EFFECT OF BRAN PARTICLE SIZE ON GUT MICROBIOTA COMMUNITY STRUCTURE AND FUNCTION

by

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To my lovely Family

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LIST OF ABBREVIATIONS

SCFA- short-chain fatty acid

PCoA- principal coordinate analysis

rRNA - ribosomal ribonucleic acid

bp - base pair

μm - micrometer

FOS - fructooligosaccharide

GOS - galactooligosaccharide

nt - nucleotide

OTU- operational taxonomic unit

ABSTRACT

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Gut microbiome composition and function are increasingly known to be linked to host health. Many factors control the gut microbiota, including mode of birth, host health status, genetics, and sanitary conditions, but diet has been one of the most-studied factors as a tool to modulate gut microbiota. Dietary fibers that act as prebiotics escape human digestion and reach the colon, where they become available to the microbes as a source of energy. Gut microbes produce various metabolites that are beneficial to the host health. Among those metabolites thought to be most important are the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate. There is high interest in studying prebiotics that increase the production of these SCFAs as they are thought have many health benefits, such as being anti-inflammatory and anti-carcinogenic.

Gut commensal preferences for these prebiotics depend on their chemical and physical properties. Other factors like the quantities of prebiotics and in what combinations they are consumed also influence gut microbial populations. There have been many studies conducted to test the effect of chemical differences amongst prebiotics on the gut microbiota, however, research studying the physical properties of dietary fiber and its effect on gut microbes is relatively scarce.

To test the hypothesis that physical size of cereal bran particles influences their fermentation by microbiota, we tested different size fractions of wheat and maize bran in *in*

vitro fermentation by fecal microbial communities. Microbes showed size-dependent bran preferences in the case of both wheat and maize bran, both in terms of community structure and metabolites production. For wheat bran, we tested 180-300, 300-500, 500-800, 850-1000 and >1700 μm fractions with fecal microbiota from three healthy donors pooled in equal amounts by weight. We saw clear, size-dependent metabolic outcomes (SCFAs production) which were accompanied by divergent microbial community structures as analyzed by 16S rRNA sequencing. We further also linked these responses to size-dependent chemical differences of wheat bran fractions. In the second study with maize bran, we tested 180-250, 250-300, 300-500 and 500-850 μm size fractions. Like in the case of wheat bran fractions, maize bran also showed size-dependent microbial community structure and metabolic outputs. Overall, this work demonstrated that bran particle size has potential as a tool for fine-tuning the gut microbiota, which in turn can alter metabolite production and have potential benefits to host health.

CHAPTER 1: INTRODUCTION

Gut Microbiota

In the womb, humans are thought to be sterile. During the process of birth, neonates begin to acquire microbiota from their mothers that populates their guts, initiating a process that continues to increase in diversity with age and subsequent introduction of new foods (Koenig et al., 2011). Human gut microbiota reaches maximal diversity and stability in middle adulthood (Spor, Koren, & Ley, 2011). Though dominated by bacteria, the adult human gut can contain members of each of the three main domains of life - *Bacteria*, *Archaea* and *Eukarya* (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The human gut houses ~10^{14} microbial cells (Clark & Coopersmith, 2007) and has also been described as natural laboratory for studying microbial evolution (Ley, Peterson, & Gordon, 2006). However, the human digestive tract separates large quantities of bacterial biomass from the main site of human digestion and nutrient absorption, which occurs mainly in the stomach and small intestine. Microbial digestion and fermentation dominates the large intestine (Walter & Ley, 2011).

Recently, the gut microbiota are increasingly thought to have direct effects on human physiology, including connections with the brain and behavior (referred to as the gut-brain axis). Studies have shown associations between gut microbiota and multiple chronic diseases, including metabolic, cardiovascular, neurologic, and autoimmune disorders (Lynch & Pedersen, 2016). Humans and their microbiota have co-evolved over long periods of time alongside concomitant changes in human dietary patterns; consequently, there is a significant potential to use diet as a tool for predictably altering the gut microbiota community (Ley, Lozupone, Hamady, Knight, & Gordon, 2008). A recent study has shown that lifestyle differences among different human populations can have a strong effect on the microbial community at the high taxonomic resolutions

level as well as with respect to overall community structure (Jha et al., 2018). This suggests that not only dietary patterns, but also other lifestyle attributes, govern the population dynamics of gut commensals. These lifestyle attributes can include occupation, level of industrialization, sanitary conditions, and others (Sonnenburg & Sonnenburg, 2014). However, we do not yet completely understand which factors exert most impact on gut microbial community structure and function. Various factors like consumption of antibiotics, different dietary patterns including the type and quantity of fiber consumption, and mode of birth are being thoroughly studied, among others (Blaser, 2016; Dominianni et al., 2015; Filippo et al., 2010; Huurre et al., 2008; Kashyap et al., 2013; Singh et al., 2017). One recent study determined that long-term changes in dietary patterns exert more significant impacts on gut bacterial enterotype (that is, a prevailing community structure common across humans); short-term dietary changes (~10 days) did not have a significant effect on enterotype partitioning (Wu et al., 2011).

These data suggest the idea that diet-microbiome interactions can be optimized to promote specific microbial community structures and metabolic outcomes. The food that we eat can have direct or indirect effects on our gut commensals. Fermentation of dietary fiber in the gut leads to the production of the short chain fatty acids (SCFAs) acetate, propionate and butyrate; these are typically regarded as the terminal end-products of fermentation in the gut. Other organic acids (e.g. succinate, lactate) are produced through fermentation by some microbes (e.g. members of genus *Bifidobacterium* are known to ferment to lactate), but they in turn get converted to SCFAs (for example, lactate can be converted to propionate) (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016; Ríos-Covián et al., 2016). SCFAs are increasingly known to have a significant range of health benefits. Butyrate is known to regulate intestinal barrier functions, inflammatory and oxidative status, immune regulation, and cell growth and differentiation (Canani et al., 2011).

Thus, increased production of SCFAs like butyrate or even greater external delivery (meaning supplementing butyrate externally) of butyrate to the distal colon may have many potential health benefits, like reducing colitis (Wong, de Souza, Kendall, Emam, & Jenkins, 2006), and propionate reduces serum cholesterol levels (Illman et al., 1988), inhibits fatty acid synthesis (Nishina & Freedland, 1990), and has anti-cancer properties like inducing apoptosis (Jan et al., 2002). Strategies to modulate the gut microbiome have increasingly also considered combining probiotic organisms with fibers (an approach referred to as "synbiotic") to improve beneficial impact; these approaches suggest the need to understand the pairing of microbiota with the fibers they are adapted to ferment.

The most widely used method for gut microbial population analysis is 16S rRNA sequencing. 16S rRNA sequencing serves as a gold standard for measuring phylogenetic diversity in microbial communities (Huse, Ye, Zhou, & Fodor, 2012). The 16S rRNA is an approximately 1500 bp-long sequence that is widely used for bacterial identification (Reller, Weinstein, & Petti, 2007). Sequencing regions of this gene is used as a fast, high-throughput, and reliable method to identify a large variety of bacteria simultaneously (Reller et al., 2007). The 16S rRNA gene has been widely used to study the microbial phylogenetic diversity and taxonomic composition of different environments including the gut; high-throughput amplicon sequencing of this gene in various human samples has been critical to measuring diversity within and between samples (alpha and beta diversity, respectively) (Kuczynski et al., 2010; Langille et al., 2013). An individual's microbiota varies across different body sites and throughout the lifespan (Costello et al., 2009). As humans adopted agricultural lifestyles instead of being hunter/gatherers, and later to industrialized food systems and now processed, sanitized food, microbial diversity is thought to have been lost and is now at concerning levels in Western nations (Sonnenburg & Sonnenburg, 2014). These

losses are thought to be driven by reductions in the consumption of microbiota-accessible carbohydrates from our diet, which act as fuel for the microbial community in our gut (Sonnenburg & Sonnenburg, 2014).

