of microbial accessible carbohydrates (MAC) during fermentation of the dietary fiber by human fecal microbiota (Dust, Gajda, Flickinger, Burkhalter, Merchen, & Fahey, Jr., 2004; Drzikova, Dongowski, Gebhardt, & Habel, 2005; Hernot, Boileau, Bauer, Swanson, & Fahey, Jr., 2008; Connolly, Lovegrove, & Tuohy, 2010). Kim & White. (2010) reported the in vitro fermentation properties of high (6.87×10^5 g/mol), medium (3.71×10^5 g/mol) and low molecular (1.56×10^5 g/mol) weight β-glucan from whole grain oats. The low molecular weight β-glucan resulted in higher amounts of propionate than its higher molecular weight counterparts during fermentation. On the other hand, Connolly et al. (2010) reported higher propionate and butyrate product during the later stages of fermentation of thick (0.85-1.00 mm) oat flakes compared with thin (0.53-0.63 mm) flakes. (Butyrate and propionate are considered beneficial short chain fatty acids (SCFA) produced by gut bacteria during fermentation (den Besten, Eunen, Groen, Venema, Reijngoud, & Bakker, 2013).) Low moisture (15%) coupled with low screw speed extrusion conditions (120 rpm) not only resulted in greatest extractability (around 3-fold) of non-starch polysaccharides in wheat bran, but also led to

the highest production of SCFA (1.4-fold) compared to untreated bran (Arcila, Weier & Rose, 2015). Extrusion processing made oat and barley extrudates more fermentable than wheat and corn (Hernot et al., 2008), with an 58% increase in the production of total SCFA during fermentation with human fecal microbiota compared with the native unprocessed whole grain. In contrast, Moen, Berget, Rud, Hole, Kjos, & Sahlstrøm (2016) showed that SCFA concentration and beneficial probiotic bacteria levels, (*Bifidobacterium* and *Lactobacillus)* were lower in pigs' feces collected after consuming extruded oat and barley diets compared to the unextruded diets. Thus, the purpose of this study was to investigate the influence of moisture content during extrusion on the in vitro fermentation of whole grain oats by human fecal microbiota.

3.3. Materials and methods

3.3.1. Starting material

Whole grain oat flour was obtained from General Mills (Minnesota, MN, USA). Moisture, non-starch polysaccharides (NSP) and total starch were measured in the whole grain oat flour following approved methods 44-15.02, 32-25.01 and 76-13.01 respectively (AACC International, 2016). A kit was used to assay total starch (K-TSTA, Megazyme, Wicklow, Ireland). Amylose: amylopectin ratio was measured using the dual wavelength iodine binding method (Zhu, Jackson, Wehling, & Geera, 2008). Protein was determined by following the approved method 46-30.01 (AACC International, 2016) using a using a nitrogen analyzer (FP528, Leco, St. Joseph, WI USA).

3.3.2. Extrusion of whole grain oat flour

Extrusion of whole grain oat flour was completed following the procedures descried by Brahma et al. (2016). In brief, extrudates were produced in duplicate at three

moisture levels (wet basis): 15%, 18% and 21%, at fixed screw speed (300 rpm) and temperature (130 ˚C). After drying, the extrudates were packaged in zip top bags and stored at 4 ℃ until further analysis.

3.3.3. In vitro digestion

Extrudates were milled using a cyclone mill (Model 4425, UDY, Fort Collins, CO, USA) equipped with a 1 mm screen. The milled samples were then subjected to in vitro digestion according Yang, Martínez, Walter, Keshavarzian, and Rose (2013). In brief, 25 g of sample was mixed with 300 mL of water and boiled for 20 min with constant stirring. The mixture was cooled, and the pH was adjusted to 2.5 with 1 M HCl followed by addition of 10 mL of 10% (w/v) pepsin (P-700; Sigma, St Louis, MO, USA) in 50 mM HCl. The mixture was then placed on an orbital shaker (150 rpm) set at 37℃ for 30 min, 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% (w/v) pancreatin (P-7545; Sigma) in sodium maleate buffer and 2 mL of amyloglucosidase (3260 U/mL; Megazyme, Bray, Ireland) were then added, and the samples were kept in a shaking water bath at 37 ℃ for 6 h. The digested slurries were then transferred into dialysis tubing (molecular weight cutoff 12,000- 14,000) (Spectrum Laboratories, Rancho Dominguez, CA, USA), and dialyzed for 3 d against distilled water at 4 ˚C with changing of the water every 3 h during the day. The retentate was frozen (-20 ˚C) overnight and then freeze-dried. The freeze-dried samples were analyzed for total starch and NSP with a sample size of 150 mg following AACCI approved method 32-25.01 (AACC International 2016). The total starch concentration in the freeze-dried sample was used to calculate the resistant starch concentration.

3.3.4. In vitro fecal fermentation and analysis

In vitro batch fecal fermentation was performed according to the methods described by Arcila et al. (2015) using separately prepared tubes containing 15 mg of digested, freeze-dried material suspended in 1 mL of sterile fermentation medium and 0.1 mL of freshly prepared, pooled fecal inoculum from 3 healthy individuals with no record of gastrointestinal abnormalities or antibiotic administration in the last 6 months. The fecal slurry was prepared by mixing the fecal samples with the sterile phosphate buffered saline (PBS, pH 7.0) in the ratio 1:9 (w/v) using a hand blender for 1 min and then filtering through four layers of cheesecloth. The fermentation medium contained (per L) peptone (2 g; Fisher Scientific, Pittsburgh, PA USA), yeast extract (2 g; Alfa Aesar, Ward Hill, MA USA), bile salts (0.5 g; Oxoid), $NaHCO_3$ (2 g), NaCl (0.1 g), K2HPO4 (0.08 g), MgSO4·7H2O (0.01 g), CaCl2·6H2O (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma), hemin (50 mg; Sigma), Tween 80 (2 mL), vitamin K (10 µL; Sigma), and 0.025% (w/v) resazurin solution (4 mL). The fermentation tubes were inoculated with 0.1 mL of fecal slurry, capped and incubated at 37 °C with orbital shaking (125 rpm) for 24 h. Samples were collected 0, 8 and 24 h during fermentation and were immediately stored at -80 °C. All steps were completed inside an anaerobic hood (Bactron X, Sheldon manufacturing, Cornelius, Oregon USA) containing 5% H_2, 5% CO_2 and 90% N_2.

For analysis, fermentation tubes were thawed and centrifuged at 10000 g for 5 min. The supernatants (0.4 mL each) were used for analysis of short/branched chain fatty acids (S/BCFA) and WE-BG, while the pellets were used for quantifying *Bifidobacterium* and *Lactobacillus* counts. Levels of SCFA were quantified by gas chromatography according to Arcila et al. (2015), whereas WE-BG was analyzed following the European

Brewery Convention method 8.11.1 using a kit (K-BGLU, Megazyme). *Bifidobacterium* and *Lactobacillus* were measured by quantitative real time PCR as described previously (Hartzell, Maldonado-Gómez, Hutkins, & Rose, 2013). Briefly, qPCR was performed using Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany), SYBR Green (Real master Mix, 5 PRIME Inc., Gaithersburg, MD, USA) with specific primers for *Bifidobacterium longum* ATCC 15705 (F: TCGCGTC(C/T) GGTGTGAAAG and R: CCACATCCAGC(A/G) TCCAC) and *Lactobacillus reuteri* MM4 (F: AGCAGTAGGGAATCTTCCA and R: ATTYCACCGCTACACATG) and annealing temperatures of 58 °C and 61°C respectively.

3.3.5. Data analysis

Extrusion was replicated in duplicates and each replicate was analyzed 4 times (S/BCFA, WE-BG, and *Bifidobacterium* and *Lactobacillus* counts). All data were reported on a dry weight basis except moisture content, which was on a wet basis. S/BCFA, WE-BG, and *Bifidobacterium* and *Lactobacillus* counts were analyzed using ANOVA by fermentation time. At each time point and for individual response variable, differences between sample means were calculated by Fisher's least significant difference, where $p < 0.05$ was considered significant.

3.4. Results and discussion

3.4.1. Oat flour composition

The total starch, protein, WE-BG, and NSP composition of the whole grain oat flour was 51.2±0.0%, 13.9±0.0%, 1.1±0.1% and 11.8±0.3%, respectively. Amylose: amylopectin ratio of the oat flour starch was 22.5:77.5±0.2, while the composition was

similar to previous reports (Dhingra, Michael, Rajput, & Patil, 2012; Kim & White, 2012; Zheng et al., 2015).

3.4.2. Composition of unprocessed oat flour and extrudates after in vitro digestion

In vitro digestion was completed on all the substrates prior to in vitro fermentation. Following digestion, all samples contained less than 5% starch (Table 3-1), which is in accordance with the ranges for RS typically present in whole grains (Yang et al., 2013). The differences in RS concentration may have been caused by a re-association of the depolymerized high molecular weight branched amylopectin due to shear degradation during different extrusion processing conditions (Lopez-Rubio, Flanagan, Shrestha, Gidley, & Gilbert, 2008; Li, Hasjim, Xie, Halley, & Gilbert, 2014). Alternatively, several recent papers have shown that freeze drying affects physicochemical properties of a substrate (e.g., Chen, Mao, Jiang, Wang, Li, & Gao, 2016; Chen, Li, Mao, Huang, Miao, & Gao, 2016; Mutlu, Kahraman, & Öztürk, 2017; Zeng, Zhu, Chen, Gao, & Yu, 2016). However, in cooked, digested starch samples, freeze drying had the least impact on RS concentration among many drying methods. Freeze drying also maintained the "open" structure of substrates consistent with undried samples (as opposed to a collapsed structure in samples dried by other means; Zeng et al., 2016). Because drying the sample was necessary for the in vitro fermentation, this method was selected for our studies. Furthermore, because all samples were subjected to freeze drying, it was assumed that the effect of freeze drying was similar among samples.

No significant difference was detected between WE-BG processed at 15% moisture compared with 21% moisture (Fig. 3-1). This trend was occurred in our previous study (Brahma et al., 2016), where we speculated that the higher WE-BG

produced at lower moisture was due to the increased severity of the extrusion process while increased levels of WE-BG at higher moisture was caused by increased hydration of the flour matrix.

3.4.3. Effects of extrusion moisture on fermentation of water-extractable β-glucan

Approximately 95% of the WE-BG was metabolized within the first 8 h of fermentation regardless of extrusion processing conditions (Fig. 3-1). Although there was no significant difference in WE-BG between samples processed at 15% moisture and those processed at 21% moisture at the beginning of fermentation, higher levels of WE-BG remained media samples after 8 h into the fermentation processed at 15% moisture compared with the extrudates processed under different conditions. After 24 h of fermentation, nearly all the WE-BG was metabolized (96-98%). These results are similar to Wood, Arrigoni, Miller, & Amadò (2002), who reported the rapid fermentation of WE-BG from both digested oat bran and purified oat β-glucan within 4 h and the complete disappearance between 4 h and 24 h of fermentation. In contrast, Kaur, Rose, Rumpagaporn, Patterson, & Hamaker (2011) compared the fermentation rates of several polysaccharides, including β-glucan, and determined that it initially fermented slowly, but more rapidly during the later stages of fermentation. These results could be due to differences in the functionality of the fecal microbiota. In our study, rapid utilization of WE-BG could be because this structurally simple polysaccharide could be utilized by many members of the microbiota (Martínez et al., 2013).

3.4.4. Effects of extrusion moisture on carbohydrate and protein fermentation metabolites

Moisture content during extrusion significantly affected the production of acetate, butyrate and total SCFA in the first 8 h of fermentation (Fig. 3-2), but not after 24 h. Extrusion moisture also affected the initial rate of SCFA production but not the rate of SCFA production during extended fermentation (Table 3-2). Microbiota grown on extrudates processed at 18% moisture had the highest production of acetate and total SCFA. These samples also had the highest rate of SCFA production compared to the other extrudates. The fecal microbiota grown on extrudates produced at 15% and 18% moisture produced the most butyrate. No significant differences among the extrudates in the production of SCFA by the microbiota occurred after 24 h of fermentation.

SCFA are absorbed by the host and thus stimulate the release of several hormones involved in energy uptake and metabolism (Byrne, Chambers, Morrison, & Frost, 2015). The SCFA also are associated with lowering the pH in the gut, which, in turn, inhibits pathogens and increases mineral bioavailability. Butyrate is also an immune modulator and plays an important role in cell differentiation, proliferation and in gut barrier function (Peng, Li, Green, Holzman, & Lin, 2009). Studies have shown higher availability of arabinoxylan to fermentation after extrusion, which favored the production of acetate (Hopkins & Macfarlane, 2003; Pollet, Craeyveld, Wiele, Verstraete, Delcour, & Courtin, 2012). The high production of butyrate from microbiota grown on 15% moisture extrudate was possibly due to the increased WE-BG. Kaur et al. (2011) reported that an increase in acetate and butyrate concentration could be attributed, in part, to the fermentation of β-glucan.

As with SCFA, the processing conditions only affected BCFA production in the first 8 h of fermentation (Fig. 3-3; Table 3-2). Bacteria grown on extrudates processed at 15% moisture content resulted in the highest production of iso-butyrate and the lowest production of iso-valerate compared to the other samples (Fig. 3-3). BCFA arise from the fermentation of branched chain amino acids from protein, which was reported in previous studies (Russell et al., 2011; Koh, Vadder, Kovatcheva-Datchary, & Bäckhed, 2016).

The data from both SCFA and BCFA indicate that during the initial stage of fermentation, the microbiota differentiated among the extrudates based on the readily fermentable carbohydrates. However, at the later stages of fermentation, bacteria were unable to differentiate among the substrates. Leading to higher initial fermentation but similar total fermentation. Possible positive implications of early fermentation allow more time for fermented products to be absorbed and exert positive effects on the human host (Teixeira, Nyman, Andersson, & Alminger, 2017). Similar extended fermentation may promote delivery of fermentable carbohydrate to the more distal parts of the colon and support beneficial saccharolytic bacteria (Rose, DeMeo, Keshavarzian, & Hamaker, 2007).

The trends in production of SCFA did not match with either RS or WE-BG concentration, but were comparable to trends for other NSP (mostly insoluble polysaccharides), although other NSP were not significantly different (Table 3-1). The lack of a match between RS, WE-BG concentration and SCFA productions was unexpected, although these fractions represent only a small portion of the total carbohydrate potentially available to the bacteria for fermentation. Thus, the differences in SCFA production were most likely due to changes to the microbial accessibility to the

other NSP. Differences in available carbohydrates from wheat bran following extrusion has been previously studied (Arcila et al., 2015). In this study, extrusion enhanced the gut microbial fermentation of wheat bran dietary fiber by providing more fermentable carbohydrates compared with the unextruded bran. The authors noted a significant increase in water-extractable NSP, but extruded bran was still only a small fraction of the total NSP (5-7%). In contrast, production of SCFA increased dramatically (40% increase). Our results are also supported by the biofuels literature where it has been shown that extrusion can open up the microstructure of lignocellulosic components and can make the insoluble fractions more accessible to enzymatic hydrolysis (Barakat, Mayer-Laigle, Solhy, Arancon, de Vriesa, & Luque, 2014; Yoo, Alavi, Vadlani, & Amanor-Boadu, 2011). Hence, it could be that the structure of the insoluble NSP was more open following extrusion and susceptible to microbial metabolism during fermentation.

Notably, the trend for BCFA production also were similar to the other NSP concentration than with the protein concentration (Table 3-1). This result indicates that the BCFA production was more dependent on the quantity of microbial available NSP than on the total protein.

3.4.5. Effects of extrusion on Bifidobacterium and Lactobacillus counts

Extrusion moisture had significant effects on both the *Bifidobacterium* and *Lactobacillus* counts at the end of 24 h fermentation but not at 8 h (Fig. 3-4). Interestingly, at the end of fermentation, the media containing extrudates processed at 15% moisture content declined in *Bifidobacterium* and increased in *Lactobacillus* compared to other extrudates. The decrease in *Bifidobacterium* might be due to either

inhibition by their own metabolites or intolerance to acids as some *Bifidobacterium* strains are not acid tolerant (Vernazza et al., 2006). Generation of fermentation end products, such as succinate or lactate, could inhibit some *Bifidobacterium* species and encourage the growth of other *Lactobacillus* species (Belenguer et al., 2006; Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & Reyes-Gavilán, 2008).

Bifidobacterium and *Lactobacillus* were of special interest due to their probiotic potential, and cereal β-glucans have previously been reported to be bifidogenic in nature (de Angelis et al., 2015). For instance, *Bifidobacterium* significantly increased in response to thicker whole grain oat flakes (0.85-0.1 mm) than thinner flakes (0.53-0.63 mm) in an in vitro study (Connolly et al., 2010). Snart et al. (2006) showed a higher enrichment of *Lactobacillus* in in the cecum of rats fed a casein-based diet rich in high viscosity β-glucans derived from barley compared to other substrates, including barley flour, oat flour, cellulose or barley β-glucans of lower viscosity. Future studies are still needed to determine changes in other members of the microbiota.

3.5. Conclusion

Overall, the current study showed the effects on the in vitro fermentation properties of whole grain oats by extrusion moisture conditions. A higher concentration of WE-BG was present in extrudates processed under 15% moisture condition after 8 h compared with other conditions. Moreover, after 8 h of fermentation, samples processed at 15% moisture resulted in the highest production of butyrate, whereas samples processed at 18% moisture resulted in the highest acetate and total SCFA production. These results indicate that the carbohydrates were more accessible to the microbiota as a result of extrusion and play an important role in the production of these beneficial SCFA during

the initial stage of fermentation. After 24 h of fermentation, *Bifidobacterium* counts were lower and *Lactobacillus* counts were higher when the fecal microbiota was grown on the samples processed at 15% moisture compared to other samples. Therefore, this study demonstrates that moisture content during extrusion significantly affects production of fermentation metabolites by the gut microbiota during the initial stages of fermentation and the concentration of probiotic bacteria during the extended fermentation.

3.6. References

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Table 3:1. Polysaccharide and protein concentrations in unprocessed whole grain oat flour and extrudates produced at different moisture contents (15%, 18%, and 21%) after in vitro digestion (% dry basis).[A]

	Polysaccharides			
Sample	RS	WE-BG	other NSP	Protein
Unprocessed	2.5±0.0[c]	1.1±0.1[c]	57.2±2.6	23.7±0.1[a]
15%	3.6±0.1[a]	1.8±0.1[a]	66.1±2.4	22.5±0.1[b]
18%	2.6±0.2[c]	1.5±0.2[b]	63.9±3.9	22.1±0.3[b]
21%	3.3±0.1[b]	1.7±0.1[a]	59.0±5.8	23.3±0.7[a]

[A] Mean± standard deviation (n=2); RS, resistant starch; WE-BG, water-extractable β-glucan; NSP, non-starch polysaccharides; protein=N × 6.25; means within column followed by different letters are significantly different ($p<0.05$).

Table 3:2. Rate of branched/short chain fatty acid (B/SCFA) production (μmol/h) during initial fermentation (0-8 h; rate 1) and during extended fermentation (8-24 h) of unprocessed whole grain oat flour and extrudates produced at different moisture contents (15%, 18%, and 21%).[A]

Sample	Acetate	Propionate	Butyrate	SCFA	iso-Butyrate	iso-Valerate	BCFA
Rate 1							
Unprocessed	2.42 ± 0.22^{bc}	0.91 ± 0.03^{b}	0.62 ± 0.03^{b}	3.96 ± 0.26^{bc}	0.02 ± 0.00^{b}	0.02 ± 0.00^{a}	0.04 ± 0.00
15%	2.51 ± 0.31^{b}	0.90 ± 0.04^{b}	0.66 ± 0.05^{a}	4.06 ± 0.36^{b}	0.02 ± 0.00^{a}	0.02 ± 0.01^{c}	0.04 ± 0.01
18%	2.87 ± 0.27^{a}	0.95 ± 0.03^{a}	0.65 ± 0.02^{a}	4.47 ± 0.30^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{ab}	0.04 ± 0.00
21%	2.25 ± 0.28^{c}	0.90 ± 0.01^{b}	0.56 ± 0.04^{c}	3.71 ± 0.32^{c}	0.02 ± 0.00^{ab}	0.02 ± 0.00^{bc}	0.04 ± 0.00
Rate 2							
Unprocessed	0.50 ± 0.14	0.26 ± 0.03	0.13 ± 0.03	0.89 ± 0.19	0.04 ± 0.01	0.07 ± 0.01	0.11 ± 0.01
15%	0.64 ± 0.14	0.29 ± 0.03	0.11 ± 0.03	1.05 ± 0.18	0.03 ± 0.01	0.06 ± 0.01	0.09 ± 0.02
18%	0.47 ± 0.52	0.24 ± 0.05	0.10 ± 0.03	0.80 ± 0.57	0.03 ± 0.00	0.07 ± 0.02	0.10 ± 0.02
21%	0.53 ± 0.12	0.26 ± 0.07	0.14 ± 0.05	0.92 ± 0.19	0.03 ± 0.01	0.07 ± 0.02	0.11 ± 0.03

[A] Mean ± standard deviation; means followed by different letters within column and rate are significantly different ($p<0.05$; n=2); due to rounding some means with the same numeric value are significantly different

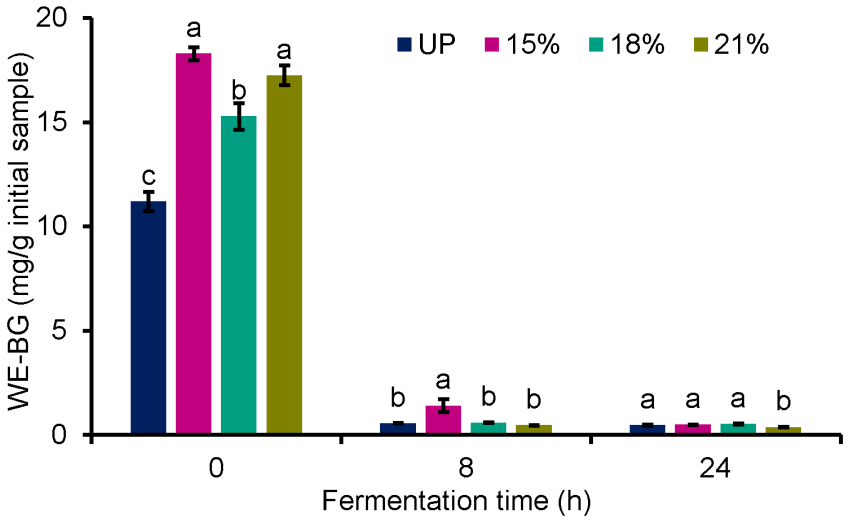

Figure 3-1. Utilization of water-extractable β-glucan during in vitro fecal fermentation of unprocessed whole grain oat flour (UP) and extrudates produced at different moisture contents (15%, 18%, and 21%); error bars show standard error; bars marked with different letters show significant differences among samples within time point ($p<0.05$, $n=2$).

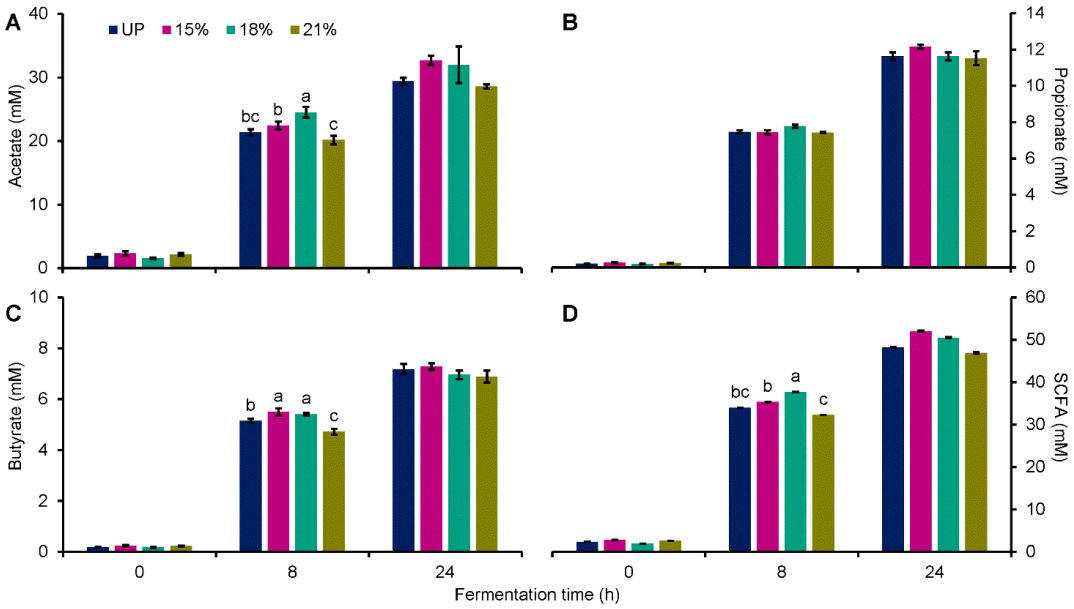

Figure 3-2. Short chain fatty acid (SCFA) production, **A)** acetate **B)** propionate **C)** butyrate **D)** total SCFA during in vitro fecal fermentation of unprocessed whole grain oat flour (UP) and extrudates produced at different moisture contents (15%, 18%, and 21%); error bars show standard error; bars marked with different letters show significant differences among samples within SCFA and time point ($p<0.05$, $n=2$).

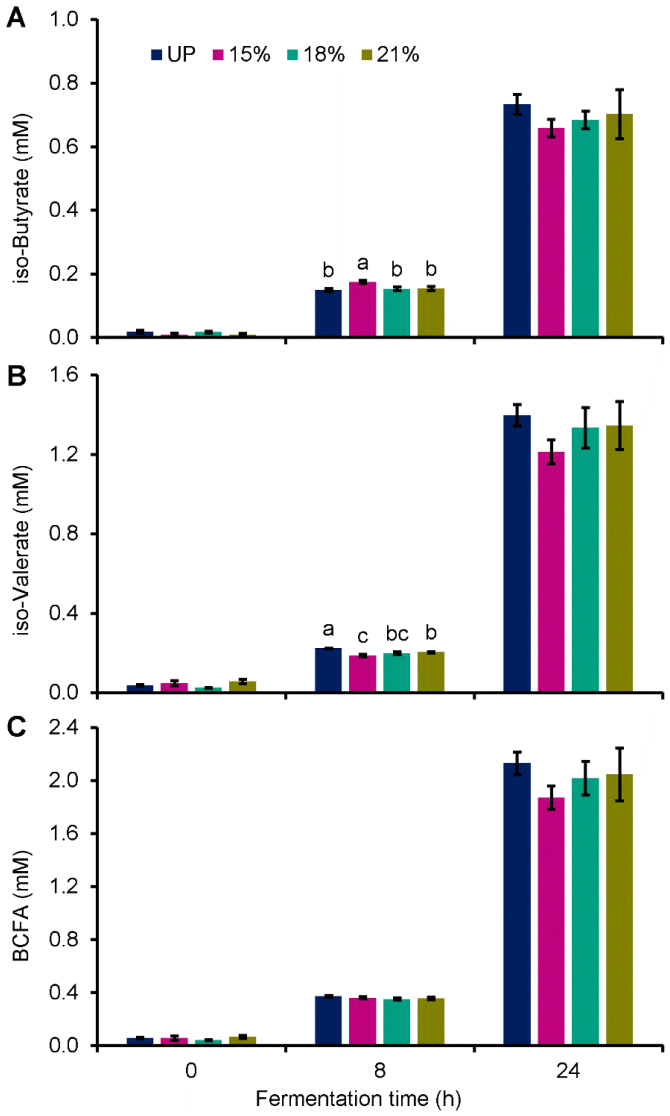

Figure 3-3. Branched chain fatty acid (BCFA) production, **A)** isobutyrate **B)** isovalerate **C)** total BCFA during in vitro fecal fermentation of unprocessed whole grain oat flour (UP) and extrudates produced at different moisture contents (15%, 18%, and 21%); error bars show standard error; bars marked with different letters show significant differences among samples within BCFA and time point ($p<0.05$, $n=2$).

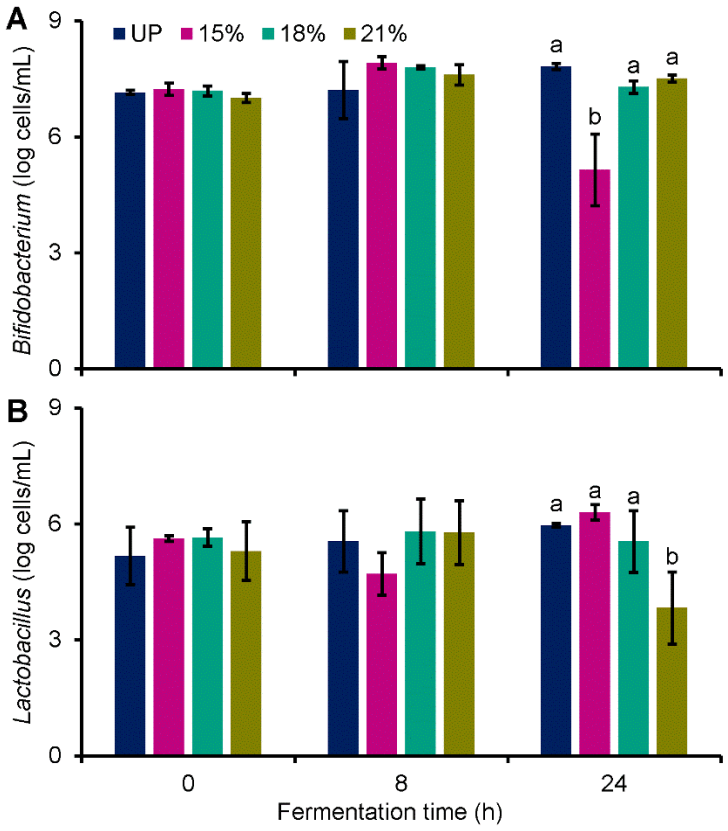

Figure 3-4. A) *Bifidobacterium* **B)** *Lactobacillus* counts during in vitro fecal fermentation of unprocessed whole grain oat flour (UP) and extrudates produced at different moisture contents (15%, 18%, and 21%); error bars show standard error; bars marked with different letters show significant differences among samples within subfigure and time point ($p<0.05$, $n=2$).