We humans have only a total of 17 enzymes that participate in the process of digestion (mainly starch), however, our gut bacteria have thousands of enzymes that can digest other complex carbohydrates and polysaccharides into SCFAs (Kaoutari, Armougom, Gordon, Raoult, & Henrissat, 2013). Because of peristalsis, the human gastrointestinal tract can be viewed as a bioreactor, where if the microbiota is not continuously provided with microbiota-accessible carbohydrates, they will eventually get washed out of our system. If microbiota are not able to access dietary carbohydrates, they will turn on our native carbohydrates, chiefly, mucus. A study conducted in mice shows that, on reduced consumption of dietary fiber, gut bacteria may start to invade and consume the mucus glycans in the intestinal lining of the mice. This, in turn, leads to reduction in the thickness of the mucus layer and, potentially, to conditions like leaky gut and other inflammatory bowel diseases (Kaoutari et al., 2013; Petersson et al., 2010). Together, it is becoming increasingly clear that gut microbiota contributes strongly to the regulation of host health and that the metabolites produced by fermentation in the colon can influence a broad range of disease conditions.

Dietary fiber

Dietary fiber is a term commonly used to describe that portion of the food which is not digestible by human enzymes. The average dietary fiber intake in the US is less than half of the FDA recommended value (Anderson et al., 2009). Dietary fiber is composed of polysaccharides or oligosaccharides diverse in structure, and the complexity of structures, both chemical and physical, and their connections with specific gut commensals have still been not thoroughly

studied (Hamaker & Tuncil, 2014a). In 2001, the AACCI (American Association of Cereal Chemists International) defined the term dietary fiber as "the edible parts of the plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine"(Dietary Fiber Definition Committee, 2001). Dietary fiber consumption is linked to changes in microbiota, especially with respect to Western dietary patterns. Multiple studies have shown that there is a significant difference in the microbiome composition of mice eating Western-like (high-fat) diets and that of individuals consuming a low-fat chow (higher fiber) diet (Isken, Klaus, Osterhoff, Pfeiffer, & Weickert, 2010; Tachon, Lee, & Marco, 2014). Food components that we eat, but which cannot be digested by our human enzymes, reach the colon and become available for the gut bacteria to process and ferment (Desai et al., 2016).

Many studies have been conducted to identify how different types of dietary fibers can potentially have effects on gut microbiota, which, in turn, have beneficial impacts on health. (Hamaker & Tuncil, 2014a; Tremaroli & Bäckhed, 2012). Several studies have been conducted to show that the consumption of dietary fiber and its fermentation in the gut reduces colon cancer incidence (O'Keefe, 2016). Further, a recent study shows that mice with polyposis had lower levels of fecal SCFAs, likely due to disturbed microbiota levels; these mice, when switched to a high-fiber diet, produced significantly higher SCFAs, which in turn led to reduced tumor load (Bishehsari et al., 2018). With respect to health benefits, it is highly likely that dietary fibers are not interchangeable due to variation in their structures.

Differences in structural properties amongst dietary fibers, such as their source, monosaccharide composition, linkage types, chain length, anomers, epimers, and association with other compounds can have effect on the capacity of microbial utilization of the fiber (Hamaker &

Tuncil, 2014a). Fermentation in the colon is also governed by environmental and host factors. Not only do the different fibers and their subtle differences affect the microbial community, but also the pH in the colon and other host factors govern microbial fiber utilization (Lopez-Siles et al., 2012). Utilization of a specific fiber by gut bacteria can also depend on the other fibers present in the environment. Some soluble fibers, when presented to the microbiota as a mix, delay the process of fermentation, while others do so as individual fibers (Tuncil et al., 2017). As delaying fermentation to the distal colon is desirable in that many gut-related diseases localize to this region, altering the kinetics of fiber fermentation is an area of significant future potential research.

The main food components that fall under the umbrella of dietary fibers include non-starch polysaccharides, animal derived products like chitin, resistant oligosaccharides and resistant starches, and others (Hamaker & Tuncil, 2014a). Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health".(Gibson & Roberfroid, 1995). Some, but not all, dietary fibers are termed as prebiotic only if they 1. Resist the process of digestion by human enzymes 2. Are fermented by the microbes in the colon 3. Support the growth or activity of one or multiple microbes in the gut (Gibson, 2004). In agricultural societies, the main sources include unrefined wheat, rice, maize, oat, and non-digestible oligosaccharides like fructooligosaccharides (FOS), galactooligosaccharides (GOS) of natural and synthetic origin (Pandey, Naik, & Vakil, 2015). A diet deficient in these and other prebiotic foods is associated with reduced microbiota diversity (Halmos et al., 2015) and diets rich in these prebiotics are associated with increased richness in microbial genes (Cotillard et al., 2013). Some research studies also show that fecal SCFA measurements are lower in low-dietary-fiber diets (Chen et al., 2013). Different types of cereals are considered amongst the most significant

sources of dietary fiber in Western countries (Lambo, Öste, & Nyman, 2005). A recent study showed the prebiotic effect of wheat bran being highly bifidogenic, by increasing levels of *Bifidobacterium* spp. under *in vitro* fecal fermentation conditions (D'hoe et al., 2018). Oat bran (Kristek et al., 2019), rice bran (Maurya et al., 2018), and maize bran (Ou et al., 2016) have also showed to have prebiotic functions, by showing bifidogenic properties, reducing tumor load, and altering the gut microbiota respectively. Most fibers induce the shift of microbial communities in the gut, however which amongst these shifts have potential health benefits is still being studied (Bindels, Delzenne, Cani, & Walter, 2015).

The health benefits of dietary fibers may be mediated most centrally by maintaining strong integrity of the gut epithelium. The human intestinal lining, together with its mucus layer, acts as a barrier between luminal bacteria and the internal systems of the body (Johansson et al., 2008). The gut microbiota and dietary fiber (prebiotics) work together towards the healthy maintenance and protection of the intestinal mucus layer (Makki, Deehan, Walter, & Bäckhed, 2018). Dietary fiber also simulates the intestinal lumen to produce mucus (McRorie & McKeown, 2017). One recent mouse study showed that the reduction in fiber consumption can potentially cause the gut microbes to consume mucus glycans which are rich in polysaccharides and this in turn can increase the pathogen susceptibility of the host (Desai et al., 2016).

Many studies to date have focused on how different chemical structures of dietary fibers can potentially modulate gut health, but the research on the effect of physical variability of fiber on the gut health is relatively scarce. One way of categorizing dietary fibers is their division into soluble and insoluble forms. Many health benefits have been attributed to the soluble form of fibers, however insoluble fibers, like those found in whole grains, have shown anti-diabetic properties (Isken et al., 2010). A recent study has also shown that the consumption of insoluble

fibers increased the release of transcription factors that are useful in regulation of hepatic fat homeostasis (Isken et al., 2010). Consequently, variation in physical structuring of fibers may exert differential health impacts independently of the chemical structures of polysaccharides.