Chapter 4 . Impact of dietary pattern of the fecal donor on in vitro fermentation properties of whole grains and brans

4.1. Abstract

Because diet influences gut microbiota composition and function, the purpose of this study was to determine how fecal donor diet impacts in vitro fermentation properties of whole grain flours and brans from corn, oats, rye, and wheat. Samples were fermented with fecal microbiota from subjects with similar energy intakes but differing in intakes of several beneficial nutrients (G1>G2). Shifts in the microbiota during fermentation were a function of diet group and time. Fecal microbiota from G1 subjects showed less decrease in diversity during fermentation, and these microbiotas were more effective in utilizing higher carbohydrate and producing butyrate compared with microbiota from G2 subjects. More carbohydrates were fermented from whole grains than brans. Rye induced high carbohydrate fermentability and butyrate production accompanied by low ammonia production, but only when using fecal microbiota from G1 subjects. Thus, diet quality influences the ability of the microbiota to ferment carbohydrates, differentiate among grains and produce butyrate.

4.2. Introduction

Dietary fibers from different whole grains (WG) and cereal brans have unique structural characteristics that may impart distinct effects on fermentation by the gut microbiota with subsequent effects on the host. Previous studies have outlined the impact of various dietary fibers on the gut microbiota (Nordlund et al., 2012; Rose et al., 2010b; Rumpagaporn et al., 2015). Cereal-derived arabinoxylans, which comprise roughly 50% of cereal dietary fiber, have different structural features that affect their fermentability by the intestinal microbiota. For instance, Rumpagaporn et al. (2015) showed that utilization of arabinoxylans by the microbiota is a complex process influenced by the degree of arabinosyl substitution and molecular weight. Rose et al. (2010b) showed that rice and corn arabinoxylans were degraded through a debranching mechanism by the microbiota, which was attributed to branched regions that were evenly distributed along the xylan backbone. In contrast, wheat arabinoxylans contained unsubstituted xylan regions that were preferentially fermented before the highly branched regions. Rye bran was shown to have the highest fermentation rate and extent compared with oat and wheat bran due to its high content of water-extractable arabinoxylan (Nordlund et al., 2012).

Other dietary fiber fractions in WG, such as fructan, β-glucan and resistant starch, can affect fermentation rate and extent (Nordlund et al., 2012). Oat bran has been shown to be more readily fermentable than wheat and rye bran, due to its high water-extractable β-glucan (Karppinen et al., 2000). A higher percentage of resistant starch in WG wheat as compared to bran resulted in higher production of acetate and butyrate and less propionate during in vitro fermentation (Hernot et al., 2008). Thus, composition and

structural details of dietary fibers in cereal grains play critical roles in regulating fermentation.

The effects of diet on the fecal gut microbiota composition have been reported previously (Costabile et al., 2008; Lappi et al., 2013; Martínez et al., 2013; Martínez et al., 2015; Russell et al., 2011). Diets rich in plant fiber could promote gut health by altering the composition and the metabolic effects of the gut microbiota. For instance, association between a plant based diet and *Prevotella* has been shown in the diets of people from Burkina Faso (non-Westerners) (Martínez et al., 2015). Another study showed a decline in *Roseburia/Eubacterium rectale* on diets rich in protein and low in carbohydrate (Russell et al., 2011). Several studies have also investigated the influence of diet from WG products on gut microbiota composition (Martínez et al., 2013; Costabile et al., 2008; Lappi et al., 2013). Lappi et al. (2013) assessed the gut microbiota composition between intake of high fiber WG rye bread and low fiber wheat bread in Finnish adults. During the 12-week intervention, *Bacteroidetes* decreased and *Clostridium* cluster IV, *Collinsella*, and *Atopobium* spp. increased. Thus, diet plays a vital role in modulating the responses of the microbiota in the human gut.

Many studies and reviews have proposed that the functionality of the gut microbiota is either equally or more important than composition (Jandhyala et al., 2015; Marchesi et al., 2016). Our previous research used an in vitro system to assess fecal microbiota functionality from different donors when grown on different carbohydrates (Yang et al., 2013; Yang & Rose, 2014). In these studies, gut microbial functionality correlated with the intake of several nutrients, in particularly dietary fiber, in the diet of the fecal donor.

Given that dietary fiber intake has an impact on functionality of the gut microbiota, we hypothesized that the gut microbiota from individuals consuming high dietary fiber diets will metabolize the dietary fibers from grains more efficiently and produce higher concentrations of beneficial metabolites compared with donors with lower dietary fiber intakes. Furthermore, due to differences in concentration and composition of dietary fibers and other components in WG and bran, we further hypothesized that these substrates will illicit different responses in gut microbiota and metabolite production during fermentation. Thus, to test our hypotheses, different WG and brans were used as substrates during in vitro fermentation using stool samples that were collected from individuals consuming diets differing in overall quality. We then analyzed bacterial communities, short/branched chain fatty acids (S/BCFA), ammonia and total carbohydrate fermented during in vitro fermentation of each substrate.

4.3. Materials and methods

4.3.1. Compositional analysis of flour and bran samples

Commercially available WG flours and brans from corn, oats, rye and wheat were obtained from General Mills (Minneapolis, MN, USA). All samples were milled using a cyclone mill (Model 4425, UDY, Fort Collins, CO, USA) equipped with a 0.5 mm screen. Compositional analysis included: moisture, protein, total starch and dietary fiber (approved methods 44-15.02, 46-30.01, 76-13.01, and 32-25.01; AACC International, 2016).

4.3.2. In vitro digestion of flour and bran samples

In vitro digestion of the samples was completed as described (Yang & Rose, 2014). In short, 25 g of sample was suspended in 300 mL of water and boiled for 20 min

with constant stirring. Once cooled, the pH was adjusted to 2.5 with 1 M HCl, and then 10 mL of 10% (w/v) pepsin (P-7000; Sigma, St Louis, MO, USA) in 50 mM HCl was added. The mixture was placed in an orbital shaker (150 rpm) at 37 °C for 30 min, followed by the addition of 50 mL of 0.1 M sodium maleate buffer (pH 6, containing 1 mM $CaCl_2$) that was adjusted to a pH of 6.9. Fifty milliliters of 12.5% (w/v) pancreatin (P-7545; Sigma) in sodium maleate buffer and 2 mL of amyloglucosidase (3,260 U/mL; Megazyme, Bray, Ireland) were then added. The samples were kept in a shaking in a water bath at 37 °C for 6 h. The digested samples were transferred into dialysis tubing (molecular weight cutoff 12,000-14,000; Spectrum Laboratories, Rancho Dominguez, CA, USA) and dialyzed for 3 d against distilled water at 4˚C with changing every 3 h during the day. Following dialysis, the samples were freeze dried and composition was re-analyzed as described (section 2.1).

4.3.3. Selection of stool donors for in vitro fermentation

Stool donors were selected from a previous study based on their dietary record data (Yang & Rose, 2014). Principal component analysis (PCA) was applied to dietary patterns of 18 subjects. Nearly 70% of the variance in the dataset was accounted for in PC1, which separated subjects by intake of several desirable macro- and micronutrients, including dietary fiber, plant protein, thiamin, riboflavin, folate, iron, magnesium and zinc. Fecal samples of the four subjects with the highest and lowest eigenvalues on PC1 were used in this study. Group 1 (G1) included subjects with the highest loadings on PC1 and consequently higher intake of many beneficial nutrients, including dietary fiber, compared with group 2 (G2) subjects (Supplementary Table 4-7-1), who had low loadings on PC1. Although subjects from both G1 and G2 had similar energy intake, G1

subjects had higher diet overall quality (Healthy Diet Indicator) scores compared with G2 subjects.

4.3.4. In vitro fecal fermentation

In vitro batch fecal fermentations were performed based on the procedure described previously (Yang & Rose, 2014). Separately prepared samples consisted of tubes containing 15 mg of digested, freeze-dried sample suspended in 1 mL sterile fermentation medium for each analysis and replicate. The fermentation tubes were inoculated with 0.1 mL of fecal slurry, capped and incubated at 37 °C with orbital shaking (125 rpm) for 24 h. The fecal slurry was prepared by mixing the fecal samples with the sterile phosphate buffered saline (PBS, pH 7.0) in the ratio 1:9 (w/v) using a hand blender for 1 min and then filtering through four layers of cheesecloth. For analysis of S/BCFA, ammonia and microbiota, tubes were removed at designated time points (0, 8 and 24 h) and immediately placed on ice. Fermentation in tubes designated for carbohydrates and microbial metabolite analysis was stopped using 20 µL of mercuric chloride (1 mg/mL). In all cases, samples were removed from the anaerobic cabinet and immediately stored at -80 °C. All steps of the fermentation were conducted in an anaerobic hood with 5% H_2, 5% CO_2, and 90% N_2.

4.3.5. Fermentation analyses

For the analysis of total carbohydrates, fermented samples were freeze-dried and the residue was analyzed for remaining carbohydrates (approved method 32-25.01, AACC International 2016). In samples designated for S/BCFA, ammonia and microbiota analysis, samples were thawed in cold water and then centrifuged at 8,000 g for 5 min. The supernatants were analyzed for S/BCFA and ammonia analysis, whereas the pellets

were evaluated for microbiota composition. S/BCFA and ammonia were quantified by gas chromatography and colorimetry, respectively, as described (Yang & Rose 2014).

4.3.6. Microbiota composition

For microbial analysis, DNA was isolated from fermentation pellets after mechanical and enzymatic bacterial cell lysis using the method based on phenol/chloroform extractions (Martínez et al., 2009). Microbiota characterization was performed by amplicon sequencing of the V5-V6 region of the 16S rRNA gene with the MiSeq (Illumina) platform using the MiSeq Reagent kit v3 (2 x 300 bp). Sequenced reads were trimmed to 250 bases (read 1), and 200 bases (read 2) (based on visual inspection of quality scores with FastQC software; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and paired-end reads were merged with the merge-illumina-pairs application (Eren et al., 2013) (P value of 0.03, enforced Q30 check, perfect matching to primers, and no ambiguous nucleotides allowed). Subsequently, the USEARCH pipeline (v7.0.100163) was used to remove chimera for generating operational taxonomic units (OTUs) (98% identity). Taxonomic assignment of reads was completed with a RDP Classifier, RDP Seqmatch and the NCBI database. An overview of the entire experimental design is presented in Fig. 1.

4.3.7. Data analysis

Fermentation responses (SCFA, BCFA, ammonia, and carbohydrate fermented) and microbiota composition (α-diversity and genus-level abundances) were analyzed using a 3-factor [diet group, grain fraction (WG or bran), grain type] repeated measures (fermentation time) ANOVA with subject within diet group as the random error using

SAS software (version 9.4, SAS Institute, Cary, NC, USA). Statistically significant differences were assessed using Bonferroni's post hoc test; an adjusted $P < 0.05$ was considered significant. Taxonomic composition of the microbiota was compared using PCA based on the average OTU abundances of the subjects in each diet group. Partial correlations between the mean microbial composition at the genus level and fermentation responses were calculated post-fermentation (time=24 h) using group, grain fraction and grain type as partial variables.

4.4. Results and discussion

4.4.1. Whole grain composition before and after in vitro digestion

Total starch, protein and dietary fiber concentration in the WG and brans were within the ranges reported previously (Table 4-1; Karppinen et al., 2000; Koehler & Wieser, 2013; Rose et al., 2010a). Other components included lipids (2-8%) and ash (2-4%) along with other compounds commonly associated with WG (Welch, 2011). Following in vitro digestion, all samples contained mostly dietary fiber. Starch contents were between 0.4-7.2%, which agree with the ranges for resistant starch common in WG (Englyst et al., 2007).

4.4.2. Characterization of outcomes from in vitro fermentation

The effects of diet group, grain fraction and grain type over time were determined using a multiple-factor repeated measures ANOVA model. Based on the ensuing results, many significant two-way interactions were evident (Supplementary Table 4-7-2). The primary term of interest was the diet group by time interaction, which identified differences in fermentation properties between the two diet groups with fermentation time. Significantly higher butyrate and carbohydrate fermentation occurred in samples

inoculated with the fecal microbiota from G1, while higher acetate, propionate and total SCFA and BCFA were produced in samples inoculated with the fecal microbiota from G2 (Fig. 4-2). This information showed that the gut microbiota from G1 subjects could utilize the fermentable carbohydrates more readily than the microbiota from G2 subjects, resulting in higher production of butyrate during the fermentation. In contrast, the microbiota from G2 produced significantly higher concentrations of other metabolites, including markers of protein fermentation.

The higher total SCFA production in the samples inoculated with fecal microbiota from G2 subjects was somewhat unexpected. Moreover, the carbohydrate fermented was inconsistent to the SCFA formed between the two diet groups. Thus, a mass balance of carbohydrates fermented to fermentation products formed was calculated. For every 1 mol of carbon from carbohydrate fermented, about 0.6 mol of carbon flowed to SCFA (acetate + propionate + butyrate), regardless of diet group (G1, 0.58±0.11 mol C in SCFA/mol C in carbohydrate fermented versus G2, 0.65±0.18 mol C in SCFA/mol C in carbohydrate fermented; $p=0.25$). Thus, SCFA were produced at the same efficiency by the microbiota from each diet group, but different metabolic pathways were used. Specifically, G1 microbiota had a greater propensity toward butyrate production, while G2 had a greater propensity toward acetate production. The implications of these results are important as butyrate has many metabolic benefits while acetate is largely absorbed and used as energy by the host (Samuel et al., 2008). These results are supported by Turnbaugh et al. (2006) who showed that dysbiosis resulted in increased SCFA production by the gut microbiota with increased energy harvest from the diet of the host.

According to our mass balance calculation, the production of SCFA accounted for only about 60% of the carbon fermented from carbohydrate. The remaining carbon may have been precursors for other metabolites produced by members of the gut microbiota, such as formic acid, succinic acid, lactic acid, carbon dioxide or methane. Our experimental approach was to focus on the major end products of microbiota fermentation that are linked to health benefits; however, based on this study, research aimed at identifying differences in production of these other metabolites is essential.

Differences between grain fraction over time (Supplementary Table 4-7-2) showed that when brans were used as substrates, less carbohydrate was fermented and more BCFA were produced (Fig. 4-3). This may be because brans had a lower percentage of resistant starch after in vitro digestion compared to WG (WG, 2.8-7.2%; bran, 0.4-2.8%; Table 4-1). Hence, the microbiota may have resorted to protein fermentation in the bran samples due to a lack of available fermentable carbohydrates. A higher percentage of resistant starch has also been reported in WG wheat flour compared with its corresponding bran fraction, with higher production of acetate and butyrate accompanied by less propionate during in vitro fermentation (Hernot et al., 2008). In another study, WG wheat modulated the gut microbiota in fecal samples compared to the wheat bran group by increasing the numbers of the genus *Bifidobacterium* and *Lactobacillus* (Costabile et al., 2008).

Among grains, significant differences were occurred for butyrate and ammonia production over time (Supplementary Table 4-7-2). When rye was used as a substrate, significantly more butyrate was produced by the microbiota compared with the other grains (Fig. 4-4). Along with oats, rye also resulted in lower ammonia production than

wheat and corn. This is in accordance with previous studies showing that grains of various types lead to differences in S/BCFA profiles during fermentation (Hernot et al., 2008; Karppinen et al.,2000; Rose et al., 2010b; Yang et al., 2013). For instance, in vitro fermentation of WG corn substrates produced no acetate, little propionate, and moderate amounts of butyrate and total SCFA when compared to WG barley, wheat, oats and rice (Hernot et al., 2008). The microbiota fermenting WG rye produced the highest butyrate and lowest propionate compared with wheat, corn, oat and rice during fermentation using fecal samples from normal and obese people (Yang et al., 2013). The possible reason for this difference could be that the higher resistant starch in both oat and rye were present compared to corn and wheat (Table 4-1), which may result in protective effects by lowering ammonia production and inhibiting protein fermentation. Moreover, rye and oats contained higher amounts of soluble dietary fiber in the form of water-extractable arabinoxylan and β-glucan, respectively (Nordlund et al., 2012), which are generally more readily available for microbial fermentation than other insoluble substrates.

Significant differences among grains depending on diet group occurred for butyrate, ammonia and carbohydrate fermentation (Supplementary Table 4-7-2). Interestingly, differences among the grains were only exhibited when using the microbiota of G1 subjects. (Fig. 4-5). In the microbiota from G1 subjects, higher fermentability of the carbohydrates from rye compared with corn and oats were detected. This could be due to a unique structural characteristic of rye as it contains high water-extractable arabinoxylans relative to other WG (Karppinen, 2010).

Differences between grain fraction among the different grain types were only significant for ammonia production (Supplementary Table 4-7-2). Both rye and oat bran

substrates resulted in significantly lower ammonia production compared to corn and wheat bran (Fig. 4-5). This could again be explained by the higher resistant starch and soluble dietary fiber in rye and oats compared with the other grains. The structural difference in starch-protein matrix could also be another contributing factor for lower ammonia production (Juntunen et al., 2003).

4.4.3. Characterization of the microbiota composition

The overall taxonomic structure of the microbiota clustered by diet group of the fecal donors and fermentation time (Fig. 4-6). At 0 h of fermentation the bacteria that drove separate clustering among diet groups included different members (OTUs) of the *Lachnospiracea incertae sedis*, *Blautia*, *Ruminococcus*, *Clostridium* XIVa, and *Anaerostipes* genera. *Blautia* utilize hydrogen, which is produced from glycan fermentation, and thus are likely to be induced by fermentation of carbohydrate-rich WG substrates (Nakamura et al., 2010). *Ruminococcus* has been linked to long-term intake of plant based dietary polysaccharides (David et al., 2014). Research has also shown that the microbiota from omnivorous diets are enriched in *Clostridium* XIVa (Kabeerdoss et al., 2012). *Anaerostipes* has been identified as a saccharolytic bacteria that forms butyrate from acetic and lactic acid (Bui et al., 2014). Notably, a member of the *Faecalibacterium* genus (OTU 827) was characteristic of samples corresponding to G1 at 0 h. This genus can account for more than 5% of the commensal microbiota of healthy human subjects and have also been shown to produce butyrate and correlated with dietary fiber intake (Chiba et al., 2015).

During the course of fermentation, the shifts in microbial communities among the two diet groups followed distinct trajectories despite receiving the same WG or bran

substrates (Fig.4-6). The shifts in G1 samples were driven by members of the *Phascolarctobacterium, Bacteroides, Dorea*, *Collinsella,* and *Clostridium* XVIII genera. Most of these genera have been identified as beneficial butyrate producers (Bui et al., 2014; 2013; Lappi et al., 2013; Martínez et al., 2013). However, elevated *Dorea* levels has also been reported in patients with irritable bowel syndrome (Rajilić-Stojanović et al., 2015). Many of the OTUs that were associated with G2 belonged to the undesirable *Enterobacteriaceae* family, which are associated with inflammatory responses and have been identified in patients with ulcerative colitis (Garrett et al., 2010). However, some of the bacteria that drove the shifts in the community during fermentation were members of genera that are generally recognized as beneficial or desirable members of the community, including *Bifidobacterium* and *Roseburia*. Thus, during the course of the fermentation, the substrates resulted in increases in potentially beneficial bacteria in both diet groups, but these genera were different between the two groups.

There was a noticeable lack of effect of the different WG or bran substrates on the overall community (Fig. 4-6). This is in accordance with other studies that claimed that WG substrates with similar compositions fail to induce significant changes in the overall gut microbiota composition but could have targeted effects on specific genera of bacteria (Walter, 2013, Walker et al., 2011).

Significant effects repeatedly occurred based on diet group, grain type and time on α-diversity using as determined by Shannon's and Simpson's indices (Supplementary Table 4-7-3). There was no apparent effect of grain fraction on diversity measures. Overall, α-diversity decreased during fermentation (Fig. 4-6), which is undesirable because it leads to reduced functionality and resilience of the gut microbiota and is associated with many

human diseases (Lloyd-Price et al., 2016). The decrease in diversity in the present study could result from growing a fecal microbial community in an in vitro fermentation system (Possemiers et al., 2004). Importantly, the microbiota from G1 subjects did not experience as a dramatic drop in diversity as the microbiota from G2 subjects during fermentation (Fig. 4-6). This can be related to the behavior of a healthy microbiota, which might have a degree of resilience to many external changes, including dietary change as well as their ability to recover a healthy functional profile following a perturbation. The stability of the microbiota could benefit the host by ensuring that beneficial functions are maintained (Lloyd-Price et al., 2016). Oats resulted in lower diversity than other grains. This could be due to the high concentration of a structurally simple fermentable polysaccharide, β-glucan, which is supported by previous study that have shown reduction in α-diversity of the gut microbiota on β-glucan (Zhong et al., 2015).

Among the dominant genera, the effects of diet group and fermentation time clearly showed the most significant differences among samples (Supplementary Table 4-7-4). On average, G1 microbiota had significantly higher abundances of *Blautia*, *Butyricicoccus*, *Collinsella*, *Coprococcus*, *Erysipelotrichaceae incertae sedis*, *Fecalibacterium*, *Lachnospiracea incertae sedis*, *Streptococcus*, *Subdoligranulum* and *Veillonella*, while G2 had significantly higher abundances of *Akkermansia*, *Alistipes*, *Bacteroides*, *Catenibacterium*, *Clostridium* XlVb, *Enterococcus*, *Klebsiella*, *Leuconostoc*, *Megamonas*, *Oscillibacter*, *Raoultella*, *Slackia* and *Stenotrophomonas* (Table 4-2). During the fermentation, *Bacteroides*, *Bilophila*, *Butyricicoccus*, *Clostridium* XlVa, *Collinsella*, *Dorea*, *Escherichia*/*Shigella*, *Parabacteroides*, and *Phascolarctobacterium* increased independent of diet group, while *Blautia*, *Clostridium* IV, *Clostridium* XlVb,

Desulfovibrio, *Faecalibacterium*, *Lachnospiracea incertae sedis*, *Lactococcus*, *Parasutterella*, *Stenotrophomonas*, *Streptococcus*, and *Syntrophococcus* decreased.

Some of the shifts in the gut microbiota composition were in agreement with results from previous WG *in vivo* trials suggesting that the in vitro system used in our experiments can help predict changes that might occur in the gut microbiota *in vivo*. For instance, *Dorea*, *Collinsella*, and *Bacteroides* increase in different WG *in vivo* studies (Costabile et al., 2008; Lappi et al., 2013). Conversely, losses in abundance in a few genera, such as *Blautia* and *Faecalibacterium*, were not anticipated. These strains may be unable to compete in the in vitro environment due to lack of certain growth factors, but the exact mechanisms leading to their reduction is difficult to elucidate (Krumbeck et al., 2015). It must be further emphasized that the shifts in *Bacteroidetes, Butyricicoccus, Collinesella, Dorea, Blautia and Lachnospiracea incertae sedis* during fermentation are comparable with a previous in vitro fermentation study (Yang, 2015).

Correlations between microbial composition at the genus level and the post-fermentation responses revealed significant associations (Table 4-2). *Bifidobacterium* and *Faecalibacterium* were not only positively correlated with butyrate production but also negatively correlated with ammonia production. Association of *Faecalibacterium* and *Bifidobacterium* with butyrate has been previously reported (Martínez et al., 2013). *Clostridium XVIII*, *Lactobacillus*, and *Turicibacter* were also negatively correlated with ammonia production. Other negative correlations were also detected for products of protein fermentation: iso-valerate (*Bifidobacterium* and *Gemmiger*) and BCFA (*Dialister*). On the other hand, genera such as *Bacteroides*, *Bilophila*, *Coprococcus*, *Desulfovibrio*, *Dorea*, *Escherichia/Shigella*, *Parasutterella*, *Phascolarcbacterium*, and *Roseburia* were

positively correlated with ammonia production. *Bilophila*, *Catenibacterium*, *Clostridium XIVb*, *Eggerthella*, and *Slackia*, also correlated positively with one or both BCFA. The reason could be these bacteria started utilizing proteins to produce protein fermentation metabolites at the end of 24 h. *Clostridium XIVa*, *Gordonibacter* and *Slackia* were positively correlated with fermentation of carbohydrates. As health-promoting bacteria continue to be identified, these data may be useful in selecting a dietary fiber from a WG or bran that could promote the growth of such bacteria.

4.5. Conclusion

This study revealed a substantial effect of diet of the fecal donor relative to their on the microbial composition and functionality by using in vitro fermentation of WG and brans. The microbiota from G1 subjects (with higher diet quality) was associated with higher diversity and abundances of some beneficial microbial genera, including *Faecalibacterium*, compared to G2 subjects. Individual grains did not differ in their impact on the overall microbial community structure during fermentation, but shifts were induced in the microbial community depending on which diet group the fecal inoculum originated. During in vitro fermentation, the microbiota of G1 subjects were better equipped to metabolize the complex carbohydrates in grains than the microbiota from G2 subjects. The microbiota from both groups produced SCFA from carbohydrate fermented with the same efficiency, but the microbiota from G1 subjects produced more butyrate while the microbiota from G2 subjects produced more acetate and propionate. Furthermore, only the microbiota from G1 subjects generated significant differences among grains, generally showing more positive effects in terms of carbohydrate fermentability, high butyrate production, and low ammonia production. Thus, in the

absence of any clinical intervention, habitual diet quality had a dramatic influence on the ability of the gut microbiota to ferment the dietary fibers in grains, differentiate among grains and produce metabolites that are beneficial to human health.

Our study does not necessarily convey that whole grain intake is the reason behind the differences between two groups, but it does support the importance of diet quality in promoting gut health. Furthermore, we expect that these data will provide preliminary evidence for future *in vivo* trials using whole grains and will stimulate commercial interest in development of whole grain products that provide the optimal impact on human health.

4.6. References

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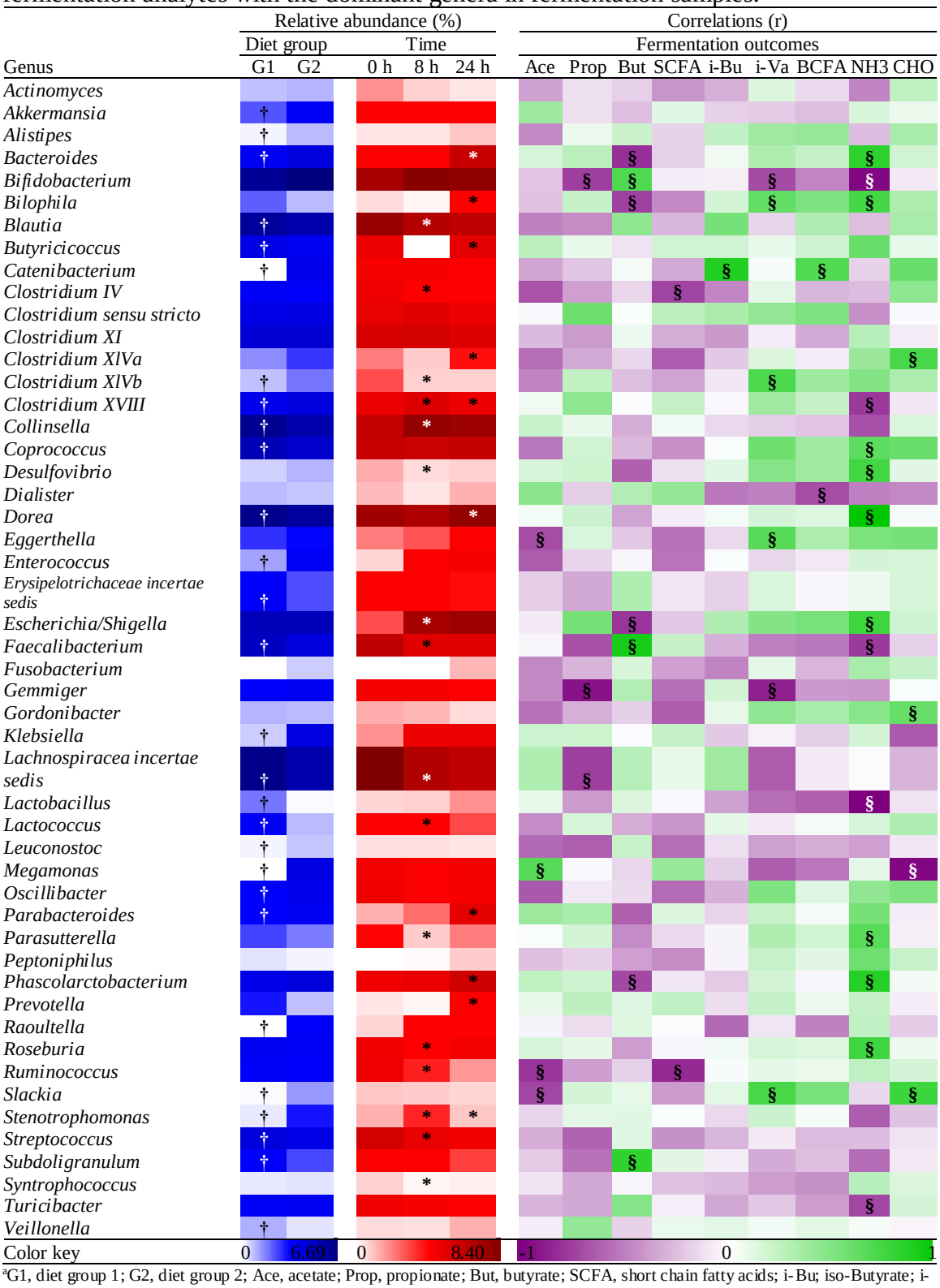
Table 4:1. Composition of grain samples before and after in vitro digestion (% dry basis except moisture, % wet basis).[A]

				Dietary fiber		
Sample	Moisture	Starch	Protein	Soluble	Insoluble	Total
Before in vitro digestion						
Corn flour	10.3±0.6	61.6±2.0	10.7±0.1	0.9±0.4	11.8±0.9	12.7
Oat flour	9.2±0.0	57.3±2.0	15.3±0.6	3.2±0.5	7.8±0.1	11.0
Rye flour	11.5±0.0	56.5±0.1	13.3±0.2	4.4±0.6	10.1±0.1	14.5
Wheat flour	11.6±0.0	52.9±0.7	19.1±0.0	0.7±0.1	11.4±1.7	12.1
Corn bran	7.4±0.0	11.4±0.7	6.12±0.0	1.3±0.5	57.7±1.8	59.0
Oat Bran	8.8±0.2	35.9±2.0	22.5±0.1	7.5±0.6	16.0±1.8	23.5
Rye bran	11.0±0.0	45.7±0.6	17.6±0.2	4.7±0.9	15.0±0.7	19.7
Wheat bran	11.1±0.1	20.8±1.0	26.4±0.1	2.7±0.2	33.8±1.9	36.5
After in vitro digestion						
Corn flour	6.8±1.1	2.8±0.1	24.6±0.1	7.7±0.9	52.1±13.6	59.8
Oat flour	5.9±0.8	3.6±0.5	21.6±0.2	23.0±2.9	41.3±5.3	64.3
Rye flour	7.7±1.2	7.2±0.1	22.1±0.1	25.2±0.2	49.9±0.3	75.1
Wheat flour	5.5±0.9	2.9±0.3	21.4±0.0	10.0±1.5	60.5±0.8	70.5
Corn bran	4.5±0.6	0.4±0.1	7.75±0.2	11.4±1.5	58.0±1.1	69.4
Oat bran	3.9±1.8	2.8±1.1	19.6±1	28.6±1.4	38.5±5.5	67.1
Rye bran	6.2±0.3	2.2±0.4	19.1±0.1	18.5±4.5	51.5±8.4	70.0
Wheat bran	4.6±0.9	1.2±0.3	13.3±0.1	10.6±0.2	53.2±2.4	63.8

[A]Values are mean ± standard deviation (n=2).