Cereal brans: sources of dietary fiber varying in particle size

Industrialization and Westernization in field agriculture and food processing, along with other coordinate changes in lifestyle, has dramatically impacted the physical form of cereal products consumed. Physiologically, the cereal kernel consists of three main parts: 1. outermost bran layer, 2. starchy endosperm and 3. germ (or embryo) (need help with citation here). The outer pericarp layer is termed as bran and the layer inner to the pericarp is called the aleurone layer (Ndolo & Beta, 2013). Cereal bran is a particularly good source of dietary fiber and can contain up to 50% dietary fiber by weight (Mudgil & Barak, 2013). Endosperm is morphologically the largest starchy component in cereal grains and is the main component in refined grains (Ndolo & Beta, 2013; Slavin, 2000). The germ is embryo, rich in nutrients that develops into a new plant. Over centuries of industrial revolution and simultaneous improvement in the efficiency of milling devices, the processing of cereal grains changed markedly. This led to overall reduction in the particle size of the grains (Cordain et al., 2005). Before the industrial revolution, unless sieved, the flour contained the entire germ and bran fractions along with endosperm, and thus had higher overall dietary fiber content (Storck & Teague, 1952). But after the invention of more-efficient milling and sieving processes, the germ and the bran were completely removed during the milling process and the flour contained mainly endosperm, which also led to smaller particulate sizes of the flour (Storck & Teague, 1952).

Cereal brans have long been known to exert health benefits. Brans from different grains are a common source to increase the dietary fiber intake, however, heartburn and bloating are

commonly reported as symptoms post-bran consumption (Cann, Read, & Holdsworth, 1984). Studies have shown that gastric emptying is delayed by the consumption of coarse bran (Grimes & Goddard, 1977; Vincent et al., 1995). A recent study showed that feeding coarse wheat bran (15%, by weight) to piglets for 6 weeks increased their microbiota richness (Kraler, Ghanbari, Domig, Schedle, & Kneifel, 2016). Another study showed that when preschool children consumed bran fiber for 4 weeks, their fecal weight and stool frequency increased significantly, alongside increased intake of iron, zinc and vitamins present in cereal bran (Williams, Bollella, Strobino, Boccia, & Campanaro, 1999)

Milling and size separation of cereal brans has the potential to significantly alter the nutrient content of different size fractions. One possible milling-induced chemical change is that processing and reduction of particle sizes of bran can significantly reduce the phytate content (Majzoobi, Pashangeh, Farahnaky, Eskandari, & Jamalian, 2014). Phytic acid or phytate is phosphate ester of inositol. Research has showed potential health benefits of phytate for its anti-cancerous properties and a key compound in increasing phosphorus.(Shamsuddin, 2002; Simons et al., 1990). For most grains, the inner part of the bran, which is closer to the endosperm, contains higher amounts of aleurone. Another study also showed that the smaller sizes of bran tend to be derived from the inner part of the grain and have less dietary fiber content compared to that of the outer layer (de Kock, Taylor, & Taylor, 1999). Aleurone (innermost layer of wheat bran) has increased fermentability than that of whole wheat bran indicated by higher SCFA production in *in vitro* assays (Amrein, Gränicher, Arrigoni, & Amadò, 2003). A similar study tested whether any difference existed between the large and small bran size fractions, and aleurone and its by-products of different sizes by fermentation using fecal microbiota (Stewart & Slavin, 2009). The authors found differences in the amount of SCFA produced between the small and large bran fractions

with small fractions producing higher amounts of total SCFAs (Stewart & Slavin, 2009). In the same study, the researchers found that there is difference in the SCFA production by fine (~69μm) and coarse (~108μm) fractions of aleurone (Stewart & Slavin, 2009). This suggests that there might be some fermentability differences amongst various bran fractions by gut microbiota based on the differences in their aleurone content. Thus, there could be some potential connections between the reduction of particle sizes of the grains and chronic disease in Western countries. Therefore, I hypothesized that the differences in SCFA production are linked to the size-based preferences for bran by certain members of the gut microbial community.

Aims

As discussed above, because of changes in cereal processing associated with industrialization and agricultural developments, the process of westernization inextricably links reduction in the total amount of dietary fiber consumed with reduction in the size of insoluble particles. The process of industrialization and westernization, and thus increases in consumption of more-refined foods, culturally co-evolves with increases in chronic disease incidence. Therefore, particle size could be a previously-overlooked contributor to westernized diseases, via alterations in the diversity, composition, or function of the gut microbial community. The overarching hypothesis of my thesis is that this particle size exerts impacts on the gut microbiome independent of changes in the total amount of dietary fiber. In this study, I addressed the specific hypothesis that reduction in the size of bran particles by modern milling techniques would affect gut microbiota community structure and function in *in vitro* fermentation.

Because wheat bran is the most widely-consumed bran in Western countries (Neyrinck et al., 2018), we began by testing the hypothesis with respect to gut bacteria preferences for different particle sizes of wheat brans, measuring community structure and metabolic function in *in vitro*

fermentations. To determine whether bran particle size effects were specific to wheat bran or general across other bran types, I tested the same hypothesis using maize brans differing in size across similar ranges as the brans above. We further milled maize brans down from the parent bran to minimize differences in chemical composition.

CHAPTER 2: WHEAT BRAN PARTICLE SIZE STUDY

Summary

Though the physical structuring of insoluble dietary fiber sources may strongly impact their processing by microbiota in the colon, relatively little mechanistic information exists to explain how these aspects affect microbial fiber fermentation. Here, I hypothesized that wheat bran fractions varying in size would be fermented differently by gut microbiota, which would lead to size-dependent differences in metabolic fate (as short-chain fatty acids; SCFAs) and community structure. To test this hypothesis, we performed an *in vitro* fermentation assay in which wheat bran particles from a single source were separated by sieving into five size fractions and inoculated with fecal microbiota from healthy donors. SCFA analysis revealed size fraction- dependent relationships between total SCFAs produced as well as the molar ratios of acetate, propionate, and butyrate. These size-dependent metabolic outcomes were accompanied by the development of divergent microbial community structures. We further linked these distinct results to subtle, size-dependent differences in chemical composition and starch availability. These results suggest that physical context can drive differences in microbiota composition and function, that fiber-microbiota interaction studies should consider size as a variable, and that manipulating the size of insoluble fiber-containing particles might be used to control gut microbiome composition and metabolic output.

Introduction

The human colon is one of the most densely-colonized microbial habitats found on earth, being home to up to tens of trillions of microbial cells (Qin et al., 2010; Sender et al., 2016); these are collectively termed the colonic microbiota. The colonic microbiota and their products are increasingly recognized as being physiologically important for human health (Marchesi et al., 2016; Sommer et al., 2017). Reductions in colonic microbiota diversity (as determined from fecal samples) have recently been found to correlate with multiple disease states, such as metabolic syndrome and type 2 diabetes (Karlsson et al., 2013), inflammatory bowel disease (Lapthorne et al., 2013), and colorectal cancer (Ahn et al., 2013). Recently, many research groups have determined that 1) this loss of species from the colonic microbiota is linked to consumption of the high-fat, low-fiber Western diet 2) that, in mice, these extinctions compound irrevocably over generations, and 3) that higher consumption of fermentable dietary fibers increases the diversity of the colonic microbiota (Sonnenburg et al., 2016). The mechanisms driving these losses in diversity remain poorly understood (Segata, 2015), which inhibits design of dietary strategies to stably increasing colonic microbiota diversity as means to prevent or treat chronic disease.

Dietary fibers are defined as the portion of food, typically of plant origin, that cannot be digested intestinally by human enzymes. Thus, these dietary compounds transit the upper gastrointestinal tract largely intact and reach the colon where, along with host-secreted glycans, they become main energy sources for the microbiota (Koropatkin et al., 2012; Hamaker and Tuncil, 2014; Li et al., 2015; Desai et al., 2016). The term *dietary fiber* encompasses 1) non-starch polysaccharides, such as arabinoxylans, pectin, cellulose and gums, 2) resistant oligosaccharides such as fructoologosaccharides and galactooligosaccharides, 3) other digestion-resistant carbohydrates such as resistant starches and dextrins, 4) animal derived carbohydrates such as chitin, chondroitin sulfate and hyaluronan, and 5) non-carbohydrate-based compounds such as

lignin, which is found in the plant cell wall (Hamaker and Tuncil, 2014). Anaerobic metabolism of dietary fibers by colonic microbiota results in short-chain fatty acids (SCFAs; specifically, acetate, propionate, and butyrate) as the predominant terminal products of fermentation; these SCFAs are increasingly understood to modulate host physiological processes and are thought to contribute to health through multiple mechanisms (Wong et al., 2006; Nicholson et al., 2012; Koh et al., 2016; Rios-Covian et al., 2016).

Cereal-derived dietary fibers (those from wheat, maize, oat, barley and rye) represent a relatively large fraction of human dietary fiber intake, which is driven in Western countries mainly by wheat consumption (Gebruers et al., 2008). The total dietary fiber content of wheat kernels reaches up to 18 % of dry matter (Gebruers et al., 2008), of which the bran portion contains the vast majority (85 %; Awika, 2011). Most of wheat bran dietary fiber is accounted for by non-starch polysaccharides such as arabinoxylans (also known as hemicellulose; ~70 %), cellulose (19 %), (1-3), and (1-4)-ß- glucan (6 %) (EFSA, 2010). Other non-starch polysaccharides such as glucomannan, arabinogalactan, and xyloglucan may also be present in small amounts (Maes and Delcour, 2002). The majority of the fibers found in wheat bran are water-insoluble due to their interactions with each other and with other cell wall components, such as proteins and phenolic compounds, via covalent bonding, hydrogen bonding and electrostatic interactions (Iiyama et al., 1994).

Recent studies have revealed that wheat bran impacts the structure and metabolic function of the colonic microbiota. For example, wheat bran was shown in an *in vitro* fermentor system to promote butyrate-producing bacteria belonging to family *Lachospiraceae*, which subsequently resulted in an increase in the butyrate concentration (Duncan et al., 2016). Similar results were also observed in two independent *in vivo* studies, in which consumption of a wheat bran-enriched

diet increased butyrate concentrations in the feces of obese (Salonen et al., 2014) and overweight (Walker et al., 2011) humans; this change was attributed to stimulation of members of *Lachnospiraceae* in the colons of these subjects. Increased butyrate formation in the large intestine through wheat bran fermentation was also reported to suppress chemically-induced tumors in rodents (Zoran et al., 1997; Compher et al., 1999).

Though it is well-known that westernization of diet is associated with reductions in total fiber intake, one commonly-overlooked correlation is a decrease in the average particle size of grain flours consumed (Cordain et al., 2005) . It is known that the physical structure of insoluble fiber sources significantly impacts fiber bioavailability and microbial activity (Jenkins et al., 1999; Stewart and Slavin, 2009; Dziedzic et al., 2016; Suriano et al., 2017), yet the size of insoluble fiber particles has not been rigorously examined for its impact upon the gut microbiota. Here, we test the hypothesis that wheat bran fractions varying in size (and, therefore, with different surface area-to-volume ratios) would be fermented differently by gut microbiota, which would lead to size-dependent differences in metabolic fate (measured as short-chain fatty acids; SCFAs) and community structure. Our results, obtained using five size fractions of sieved wheat bran particles, reveal that there is a direct relationship between metabolic outcomes (SCFA production) and wheat bran size fraction. Surprisingly, the relative abundances of observed operational taxonomic units (OTUs, surrogates of microbial species) are also directly linked to size fractions. Broadly, these data suggest that controlling the size of insoluble fiber-containing particles might be potentially be employed in future dietary strategies to modulate the diversity, composition, and metabolic outcomes (e.g., SCFA production) of the gut microbiome.

Materials and Methods

Wheat brans

Wheat brans were a generous gift of The Mennel Milling Company (Fostoria, OH). We obtained various sizes of wheat bran by sieving the wheat bran through various screens (sizes 180, 300, 500, 800, 850, 1000, and 1700 µm) using a sieving machine (Portable Sieve Shaker Model RX-24, sieving machine and screens both from W.S. Tyler Combustion Engineering, Inc., Mentor, OH). Before analyses, we treated the wheat bran fractions with alpha-amylase, pepsin and pancreatin enzymes *in vitro* to simulate digestion through the upper gastrointestinal tract digestion as previously described (Lebet et al., 1998a; Rose et al., 2010; Tuncil et al., 2017b). In order to determine whether the size of the coarsest brans reduce enzyme accessibility to starch (for the analysis of Fig. 7), I reduced the size of the coarsest bran (> 1700 µm) to < 500 µm using a cyclone mill (Brand and company info here) equipped with a 500 µm screen before I determined the total starch content.

Sugar composition analysis

We determined neutral monosaccharide composition of the digested wheat brans as their alditol acetate derivatives using separation by gas chromatography on a capillary column (SP2330, SUPELCO, Bellefonte, PA) coupled with mass spectrometry (GC/MS; models 7890A and 5975C inert MSD with a Triple-Axis detector, Agilent Technologies, Inc., Santa Clara, CA), as previously described (Pettolino et al., 2012). Helium was used as a carrier gas. We set the GC/MS conditions as: injection volume of 2µl with a split ratio of 1:2; injector temperature at 240 ºC; detector temperature at 300 ºC; the gradient temperature program set was 160 ºC for 6 min, then 4 ºC /min to 220 ºC for 4 min, then 3 ºC /min to 240 ºC for 5 min, and then 11 ºC /min to 255 ºC for 5 min.

We determined the total starch contents of the samples using total starch assay kit (product code: K-TSTA) according to the manufacturer instructions (Megazyme International, Wicklow, Ireland).

In vitro fermentation

We performed *in vitro* fermentation assays as previously described (Lebet et al., 1998b; Tuncil et al., 2017b) in an anaerobic chamber (BACTRONEX Anaerobic Chamber, Shel Lab, Cornelius, OR) under an 85% N_2, 5% CO_2, and 10% H_2 atmosphere. Briefly, we prepared carbonate-phosphate buffer and sterilized by autoclaving at 121 ^{o}C for 20 min. We then cooled the buffer to room temperature, removed oxygen by bubbling with carbon dioxide, and added cysteine hydrochloride (0.25 g/L of buffer) as a reducing agent. We then immediately placed the buffer into the anaerobic chamber overnight. We weighed the wheat brans (50 mg) into 25 mL Balch tubes (Chemglass Life Sciences, Vineland, NJ) for each time point (0, 6, 12, 24 and 48 h). We autoclaved the tubes containing the substrates and then transferred into the anaerobic chamber.

The following day, we added 4 ml of carbonate-phosphate buffer to each Balch tube and collected fecal samples from three healthy donors who were consuming their routine diets and had not taken antibiotics for at least 3 months. We tightly sealed the fecal samples in plastic tubes, kept on ice prior to rapidly being transferred into the anaerobic chamber, and used within 2 h of collection. We homogenized the fecal samples with carbonate-phosphate buffer [feces:buffer 1:3 (w/v)], followed by filtration through four layers of cheese cloth. We then pooled the filtered fecal slurries, and then inoculated each tube with 1 ml of pooled fecal slurry. We then immediately closed the tubes with butyl rubber stoppers (Chemglass Life Sciences), sealed with aluminum seals (Chemglass Life Sciences), and incubated at 37 ^{o}C in a shaking water bath (150 rpm). We used test tubes containing no substrate and FOS as a blank and fast-fermenting fiber control,

respectively, at each time point. We performed all analyses in triplicate. Protocols involving human stool collection and use were reviewed and approved by Purdue University's Institutional Review Board (IRB protocol #1701018645).