Table 4:2. Diet group and fermentation time mean abundances and correlations of fermentation analytes with the dominant genera in fermentation samples. [a]



[a]G1, diet group 1; G2, diet group 2; Ace, acetate; Prop, propionate; But, butyrate; SCFA, short chain fatty acids; i-Bu; iso-Butyrate; i-Va, iso-Valerate; BCFA, branched chain fatty acids; NH3, ammonia; CHO, carbohydrate fermented; †significantly different from G2; *significantly different from the previous time point for that genus; §significant correlation between abundance of the indicated genus and fermentation outcome at 24 h of fermentation ($p<0.05$).

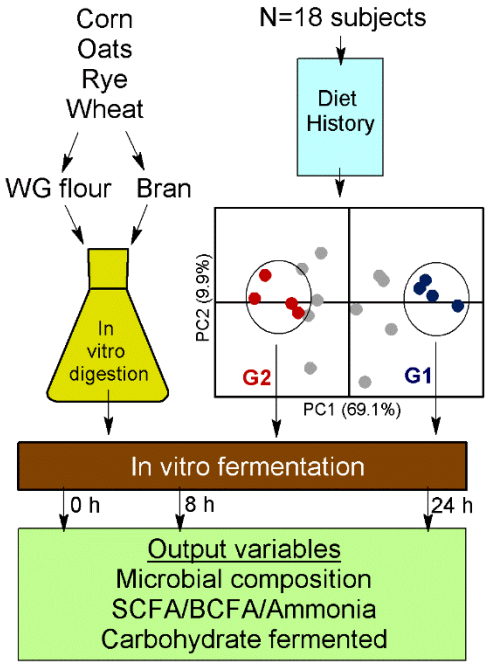


Figure 4-1. Flow chart of the study design; the inset principal components plot was based on subjects' diet history; group 1 (G1) subjects consumed a higher quality diet than group 2 (G2) subjects (S/BCFA = short/branched chain fatty acids).

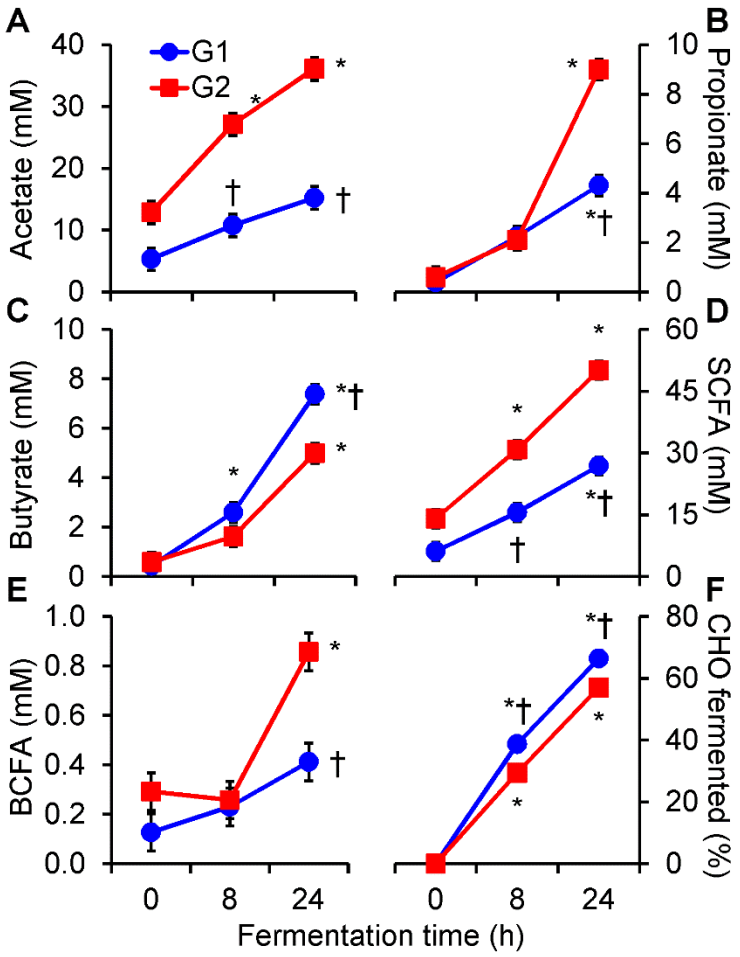


Figure 4-2. Fermentation outcomes with significant group by fermentation time interactions: **A)** acetate; **B)** propionate; **C)** butyrate; **D)** short chain fatty acids (SCFA); **E)** branched chain fatty acids (BCFA); **F)** carbohydrate (CHO) fermented; *significantly different from previous time point; †significantly different from G2 at that time point.

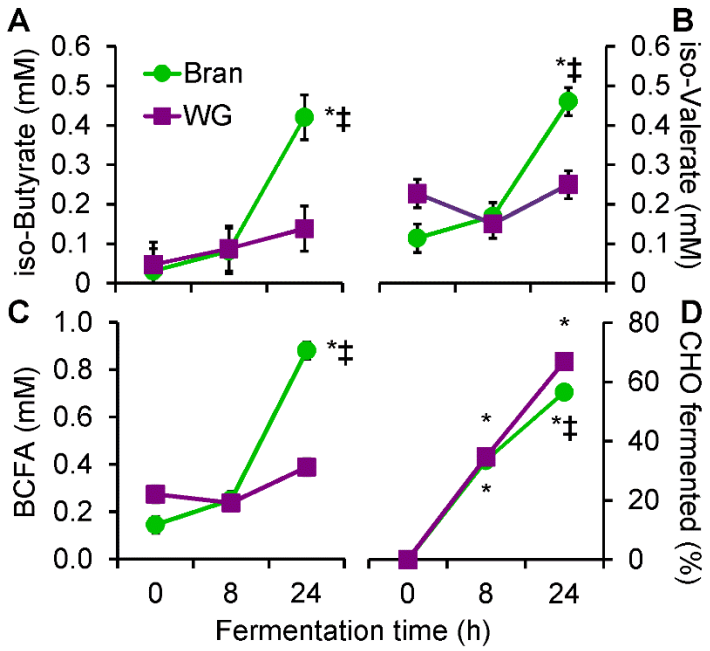


Figure 4-3. Fermentation outcomes with significant grain fraction by fermentation time interactions: **A)** iso-butyrate; **B)** iso-valerate; **C)** branched chain fatty acids (BCFA); **D)** carbohydrate (CHO) fermented; *significantly different from previous time point; ‡significantly different from whole grain (WG) at that time point.

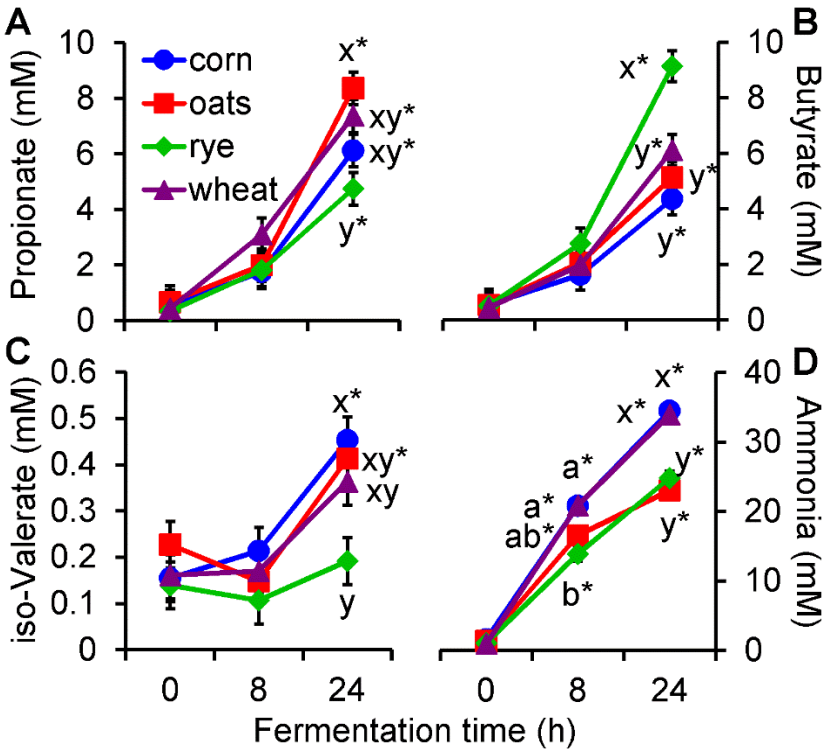


Figure 4-4. Fermentation outcomes with significant differences among grain type by fermentation time: **A)** propionate; **B)** butyrate; **C)** iso-valerate; **D)** ammonia; *significantly different from previous time point; ^{abxy}points marked with different letters are significantly different among grain type within time point.

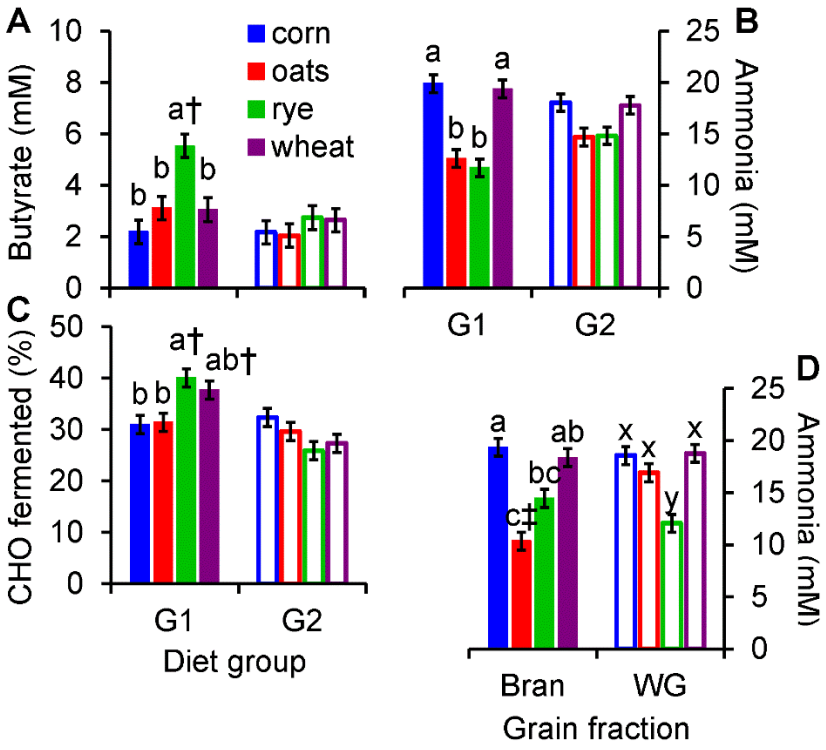


Figure 4-5. Fermentation outcomes with significant diet group by grain type interactions: **A)** butyrate; **B)** ammonia; **C)** carbohydrate fermented; and significant grain fraction by grain type interaction: **D)** ammonia; †significantly different from G2 for that grain type; ‡significantly different from whole grain (WG) for that grain type; [abxy]bars marked with different letters are significantly different among grain type.

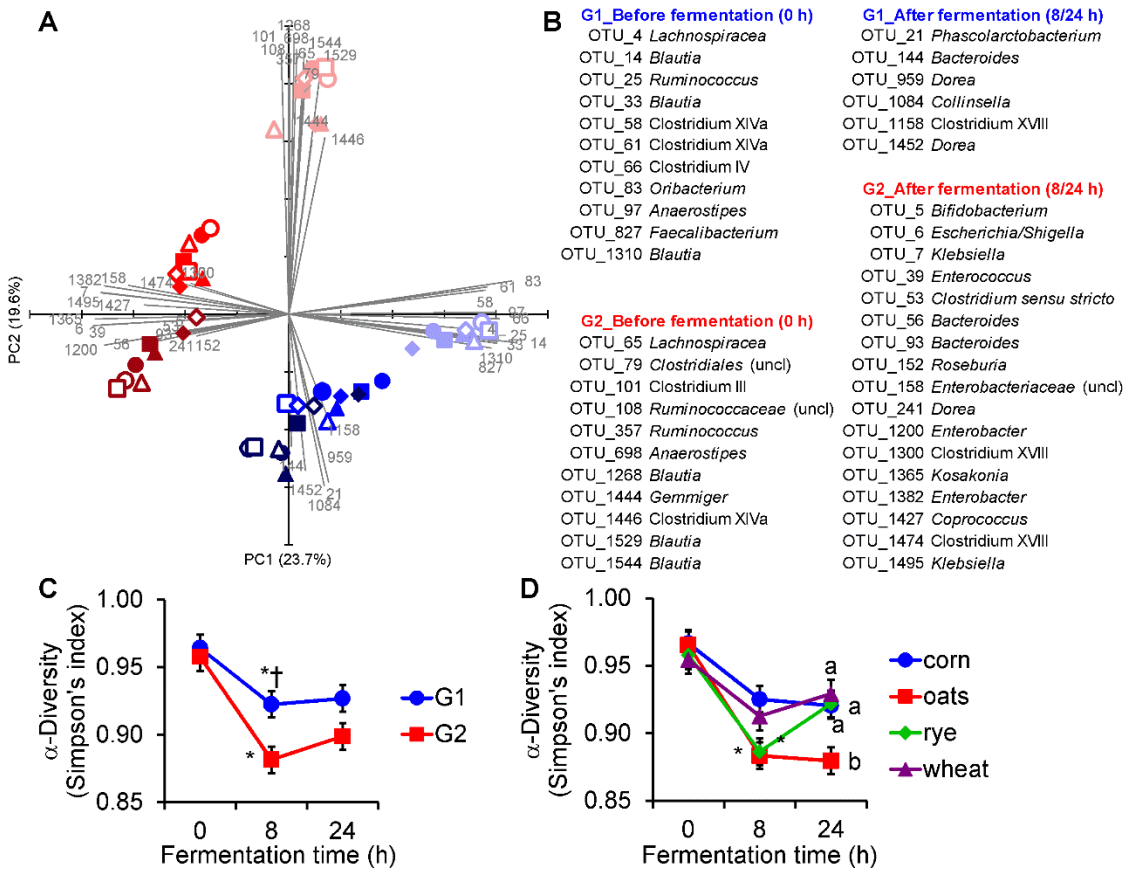


Figure 4-6. Overall bacterial community structure of samples during fermentation; **A)** varimax rotated principal components plot based on average OTU abundance of subjects; shapes represent substrates (circle, corn; square, oats; diamond, rye; triangle, wheat; shaded, bran; open, whole grain); colors represent diet groups (blue, group 1; red, group 2); color shading represented fermentation time (light, 0 h; medium, 8 h; dark, 24 h); Eigenvectors of OTUs that were associated with each diet group are plotted with OTU numbers; **B)** assignments of OTUs appearing in A; α-diversity plots of **C)** diet group and **D)** grain type by fermentation time; †significantly different from G2; *significantly different from the previous time point for that sample; [ab]points marked with different letters are significantly different among grain type within time point.