Total gas production and sample collection for SCFA and DNA analysis

At each time point, we measured total gas production with a graduated syringe by passing the needle through the rubber stopper and then we opened the tubes. We collected two aliquots from each tube for DNA extraction (1 ml) and SCFA analysis (0.4 ml). We stored the samples collected for DNA extraction immediately at -80 $^{\circ}$C until further analysis. We prepared 100 ml of an internal standard mixture (by combining 157.5 µl of 4-methylvaleric acid, 1.47 ml of 85% phosphoric acid, 39 mg of copper sulfate pentahydrate in a final volume of 25 ml ultrapure water) and added immediately to samples collected for SCFA analysis, which we then vortexed and stored at -80 $^{\circ}$C until analysis.

SCFA analysis

We performed SCFA analyses as previously described (Tuncil et al., 2017b). Briefly, we thawed the samples at room temperature and centrifuged at 13,000 rpm for 10 mins. Then, we analyzed the supernatants (4 µl) using a gas chromatography (GC-FID 7890A, Agilent Technologies Inc.) on a fused silica capillary column (Nukon^{TM}, SUPELCO No: 40369-03A, Bellefonte, PA) under the following conditions: Injector temperature at 230 $^{\circ}$C; initial oven temperature at 100 $^{\circ}$C; temperature increase of 8 $^{\circ}$C/min to 200 $^{\circ}$C with a hold for 3 min at final temperature. Helium was used as a carrier gas at 0.75 ml/min. We used acetate (catalog number: A38S), propionate (catalog number: A258), and butyrate (catalog number: AC108111000)

purchased from Fisher Scientific (Hampton, NH) as external standards and 4-methylvaleric acid (catalog number: AAA1540506, Fisher Scientific) as an internal standard for quantification.

DNA extraction

We carried out the DNA extraction from the samples using FastDNA SPIN® kit for Feces (product code: 116570200) according to the manufacturer's instructions (MP Biomedical, Santa Ana, USA) with the following modifications: 1. We centrifuged the samples stored for DNA extraction 13,000 rpm for 10 mins, and discarded the supernatants. 2. We homogenized the pellets with phosphate buffer and transferred into the Lysing Matrix E tube. Subsequently, the rest of the isolation was performed according to the manufacturer's instructions.

16S rRNA sequencing

We amplified the V4-V5 region of the 16S rRNA gene by PCR using the universal bacterial primers: 515-FB (GTGYCAGCMGCCGCGGTAA) and 926-R (CCGYCAATTYMTTTRAGTTT) (Walters et al., 2016). The PCR solution included 2 µl of template DNA (containing > 5 ng/µl DNA), 0.625 µmole of each primer, 12.5 µl of HiFi hot start ready mix (catalog number: KK2602, Kapa Biosystems, Wilmington, MA), and 0.016 µg of BSA (catalog number: BP9706100, Fisher Scientific, in a buffer containing Tris-HCl (final concentration in the reaction 0.8 mM; catalog number: BP1758, Fisher Scientific), KCl (final concentration 4 mM; catalog number: P217, Fisher Scientific), EDTA (4 µM final concentration; catalog number: S311, Fisher Scientific) and glycerol (final concentration 1.6 %, catalog number: G33, Fisher Scientific) in a final reaction volume of 25 µL. The cycling parameters comprised of an initial denaturation at 98 °C for 3 minutes, followed by 22 cycles of denaturation at 98 °C for 10 seconds, annealing at 50 °C for 30 seconds and extension at 72°C for 30 seconds. Final

extension was performed at 72°C for 10 minutes, after which products were held at 4 °C. We removed the unincorporated dNTPs and primers using the Axygen AxyPrep PCR Clean-up Kit (Axygen Scientific, an imprint of Corning Life Sciences, Tewksbury, MA) according to the manufacturer's instructions. We barcoded PCR products using the TruSeq dual-index approach, purified again cleaned up (removed the unincorporated dNTPs and primers) using the AxyPrep PCR Clean-up Kit, quantitated via Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA), and pooled. We performed quality control for pools by running 1 µL of each pool on an Agilent Bioanalyzer with a High Sensitivity Chip (Agilent, Santa Clara, CA) followed by quantitation for pool loading via the KAPA Library Quantification Kit for Illumina Platforms. Sequencing was performed using on an Illumina MiSeq run with 2 x 250 cycles and V2 chemistry (Illumina, Inc., San Diego, CA) at the Purdue Genomics Core Facility.

Sequence processing and community analysis

We processed the sequences using mothur v.1.39.3 according to the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP [accessed 9/6/2017]) (Schloss et al., 2009; Kozich et al., 2013) with the following modifications: 1. We assembled contigs and assigned to groups permitting no errors within the primer region, 2. We initially screened the sequences for a maximum length of 411 nt, zero maximum ambiguous bases and a maximum homopolymer length of 9 nt, then aligned to the mothur-formatted SILVA reference alignment (Quast et al., 2013) across positions 11894 to 27656. We classified the sequences using the mothur-formatted version 16 of the Ribosomal Database Project (Cole et al., 2014) training set, to which species epithets had been added to the reference taxonomy using a custom Perl script. We classified the sequences to the species level, where possible, at a bootstrap cutoff of 95 to restrict classification to only very high-confidence classifications. Sequences that classified within domain *Eukarya*, as chloroplasts

or mitochondrial sequences, or with unknown classification at the domain level we removed these from further processing. OTU classifications at the species level are reported as the percentage of reads that were classified within a given species at a bootstrap value of 95 or greater. We calculated α-diversity metrics using the nseqs, coverage, invsimpson, simpsoneven, chao, and shannon calculators and β-diversity metrics using the braycurtis and thetayc calculators as implemented in mothur. We generated LEfSe-formatted files using mothur using make.lefse(), and performed linear discriminant analysis using LEfSe v.1.6 (Segata et al., 2011).

Statistical analyses

All analyses were performed in triplicate. Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, Inc. La Jolla, CA). Analysis of variance (ANOVA) was performed at α = 0.05 significance level to determine differences among the samples and controls. Tukey's multiple comparison test at α = 0.05 was used to see whether mean differences were statistically different. Linear regression model was computed using Prism version 7.0 to see the relationship between wheat bran particle size and metabolic outcomes (SCFAs, and OTU abundances).

Results

Wheat bran particle size strongly influenced the amounts and proportions of SCFAs produced

We tested whether wheat bran particle size would impact the metabolism and community structure of fecal microbiota through *in vitro* batch fermentations of wheat bran size fractions (separated by sieving from an identical initial source) that we had previously enzymatically digested enzymatically *in vitro* to mimic transit through the stomach and small intestine and

inoculated with fecal microbiota from three healthy donors. To evaluate the metabolic outcomes, we measured the production of the terminal SCFAs most abundantly found in fecal samples (acetate, propionate, and butyrate) at 0, 6, 12, 24, and 48 h time points post-inoculation with fecal microbiota. We tested the following particle size fractions 1) 180 - 300 μm (the finest bran), 2) 300 – 500 μm, 3) 500 – 800 μm, 4) 850 – 1000 μm, and 5) >1700 μm (the coarsest bran). We also included the rapidly-fermentable, highly-butyrogenic soluble fiber, fructooligosaccharide (FOS) (Tuncil et al., 2017b), as a positive control for fermentation. As we hypothesized, different wheat bran size fractions not only impacted the rate and absolute amounts of SCFAs produced by fecal microbiota (**Fig. 1**) but also significantly ($p < 0.05$) influenced the molar ratios of the SCFAs (**Fig. 2**). At all time points, fermentation of FOS generated the highest amount of total SCFA, acetate, and propionate (**Fig. 1**). Among bran fractions, the finest bran produced the highest amount of total SCFA, acetate, and propionate at all the time points, and gradual decreases in the amounts of these microbial byproducts were observed as the particle size increased. We also observed the same trend for the butyrate production up to 24 h post-inoculation within the bran fractions; after 24 h post-inoculation, fermentation of the finest bran fraction generated butyrate concentrations indistinguishable from FOS, which is widely-regarded as a highly-butyrogenic fiber (Tuncil et al., 2017b). Interestingly, although the coarsest bran generated the lowest amount of butyrate in the first 24 h post-inoculation, by 48 h post-inoculation butyrate concentrations in coarse bran fermentations were also indistinguishable from those of FOS ($p < 0.05$) (**Fig. 1**).
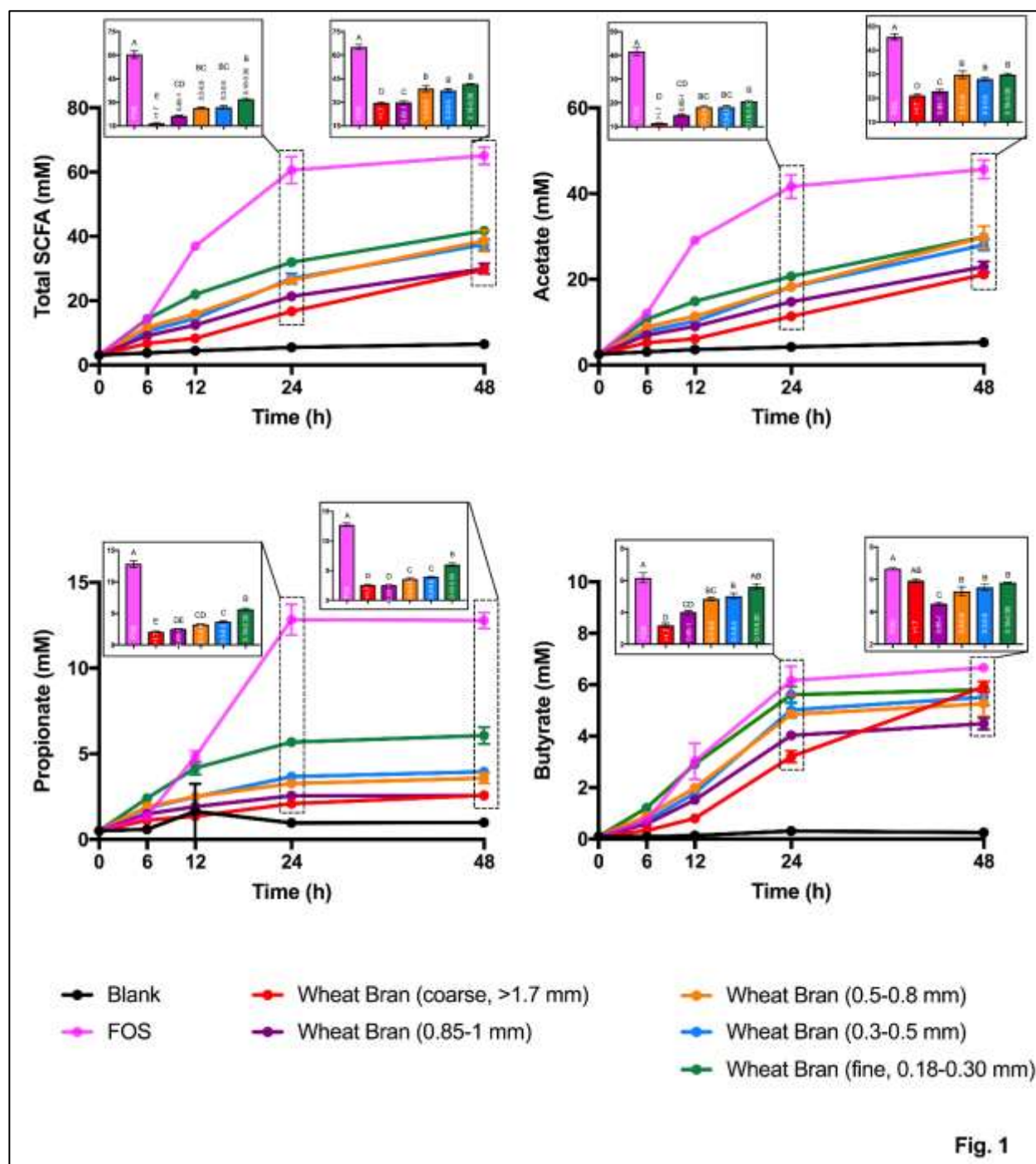
Figure 1: Short-chain fatty acid (SCFA) production by fecal microbiota in in vitro fermentations over time.

FOS (fructooligosaccharide) was used as a fast-fermenting, butyrate-producing positive control. The blank did not contain any carbon substrate. Total SCFA is the sum of acetate, propionate, and butyrate. Error bars represent the standard error of the mean of three separate replicates. Mean values with the same letter are not significantly different (Tukey's multiple comparisons test, $P < 0.05$).
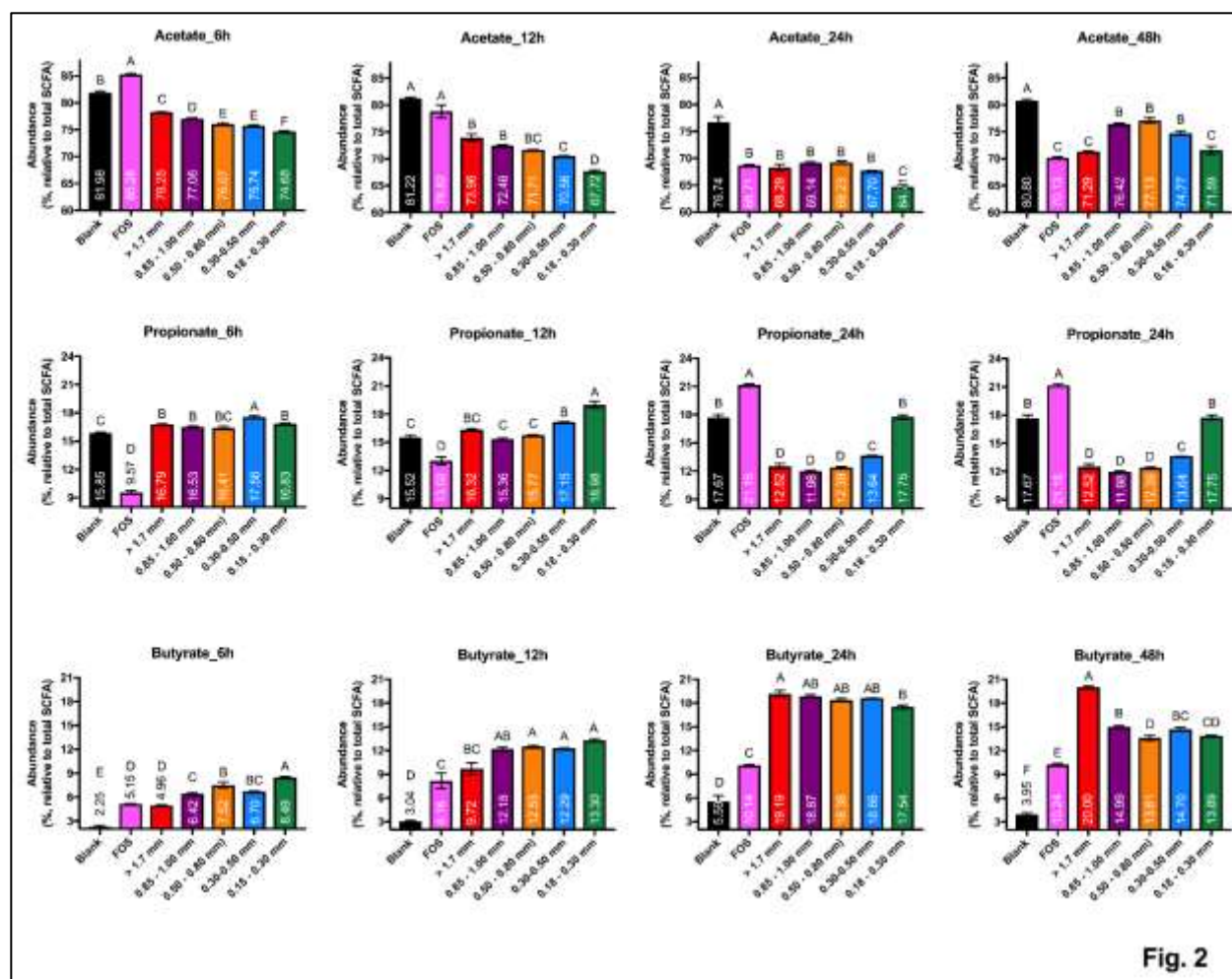
Figure 2: Relative abundances of acetate, propionate and butyrate (relative to total SCFA) produced by fecal microbiota in in vitro fermentations over time.