4.7. Supplementary Materials

Supplementary Table 4-7-1. Age, gender, body mass index (BMI), and nutrient intakes of fecal donors. [A]

		G1		G2		
Nutrient	Unit	Mean	Range	Mean	Range	p-value
Age	y	27.75	21–33	24.75	23–27	0.386
Gender	M/F	3/1		2/2		0.465
Body mass index	kg/m^2	23.78777	19.8–30.7	24.00387	20.6–26.5	0.941
Total carbohydrate	g	315.45	173–408	173.1375	135–219	0.040
Dietary fiber	g	31.3075	24.7–37.1	10.945	7.75–15.2	0.001
Soluble fiber	g	12.1375	7.37–20.0	3.4575	2.39–4.75	0.033
Insoluble fiber	g	19.15	12.6–25.7	7.45	5.12–10.4	0.009
Protein	g	109.015	89.3–127	71.31	63.4–83.1	0.014
Vegetable protein	g	42.325	34.6–62.7	15.355	13.0–18.1	0.008
Thiamin	mg	2.8825	2.51–3.72	1.195	1.05–1.26	0.001
Riboflavin	mg	4.755	4.20–5.38	2.11	1.92–2.33	0.000
Niacin	mg	41.995	33.4–47.8	16.9325	12.2–19.1	0.000
Vitamin B-6	mg	3.8	3.28–4.25	1.68	1.29–2.03	0.000
Folate	µg	781.5375	671–1065	320.405	227–382	0.004
Vitamin B-12	µg	10.41	7.88–12.8	6.8075	5.60–9.34	0.041
Copper	mg	1.855	1.55–2.37	1.185	1.00–1.35	0.015
Iron	mg	30.18	23.6–44.8	12.005	9.26–16.1	0.012
Magnesium	mg	507.74	437–648	250.3875	192–280	0.003
Phosphorous	mg	2099.073	1437–2718	1172.025	1091–1269	0.017
Potassium	mg	4415.708	4024–4793	2484.533	1894–3044	0.001
Selenium	µg	147.82	123–185	92.925	79.3–101	0.013
Sodium	mg	3870.22	2368–4481	2204.75	2010–2382	0.017
Zinc	mg	19.9475	17.1–25.2	10.8875	9.46–14.7	0.006
Phytic acid	mg	1153.583	805–1797	364.1325	246–464	0.013
Choline	mg	470.015	439–505	338.1875	296–384	0.002

[A] Only nutrients with significant differences are shown; other nutrients analyzed included energy, fat, saturated fat, trans fat, monounsaturated fat, polyunsaturated fat, ω-3 fatty acids, cholesterol, available carbohydrate, total sugars, added sugars, fructose, sucrose, animal protein, vitamin A, vitamin D, vitamin E, vitamin K, vitamin C, calcium, manganese, alcohol, caffeine, oxalic acid, and betaine; [B] t-test except for gender where a chi squared test was used.

Supplementary Table 4-7-3. ANOVA results (F-values) for microbial diversity variables.[1]

Effect	Shannon's index	Simpson index	Observed species
D	8.36*	18.9**	1.34
P	4.84	3.2	2.74
G	3.47*	4.57*	1.52
T	59.9***	38.7***	16.2***
D*T	1.37	3	1.27
P*T	2.56	1.38	0.92
G*T	2.04	2.47*	1.12
D*P	0.02	0	0.19
P*G	1.94	2.19	1.16
D*G	0.51	0.37	0.06
D*P*G	0.54	0.32	0.46
D*P*T	1.56	1.1	0.95
D*G*T	0.38	0.71	0.79
P*G*T	1.14	1.6	0.57
D*P*G*T	0.14	0.73	0.5

[1] D, diet group; P, flour/bran; G, grain type; T, time; CHO, carbohydrate fermented; *p<0.05; **p<0.01; ***p<0.001

Supplementary Table 4-7-4. ANOVA results (F-values) for dominant 50 genera.[1]

Effect	Actinomyces	Akkermansia	Alistipes	Bacteroides	Bifidobacterium	Bilophila	Blautia	Butyricicoccus	Catenibacterium	Clostridium IV	Clostridium sensu stricto	Clostridium XI
D	1.62	7.73*	14.56**	12.51*	3.29	5.87	12.99*	14.76**	23.95**	4.16	0.16	0.06
F	1.28	1.75	5.81	0.17	0.85	2.59	5.8	2.06	0.14	1.98	3.49	0.11
G	0.34	0.79	0.12	1.23	3.07	5.29**	1.52	1.04	0.16	0.79	1.14	0.28
T	24.67***	0.19	2.52	55.31***	3.09	20.86***	21.33***	15.11***	0.4	23.51***	1.74	1.03
D*T	1.66	0.96	0.14	4.65*	1.67	4.64*	0.19	0.57	0.4	1.15	1.13	1.55
F*T	0.5	0.08	0.65	0.69	0.06	2.4	1.59	4.36*	0.06	1.11	0.58	0.61
G*T	0.82	0.08	0.36	1.88	0.32	5.85***	1.9	0.61	0.36	0.35	0.37	0.21
D*F	0	1.48	3.46	0.79	0.24	0.1	0.32	3.58	0.14	0.16	0	0.32
F*G	0.27	0.65	0.51	0.68	0.44	2.65	0.1	0.5	0.16	0.1	0.16	0.52
D*G	0.28	0.11	0.35	1.59	0.37	0.39	0.28	1.27	0.24	1.83	0.28	0.88
D*F*G	0.13	0.19	0.03	0.46	0.15	0.77	0.1	0.37	0.24	0.1	1.22	0.3
D*F*T	0.03	0.02	0.6	0.14	0.17	0.03	0.2	1.65	0.06	0.46	0.38	0.02
D*G*T	0.23	0.08	0.51	0.46	0.16	2.51*	0.45	0.33	0.36	0.2	0.44	0.2
F*G*T	0.46	0.12	0.31	1	0.27	0.47	0.48	0.62	0.29	0.14	0.14	0.41
D*F*G*T	0.13	0.13	0.18	0.18	0.08	0.79	0.34	0.5	0.29	0.07	0.07	0.15

[1] D, diet group; F, grain fraction; G, grain type; T, time; CHO, carbohydrate fermented; *p<0.05; **p<0.01; ***p<0.001

Supplementary Table 4-7-4 (continued). ANOVA results (F-values) for dominant 50 genera.[1]

Effect	Clostridium XIVa	Clostridium XIVb	Clostridium XVIII	Collinsella	Coprococcus	Desulfovibrio	Dialister	Dorea	Eggerthella	Enterococcus	Erysipelotrichaceae incertae sedis	Escherichia/ Shigella	Faecalibacterium
D	4.47	7.37*	9.26*	15.07**	10.15*	3.66	0.32	7.26*	0.54	12.44*	7.07*	0.03	21.84**
F	3.72	0.36	0.32	0.01	2.57	1.22	0.74	0.07	6.46*	0.19	0.51	7.1*	0.15
G	3.98*	0.38	3.08	0.37	1.38	1.17	0.05	3.5*	1.82	0.01	0.36	6.35**	2.02
T	8.58**	12.37**	5.35*	17.28***	0.33	4.62*	1.87	5.64*	4.27*	4.89*	0.32	78.3***	17.87***
D*T	1.74	3.19	0.86	1.11	0.85	1.71	0.01	3.55	2.52	2.27	0.75	1.17	0.96
F*T	3.41	0.64	0.77	0.07	1.74	0.64	0.62	2.56	1.95	0.13	0.23	1.9	0.55
G*T	5.05***	0.4	1.36	0.67	0.34	0.79	0.32	1.07	0.52	0.04	0.12	1.68	1.4
D*F	0.68	0.13	0.17	0	0	0.27	0.14	0.44	0.1	0.14	0.05	4.19	0.36
F*G	1.06	0.55	0.48	0.25	0.18	0.76	0.21	0.14	0.43	0.24	0.16	1.6	1.69
D*G	5.52**	0.12	0.51	0.22	0.61	0.03	0.33	1.6	1.66	0.06	0.46	2.39	0.44
D*F*G	2.6	0.47	0.06	0.04	0.33	0.11	0.35	0.61	0.31	0.17	0.17	1.86	0.4
D*F*T	0.77	1.06	0.45	0.76	0.15	0.19	2.03	0.09	0.93	0.42	0.33	2.52	0.22
D*G*T	1.58	0.27	0.53	0.13	0.09	0.3	0.6	0.26	0.13	0.12	0.06	0.52	0.18
F*G*T	5.68***	0.23	0.18	0.4	0.36	0.23	0.4	0.71	0.26	0.1	0.09	1.13	0.54
D*F*G*T	2.53*	0.17	0.12	0.59	0.31	0.11	0.26	0.39	0.22	0.12	0.11	0.47	0.15

[1] D, diet group; F, grain fraction; G, grain type; T, time; CHO, carbohydrate fermented; *p<0.05; **p<0.01; ***p<0.001

Supplementary Table 4-7-4 (continued). ANOVA results (F-values) for dominant 50 genera.[1]

Effect	Fusobacterium	Gemmiger	Gordonibacter	Klebsiella	Lachnospiracea incertae sedis	Lactobacillus	Lactococcus	Leuconostoc	Megamonas	Oscillibacter	Parabacteroides	Parasutterella
D	2.27	4.15	0.05	14.93**	13.47*	10.53*	83.75***	20.52**	22.1**	16.8**	10.23*	1.07
F	1.8	0.46	2.58	0.11	0.08	0.64	0.04	0.33	0.01	2.14	0.59	0.39
G	0.92	0.92	0.92	0.03	0.35	1.29	0.44	0.73	0.15	1.83	1.99	1.01
T	2.38	3.04	5.33*	3.01	20.92***	1.49	9.89**	0.16	0.31	2.29	86.87***	5.19*
D*T	2.35	0.02	0.91	2.54	2.43	2.01	4.91*	0.16	0.31	0.46	3.41	0.26
F*T	1.87	0.03	1.38	0.09	0.11	0.46	0.01	0.75	0.23	3.18	1.59	0.03
G*T	0.89	0.16	1.17	0.1	0.45	1.1	0.18	0.12	0.1	0.45	3.24*	0.87
D*F	1.91	0.62	0.57	0.04	0.03	0.91	0.35	0.55	0.01	0.01	0.89	0.22
F*G	0.86	0.32	1.01	0.05	0.06	1.12	0.15	0.52	0.15	0.15	1.73	0.27
D*G	0.71	0.12	2.33	0.05	0.16	1.98	0.05	0.27	0.15	2.82	4.89*	0.48
D*F*G	0.74	0.3	0.81	0.04	0.14	1.85	0.08	0.4	0.15	0.46	1.2	0.04
D*F*T	1.95	0.01	0.25	0.09	0.06	0.32	0.38	0.38	0.22	0.34	1.64	0.01
D*G*T	0.88	0.22	0.35	0.1	0.15	1.2	0.18	0.04	0.1	0.18	1.22	0.04
F*G*T	0.72	0.18	0.19	0.09	0.05	1.82	0.07	0.27	0.21	0.32	5.23***	0.55
D*F*G*T	0.75	0.07	0.15	0.09	0.16	1.88	0.07	0.09	0.21	0.15	1.08	0.17

[1] D, diet group; F, grain fraction; G, grain type; T, time; CHO, carbohydrate fermented; *p<0.05; **p<0.01; ***p<0.001

Supplementary Table 4-7-4 (continued). ANOVA results (F-values) for dominant 50 genera.[1]

Effect	Peptoniphilus	Phascolarcto-bacterium	Prevotella	Raoultella	Roseburia	Ruminococcus	Slackia	Stenotrophomonas	Streptococcus	Subdoligranulum	Syntrophococcus	Turicibacter	Veillonella
D	0.35	2.89	2.16	15.74**	3.2	1.31	32.37**	28.98**	9.79*	31.87**	0.03	0.61	7.39*
F	2.56	0.73	0.01	0.01	0.03	0	0.04	2.31	0.74	0.12	0.9	0.66	4.2
G	2.11	1.28	0.66	0.09	1.34	0.5	0.82	0.63	0.62	0.5	0.92	0.57	0.65
T	2.27	8.13**	4.78*	2.59	4.91*	29.04***	0.31	7.81**	27.14***	5.81*	17.66***	8.96**	2.99
D*T	0.46	0.9	3.08	2.58	3.71	0.1	0.42	5.19*	1.33	0.04	0.3	2.56	2.7
F*T	2.43	0.03	0.04	0.03	0.65	0.68	0.93	0.26	0.44	0.27	0.93	0.06	1.56
G*T	1.97	0.59	0.7	0.14	0.79	0.3	0.16	0.52	0.3	0.61	0.4	0.26	1.04
D*F	0.44	0.19	0.5	0.01	0.94	0	0.04	2.36	1.88	0.05	1.83	0.39	0.54
F*G	0.4	0.03	0.62	0.09	0.42	0.37	0.67	0.51	0.57	0.1	0.99	0.46	0.37
D*G	2.07	0.26	0.36	0.07	0.22	0.33	0.72	0.29	0.23	0.77	0.54	0.01	0.74
D*F*G	0.37	0.14	0.45	0.07	0.22	0.16	0.61	0.39	0.13	0.17	0.37	0.14	0.49
D*F*T	0.36	0.1	0.4	0.03	0.17	0.11	0.64	0.24	0.17	0	0.86	0.32	0.74
D*G*T	0.45	0.02	0.62	0.14	0.24	0.19	0.18	0.51	0.08	0.25	0.58	0.17	1.02
F*G*T	1.94	0.19	0.33	0.09	0.25	0.15	0.29	0.19	0.3	0.1	0.15	0.16	0.37
D*F*G*T	0.45	0.19	0.49	0.09	0.11	0.11	0.25	0.23	0.24	0.09	0.18	0.14	0.46

1 D, diet group; F, grain fraction; G, grain type; T, time; CHO, carbohydrate fermented; *p<0.05; **p<0.01; ***p<0.001

Chapter 5 . Overall Conclusions

Whole grains are rich sources of fibers and phytochemicals in our diet and the structural diversities make the whole grain carbohydrates an excellent resource for the human intestinal microbiota. However, for the dietary fibers to support a healthy gut microbiome, they must be accessible for metabolism by the gut microbiota. Unfortunately, microbiota accessible carbohydrates (MAC) are very low in whole grains. Although food and diet have been strongly associated with health and disease, a critical knowledge gap exists on

- how diet shapes the bacterial populations
- the specific bacteria that may be enriched to support host health,
- processing-associated changes in whole grain foods/diet that could benefit gut health
- identifying new areas of research which may contribute to a better understanding of the underlying mechanisms of whole grain-gut microbiota interactions linked to human host health.