FOS (fructooligosaccharide) was used as a fast fermenting-butyrate producing comparator. The blank did not contain any substrate. Error bars represent the standard error of the mean of three separate replicates. Mean values with the same letter are not significantly different (Tukey's multiple comparisons test, $P < 0.05$). Total SCFA is the sum of acetate, propionate, and butyrate.

In terms of SCFA molar ratios, at early time points (6 and 12 h post-inoculation) there was a direct relationship between the proportion of acetate and size fraction, with the coarsest wheat bran producing the highest proportion of acetate and the finest one generating the lowest proportion (**Fig. 2**). However, this relationship was not maintained over increasing incubation times. At later time points (24 h post-inoculation and later), we observed an inverse relationship

between bran size fraction and propionate production, with the smallest particles generating significantly more propionate than larger ones. The relationship between particle size fraction and butyrogenesis was temporally complex; we observed an inverse relationship between butyrate molar ratio and wheat bran particle size fraction at early time points (6 and 12 h post-inoculation) with the coarsest wheat bran generating the lowest proportion of butyrate and the finest one the highest. Surprisingly, this inverse relationship reversed after 12 h post-inoculation, at which a direct relationship between relative abundance of butyrate and wheat bran particle size fraction emerged, with the highest proportion of butyrate produced from the coarsest bran. The relative abundance of butyrate produced from the coarsest bran at 48 h post-inoculation reached 20 %, which was almost double the relative abundance of butyrate generated from FOS (10.24 %) (**Fig. 2**). These data clearly indicated that particle size fraction dramatically influenced the metabolic outcome of wheat bran fermentation by fecal microbiota.

Wheat bran particle size fraction significantly impacted the fecal microbiota community structure

To determine whether the observed alterations in metabolism of wheat bran size fractions were accompanied by shifts in the microbiota, we assessed the effects of wheat bran size fraction on colonic microbiota composition by amplicon sequencing targeting the V4 and V5 region of the bacterial 16S rRNA gene (using primers 515FB and 926R) from genomic DNA extracted at 24 and 48 h post-inoculation. Sequences were clustered into OTUs defined at the 97% identity level, from which we calculated α- and β-diversity metrics. Fermentation of FOS and bran fractions resulted in very different microbial community structures over time (Fig. 3). Within bran-consuming cultures, the microbiota associated with distinct size fractions were significantly different across both time points (AMOVA, $p < 0.001$), although we observed no significant

differences ($p < 0.05$) in α-diversity metrics. Even though size fraction clusters were not clearly resolved after 24 h post-inoculation, (Fig. 3a), clear separations between microbiota consuming different bran size fractions were evident 48 h post-inoculation. Especially evident were demarcations of microbiota growing on the finest wheat bran treatment and those consuming the coarsest bran (Fig. 3b). Taken together, these data suggest that divergent communities arise as colonic microbiota ferment wheat bran particles of differing size fractions.
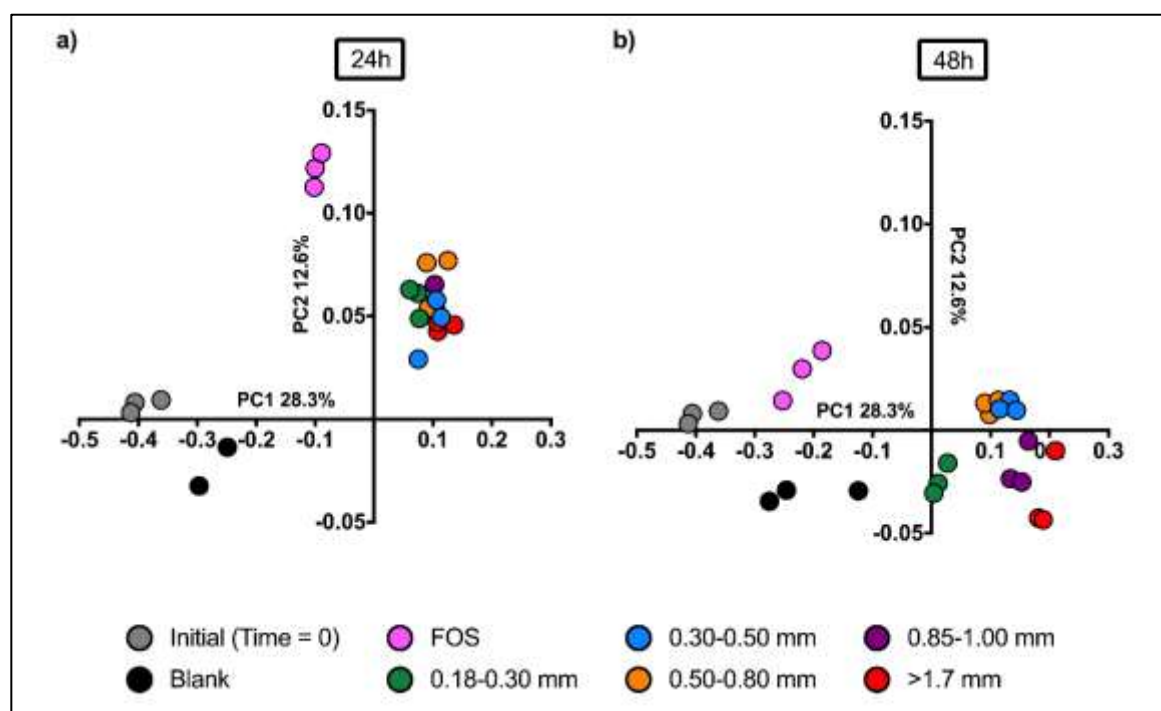
Figure 3: Principal component analysis of community structures associated with wheat bran size fractions, as determined by 16S rRNA gene amplicon sequencing.
Bray-Curtis dissimilarity of fecal microbiota was based on the relative abundances of OTUs at a 97% identity level after *in vitro* fermentation for **a)** 24 h and **b)** 48 h. FOS (fructooligosaccharide) was used as a fast-fermenting, butyrate-producing positive control. The blank did not contain any substrate.