The following chapters helped answer some of these questions.

Chapter 1 reviewed the impact of whole grain foods and components of whole grain intervention studies on human metabolic health and the gut microbiota. Answers to these questions are important to establish the fundamentals to develop whole grain based food products that can modulate the human gut microbiota. Chapter 2 identified the effects extrusion processing conditions can influence the physicochemical properties and digestion profiles of starch in whole grain oats. Moderate extrusion screw speed tended to increase SDS and diminish RDS, whereas moisture contents significantly affected both RS and extractable β-glucan concentration, with the lower moisture content tending to

increase both. This strategy implied that extrusion may enhance the proportion of β-glucan dietary fiber and RS available for fermentation by the microbiota. Moreover, increased SDS and diminished RDS could indicate that oats extruded under moderate screw speed may have potential lower glycemic index. As extrusion moisture was determined to play a critical role in increasing the fractions of resistant starch (RS), slowly digestible starch (SDS) as well as water-extractable β-glucan in extruded whole grain oats, Chapter 3 reported the impact of extrusion moisture on in vitro fermentation characteristics of extruded whole oats. The significance of this study was that information was obtained on the extrusion moisture and its impact on the production of acetate, butyrate and total SCFA during the initial stages of fermentation and affecting the probiotic *Bifidobacterium* and *Lactobacillu*s counts during extended fermentation. In Chapter 4, different whole grains and brans as substrates for in vitro fermentation to establish whether dietary habits of a fecal donor impacted gut microbial composition and their functionality. This study demonstrated that the microbiota from subjects with higher habitual diet quality had the ability to efficiently degrade the dietary fibers in grains, differentiate among grains and produce beneficial metabolites linked to human health.

In summary, the studies presented this dissertation shows that whole grains are versatile substrates to increase the rate of fermentation in the human gut and produce beneficial metabolites linked to human health. Based on the accumulated data, it was also determined that a high habitual diet quality must be maintained to support the growth of microbiota that are more capable in fermenting the non-digestible carbohydrates to promote gut health. Thus, it is expected that the information provided by these studies

will stimulate commercial interest in the development of whole grain based products with the most impact on human health.

Appendix

Appendix 1. Impact of various treatments on *in vitro* fermentation characteristics of soluble carbohydrates by human fecal microbiota

1. Abstract

A well-preserved or well-processed fecal sample or inoculum is critical to execute an in vitro fermentation experiment. Both the storage and freshness of fecal sample necessary to complete multiple studies with the same microbiota for a wide variety of substrates over a long period. Thus, the objective of this research was to identify the effects of different conditions, such as before/after storage, pooled/unpooled stool samples, and treatment with/without glycerol, on in vitro fermentability of a mixture of soluble carbohydrates (pectin, arabinogalactan, xylan and waxy corn starch). Before and after storage, samples affected carbohydrate fermentability after 24 h fermentation and the production of majority of metabolites, such as acetate, propionate and total SCFA after 8 h fermentation, but not after 24 of fermentation. After 8 h fermentation, microbiota from pooled fecal samples produced more propionate than the unpooled samples, whereas after 24 h fermentation, microbiota from unpooled samples produced more butyrate than the pooled. Samples treated with/without glycerol did not significantly influence either carbohydrate fermentability or production of metabolites. Thus, freezing at -80 °C could be one of the best practices to preserve the human stool samples for in vitro fermentation studies, however microbial analysis is needed to confirm our results.

2. Introduction

The composition of the gut microbiota is critical in understanding the relationship between human health and disease. Compelling evidence has been reported

that bacterial colonization plays a central role in the development and regulation of the host immune system (Hansen et al., 2014), and metabolic activity (Aguirre et al., 2016) with the disruption of the static microbial balance leading to a phenomenon, referred to as dysbiosis (Choo et al., 2015). Dysbiosis has been associated with obesity, chronic gastrointestinal inflammatory diseases, Type I and II diabetes and carcinogenesis (Bäckhed et al., 2004; Boulangé et al., 2016; Harley & Karp, 2012). In addition, the microbiota also plays an important metabolic function by assisting in the extraction of energy and nutrients, such as short chain fatty acids (SCFA) and amino acids from non-digestible carbohydrates in the diet (Carding et al., 2015). Food composition therefore affects the fermentation characteristics, which in turn impacts the composition and activity of the gut microbiota as shown by multiple studies using a variety of substrates (Brahma et al., 2017; Dura et al., 2017; Karataş et al., 2017; Saman et al., 2017; Yang et al., 2013; Yang & Rose, 2014, Vanegas et al., 2017; Vetrani et al., 2016).

However, most of the studies were performed in an in vitro setting as this approach lends itself to flexibility in experimental design and cost-effectiveness compared to human clinical trials. Hence, to perform reproducible and reliable experiment data, a well-preserved or well-processed fecal sample or inoculum is critical to execute multiple in vitro fermentation experiments using the same microbiota for a wide variety of substrates over a long period (Aguirre et al., 2015). Only limited in vitro studies have addressed changes in microbial activity and composition induced by storage of fecal microflora (Aguirre et al., 2015; Choo et al., 2015; Fouhy et al., 2015; Gaci et al., 2017; Hubálek, (2003); Metzler-Zebeli et al., 2016; Prates et al., 2010; Rose et al., 2010; Tedjo et al., 2015). Aguirre et al. (2015) studied the effects of four treatments of human

feces as inoculum for in vitro fermentation, as an alternative to fresh fecal samples. The first treatment, which was used as a reference, consisted of fresh feces resuspended in dialysate solution + glycerol; the second treatment was fresh feces resuspended in dialysate solution+ glycerol and then stored at−80 °C; the third treatment was fecal sample frozen with 1.5 g glycerol and the last treatment was frozen fecal samples. The authors reported that SCFA production by the microbiota was significantly affected by the various treatments. Finally, the authors concluded that fresh feces resuspended in dialysate solution+ glycerol and then stored at−80 °C could be used as a substituent to fresh feces for in vitro fermentation studies, as the results obtained from both the treatments showed high similarities. Hubálek. (2003) and Prates et al. (2010) also recommended the use of glycerol when processing and storing stocks of bacterial cultures, as glycerol acts as a cryoprotectant which has the potential to preserve the viability of the cells. Thus, the purpose of this study was to determine the effects of various handling conditions of fecal samples, that included before/after freezing storage, pooled/unpooled and treatment with/without glycerol affected, on in vitro human fecal fermentation properties of soluble carbohydrates.

2. Materials and methods

2.1. Collection and processing of stool samples

Stool samples were collected fresh from three healthy individuals with no record of gastrointestinal abnormalities or antibiotic administration in the last 6 months. Four fecal slurries were prepared, the first three using the stool samples from each of the three individuals and the forth was a pooled, which consisted of equal weights of stool sample from all the three individuals and mixed together. The slurries were prepared with

the sterile phosphate buffered saline (PBS, pH 7.0) with and without 10% glycerol, in the ratio 1:9 (w/v) using a hand blender for 1 min and then filtered through four layers of cheesecloth. Samples with PBS containing 10% glycerol were stored -80 ℃ for 10 weeks.

2.2. Preparation of substrates

Pectin citrus powder (Alfa Aesar, Ward Hill, MA), Arabinogalactan from Larch wood (TCI America, Portland, OR), Xylan from Beech wood (TCI America, Portland, OR) and waxy corn starch were mixed in equal proportions to 200 mL water to make a solution of soluble substrates (2% of total carbohydrates). The solution was autoclaved to avoid precipitation of substrates and was used directly in the in vitro fermentation system without conducting the in vitro digestion step.

2.3. In vitro fermentation

Separate in vitro batch fecal fermentations were performed using fresh fecal slurries and after 10 weeks using stored fecal slurries, as per the methods described by Arcila et al. (2015) with modifications. For each fermentation experiment, all the tubes contained 0.5 mL of each of carbohydrate solution and the media. The tubes were separated based on the 0.1 mL inoculation of fecal slurry containing PBS with and without 10% glycerol. The substrate solution was not hydrated with the media overnight because it was completely soluble. The 2X fermentation medium was prepared with (per L) peptone (4 g; Fisher Scientific, Pittsburgh, PA USA), yeast extract (4g; Alfa Aesar, Ward Hill, MA USA), bile salts (1.0 g; Oxoid), NaHCO3 (4 g), NaCl (0.2 g), K2HPO4 (0.16 g), MgSO4·7H2O (0.02 g), CaCl2·6H2O (0.02 g), L-cysteine hydrochloride (1.0 g; Sigma), hemin solution (2 mL; 0.025 g in 5 mL of 1 M NaOH; Sigma), Tween 80 (4

mL), vitamin K (20 µL; 0.1 g in 9.9 mL of ethanol and mixed thoroughly; Sigma), and 0.025% (w/v) resazurin solution (8 mL). The pH of the media was adjusted to 6.8 and then filtered to sterilize before transferring to the anaerobic hood. All the steps prior to inoculation, capping and transferring the tubes to vial-storage cardboard boxes, were carried out inside an anaerobic hood (Bactron X, Sheldon manufacturing, Cornelius, Oregon USA) containing 5% H2, 5% CO2, and 90% N2. The tubes were then immediately taken out of the hood and transferred to an anaerobic incubator set at 37 °C with orbital shaking (125 rpm) for 24 h. Samples were collected after 0, 8 and 24 h of fermentation and were immediately transferred on ice to a refrigerated centrifuge at 8000 g for 5 min to separate the pellets and the supernatant. Both the fractions were collected in separate tubes and were stored at -80 °C until further analysis.

2.4. Fermentation analysis

For the analysis of total carbohydrates, frozen supernatants were thawed and analyzed for soluble carbohydrates. In short, microcentrifuge tubes containing 200 µL of supernatant was combined with 200 µL of myo-inositol (1 mg/ml containing 3% antifoam B) and 200 µL of 1.2 M sulfuric acid; covered with aluminum foil and pressure cooked on HIGH (15 psi) for 1 h to form the hydrolysate syrup. The remaining steps were similar to the approved method 32-25.01, AACC International, 2016. For the analysis of S/BCFA, 0.4 mL of supernatant was used for quantification by gas chromatography (Arcila et al., 2015).

2.5. Data analysis

Fermentation responses (SCFA, carbohydrate fermented) were analyzed using a three factor (pooled/unpooled, before/after storage, glycerol/non-glycerol) repeated

measures (fermentation time) ANOVA using SAS software (version 9.4, SAS Institute, Cary, NC, USA). At each time point, differences between sample means were calculated by Fisher's least significant difference, and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Effects of treatment factors on carbohydrate fermentability

Samples treated/not treated with glycerol or pooled/unpooled faecal samples did not significantly affect the percentage of carbohydrates utilized at the end of fermentation (Fig.1 A & B). However, a significant difference occurred when the fecal samples were stored before and after the end of fermentation. More specifically, carbohydrate fermentability measured in fecal samples before storage were higher than the after-storage samples (Fig. 1C). The fresh fecal samples were treated with buffer, made into a slurry and used as substrates for fermentation and the same slurries were stored at -80 °C and further used as substrates for fermentation after 10 weeks, instead of using the stored raw fecal samples and processed as before to represent the similar processing techniques for both before and after storage samples. Another explanation for these results is that the soluble carbohydrates were used as the fermentation substrates making it possible for utilization of soluble fibers by microbiota in the first half of fermentation followed by metabolizing the substances already present in the post-fermentation media. Higher carbohydrate fermentability could be attributed to higher microbiota abundance and diversity (Sonnenberg & Sonnenberg, 2014). Hsieh et al. (2016) reported that certain taxa could be at risk due to under or over sampling of protocol differences. Although controversial studies claim that relative proportions and absolute abundances of gut microbial community are altered by freezing storage (Bahl et

al., 2012; Cardona et al., 2012), while others claim no or little impact of frozen conditions on gut microbial community (Carroll et al., 2012; Fouhy et al., 2015). For instance, Metzler-Zebeli et al. (2016) reported that freezer-stored fecal samples from pigs resulted in lesser abundance of total bacteria compared to the fresh fecal samples. However, other researchers also concluded that their results could only partly be applied to human stool samples. Cardona et al. (2012) indicated that random shearing and fragmentation could occur during freezing storage, which might affect *Bacteroidetes, Firmicutes, Actinobacteria* and *Proteobacteria* abundances in human stool samples, as was evident from next-generation sequencing (*i.e.*, 454-sequencing) analysis. This trend could also explain the high carbohydrate fermentability from fecal samples before storage compared to after storage, as observed in the current study.

3.2. Effects of treatment factors on SCFA production

Pooled/unpooled significantly affected the production of propionate and butyrate after 8 and 24 h fermentation, respectively (Fig. 3 & 4C). After 8 h fermentation, microbiota from pooled fecal samples produced more propionate than the unpooled samples, whereas after 24 h fermentation, microbiota from unpooled samples produced more butyrate. Although studies have shown that pooled fecal samples for in vitro studies can provide a representative and reproducible bacterial community to that of fecal samples from individual, these two factors can also account for significant changes in the functional aspect of the gut microbiota (Aguirre et al., 2014; McDonald et al., 2013; Venema et al., 2003). Variations in production of microbial metabolites could be attributed to either the composition of the gut microbial community present in the combined feces or could be due to metabolic cross-feeding occurring during in vitro

systems (Kovatcheva-Datchary et al., 2009; Maathuis et al., 2012; Wintermute & Silver, 2010). No significant effects of the treatment with/without glycerol were apparent on SCFA production (Fig. 2, 3 & 4B). In particular, microbiota from before/after storage samples significant effected the production of acetate, propionate and total SCFA. This trend agrees with the data from carbohydrate fermentability, where higher carbohydrate was utilized from before storage samples than after storage. At the end of 8 h fermentation, microbiota from before storage samples produced more acetate, propionate and total SCFA than that of after storage samples. Interestingly, after 24 h fermentation, there was no difference in the production of acetate, butyrate and total SCFA between before and after storage samples with the notable exception of propionate (Fig. 2, 3 & 4A).

4. Conclusions

This study showed that before and after storage samples could affect carbohydrate fermentability after 24 h fermentation, which, in turn, may also impact the production of majority of metabolites such as acetate, propionate and total SCFA after 8 h fermentation, but not after 24 of fermentation, which indicates that freezing at -80 °C could be one of the best practices to preserve the human stool samples for in vitro fermentation studies. After 8 h fermentation, microbiota from pooled fecal samples produced more propionate than the unpooled samples, whereas after 24 h fermentation, microbiota from unpooled samples produced more butyrate. Samples treated with/without glycerol did not significantly influence either carbohydrate fermentability or production of metabolites. However, microbial analysis needs to be further performed to draw definitive conclusions.

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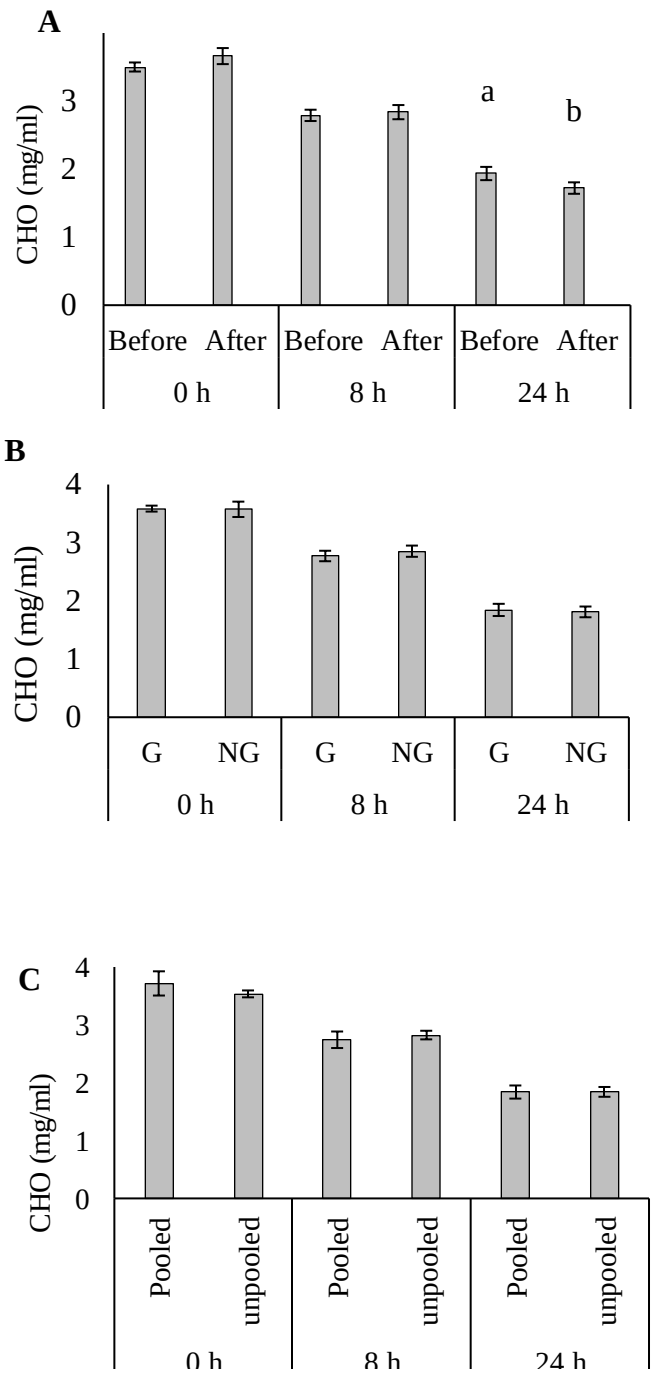

Figure 1. Means of significant treatment effects [before/after (A), glycerol/non-glycerol (B), pooled/unpooled (C)] on carbohydrate fermentability of soluble substrates; error bars show standard error (n=2); bars marked with different letters are significantly different ($p<0.05$).

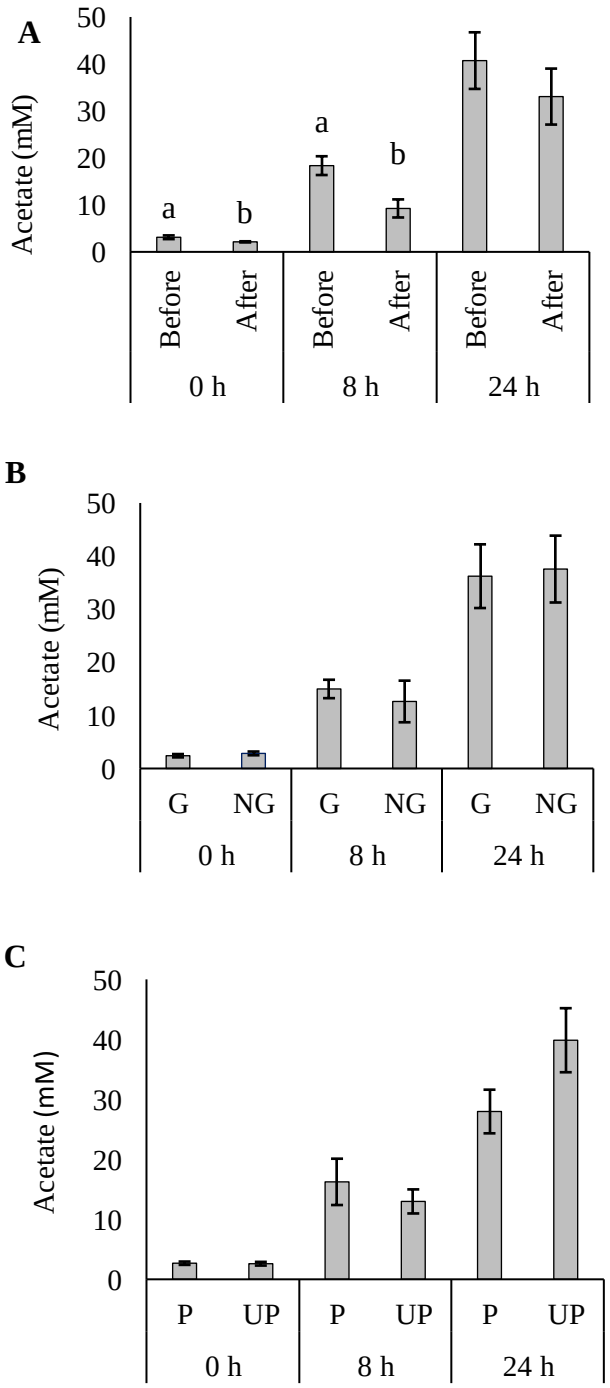

Figure 2. Means of significant treatment effects [before/after (A), glycerol/non-glycerol (B), pooled/unpooled (C)] on acetate production; error bars show standard error (n=2); bars marked with different letters are significantly different ($p<0.05$).

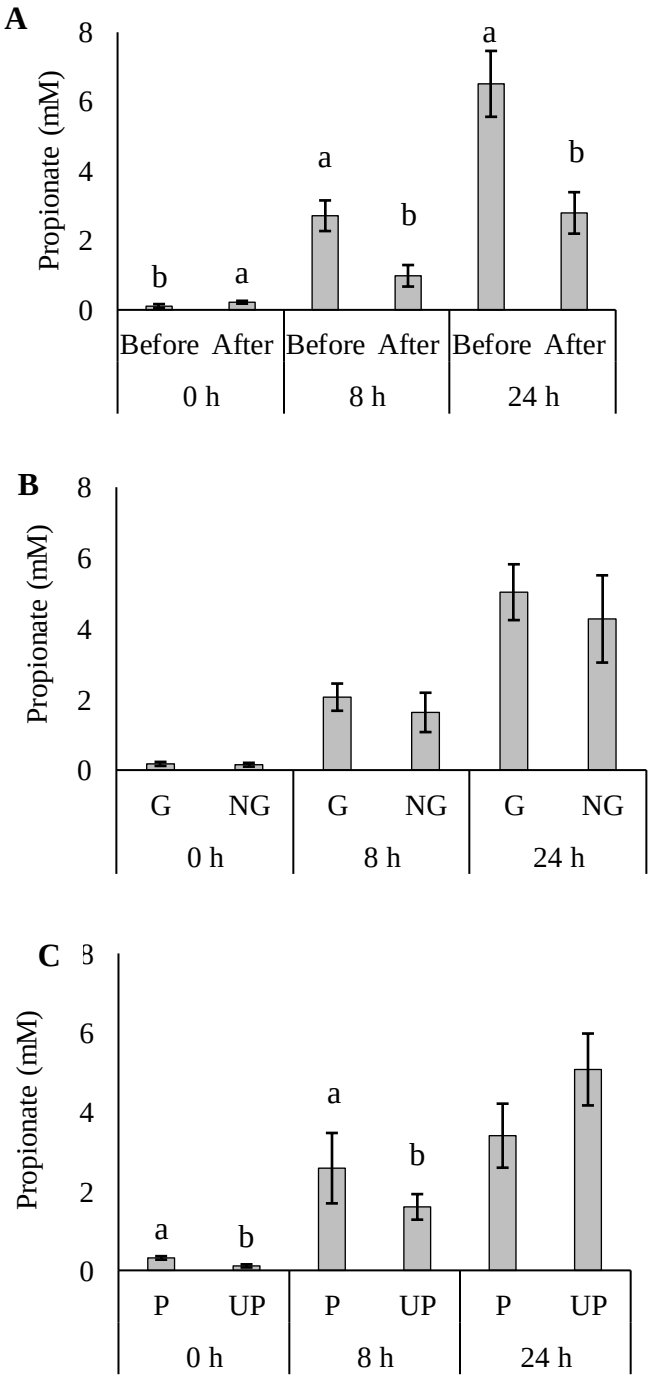

Figure 3. Means of significant treatment effects [before/after (A), glycerol/non-glycerol (B), pooled/unpooled (C)] on propionate production; error bars show standard error (n=2); bars marked with different letters are significantly different ($p<0.05$).

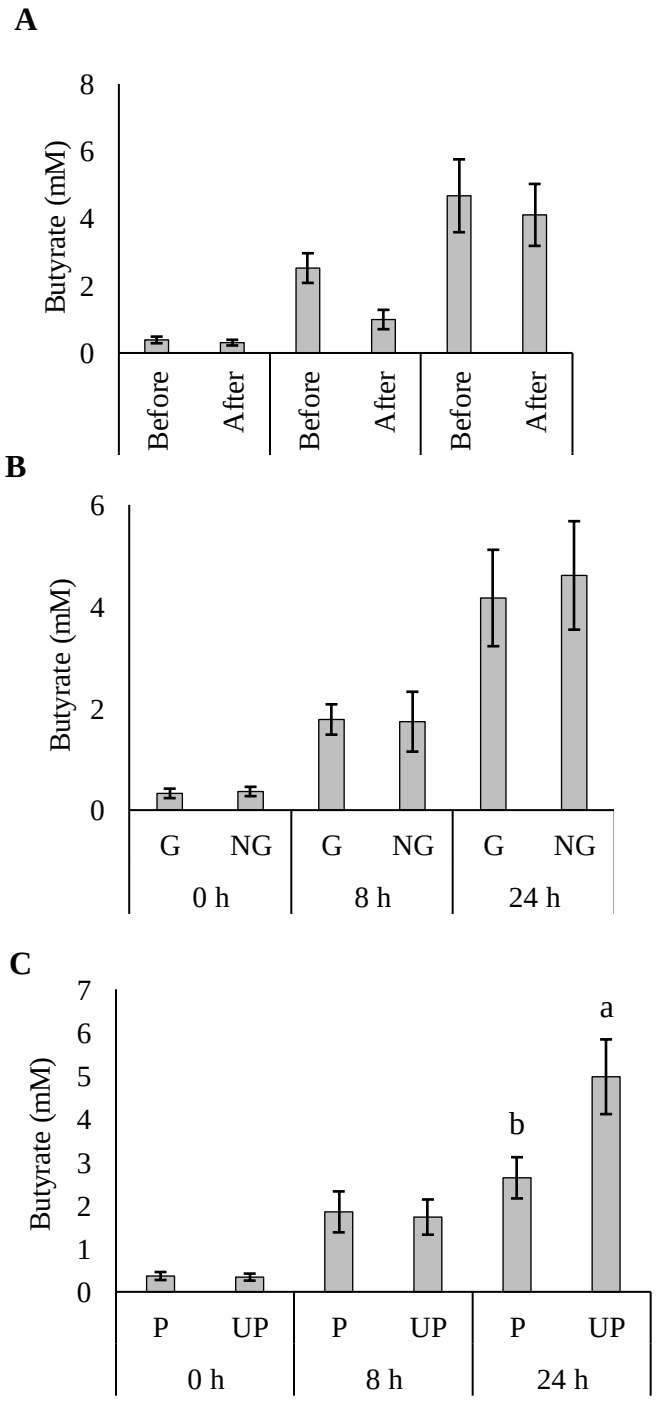

Figure 4. Means of significant treatment effects [before/after (A), glycerol/non-glycerol (B), pooled/unpooled (C)] on butyrate production; error bars show standard error (n=2); bars marked with different letters are significantly different ($p<0.05$).

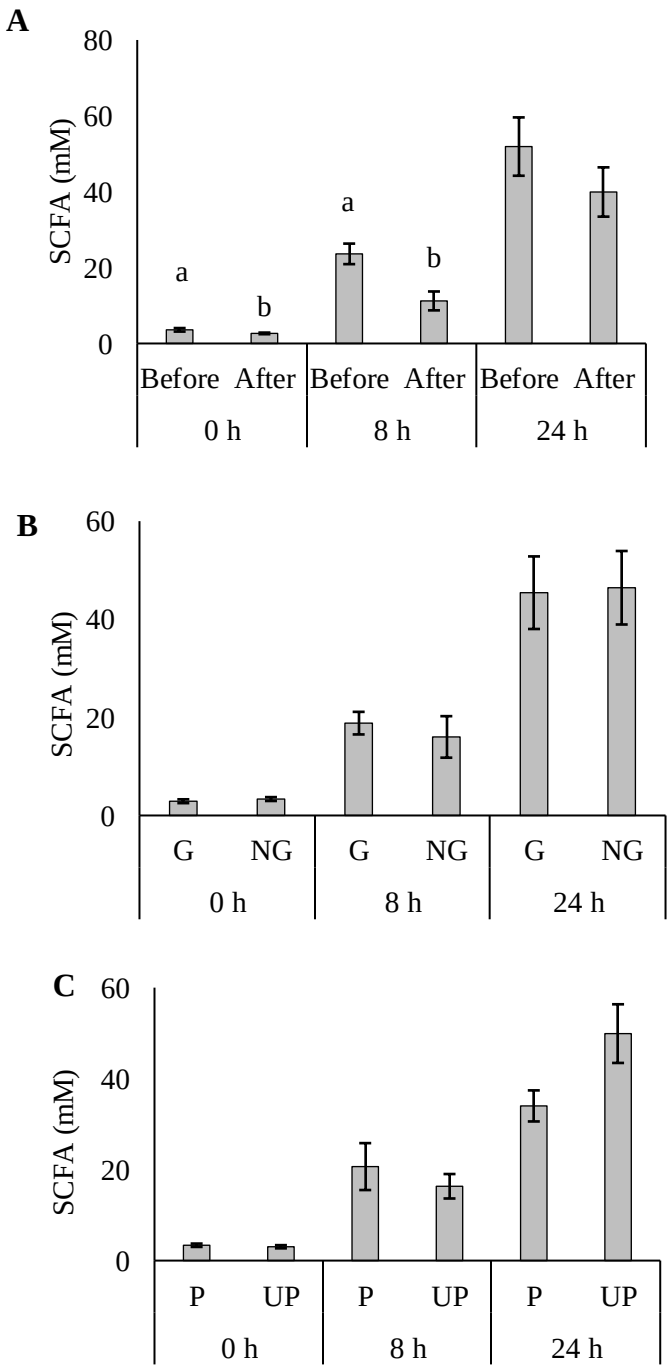

Figure 5. Means of significant treatment effects [before/after (A), glycerol/non-glycerol (B), pooled/unpooled (C)] on total SCFA production; error bars show standard error (n=2); bars marked with different letters are significantly different ($p<0.05$).