The selective effect of bran particle size fraction operates at fine taxonomic resolutions

Though particle size fraction resulted in significant differences in abundances of taxa at the family level and higher, the selective effects of particle size also occurred at the genus or species

level. In general, overrepresentation of phylum *Firmicutes* in association with the coarsest brans was driven by increases in members of *Lachnospiraceae*, whereas relative increases in members of *Bacteroidaceae* drove increased representation of phylum *Bacteroidetes* associated with the finest brans. However, within genus *Bacteroides,* distinct OTUs increased in abundance in response to differential bran size fractions. The most obvious change within bran treatments was a 20-fold increase in the relative abundance of OTU6 *Bacteroides* (of which 59% of reads within the cluster could be classified as *B. intestinalis*) with the coarsest bran treatment. Similarly, we observed 8-, 4-, 20,- and 23-fold increases over the initial microbiota in the relative abundances of OTU5 *Roseburia*, OTU8 *Coprococcus eutactus*, OTU12 *Lachnospiraceae* and OTU15 *Lachnospiraceae*, respectively, in fermentations of the coarsest bran. In contrast, growth on wheat bran caused dramatic decreases in the relative abundances of *Bifidobacterium*-related OTUs; the coarsest wheat bran treatment resulted in 5-, and 3-fold decreases in OTU11, and OTU7, respectively, compared to the inoculum (**Figs. 4**).

More surprisingly, we observed either direct or inverse relationships between individual OTUs and wheat bran particle size fraction. For example, there was an inverse relationship between OTU3 *Bacteroides* (of which 67% of reads could be classified within *B. dorei*) and wheat bran particle size fraction at both 24 and 48 h post-inoculation, such that increasing particle size increased led to decreasing relative abundance of this OTU (**Figs. 4**). Conversely, at 48 h post-inoculation time point, OTU5 *Roseburia* (of which 69% of the reads could be classified within *R. faecis*), OTU6 *Bacteroides* (of which 59% of the reads could be classified within *B. intestinalis*), OTU8 *Coprococcus eutactus*, OTU12 *Lachnospiraceae* and OTU15 *Lachnospiraceae* displayed a direct relationship with wheat bran size fraction, such that the relative abundances of these species gradually increased with increasing wheat bran particle size (**Figs. 4**).
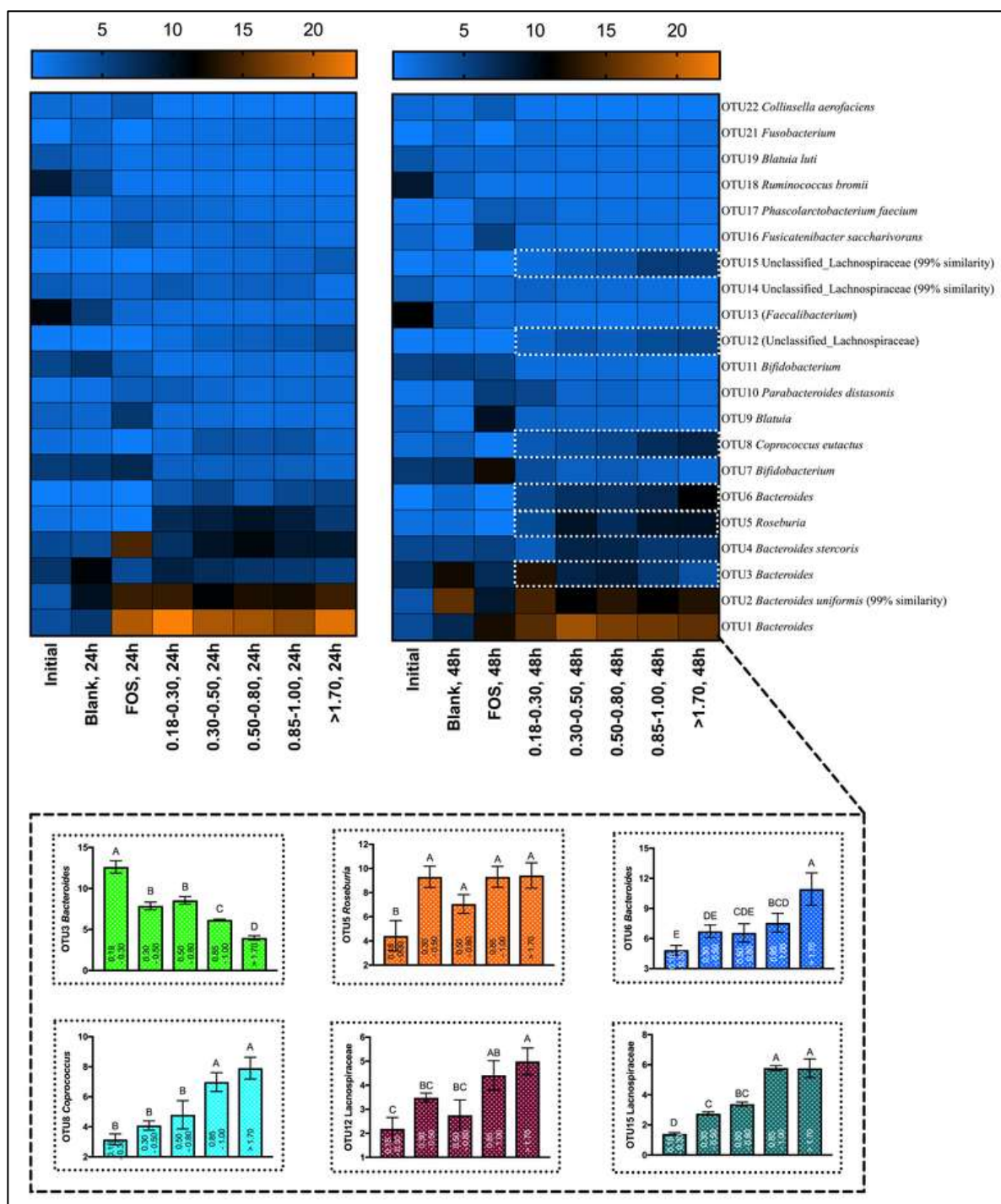
Figure 4: Relative abundances (percentage of sequences) based on the top 50 OTUs in each sample.
Error bars represent the standard error of the mean of three separate replicates. Mean values with the same letter are not significantly different (Tukey's multiple comparisons test, $P < 0.05$).

To identify the specific bacterial taxa representative of the extremes in wheat bran particle size fractions, we compared the microbial compositions of the coarsest and the finest wheat bran treatments at 48 h post-inoculation using the linear discriminant analysis effect size (LEfSe) method (**Fig. 5**). Members of *Lachnospiraceae* (specifically, *Coprococcus eutactus* and *Roseburia hominis*) were shown to be discriminators for the coarsest bran treatment, whereas members of *Bacteroides* and its parent taxa were differentiators for the finest bran treatment (**Fig. 5a,** LDA > 4). However, within genus *Bacteroides*, OTU6 (attributed in Fig. 7 as *Bacteroides intestinalis*) and OTU4 (attributed in Fig. 7 as *Bacteroides stercoris*) were differentiators for the coarsest bran particles. These relationships suggest that stable relationships exist between bran particle size and individual species (especially members of *Bacteroides*) within the colonic microbiota.
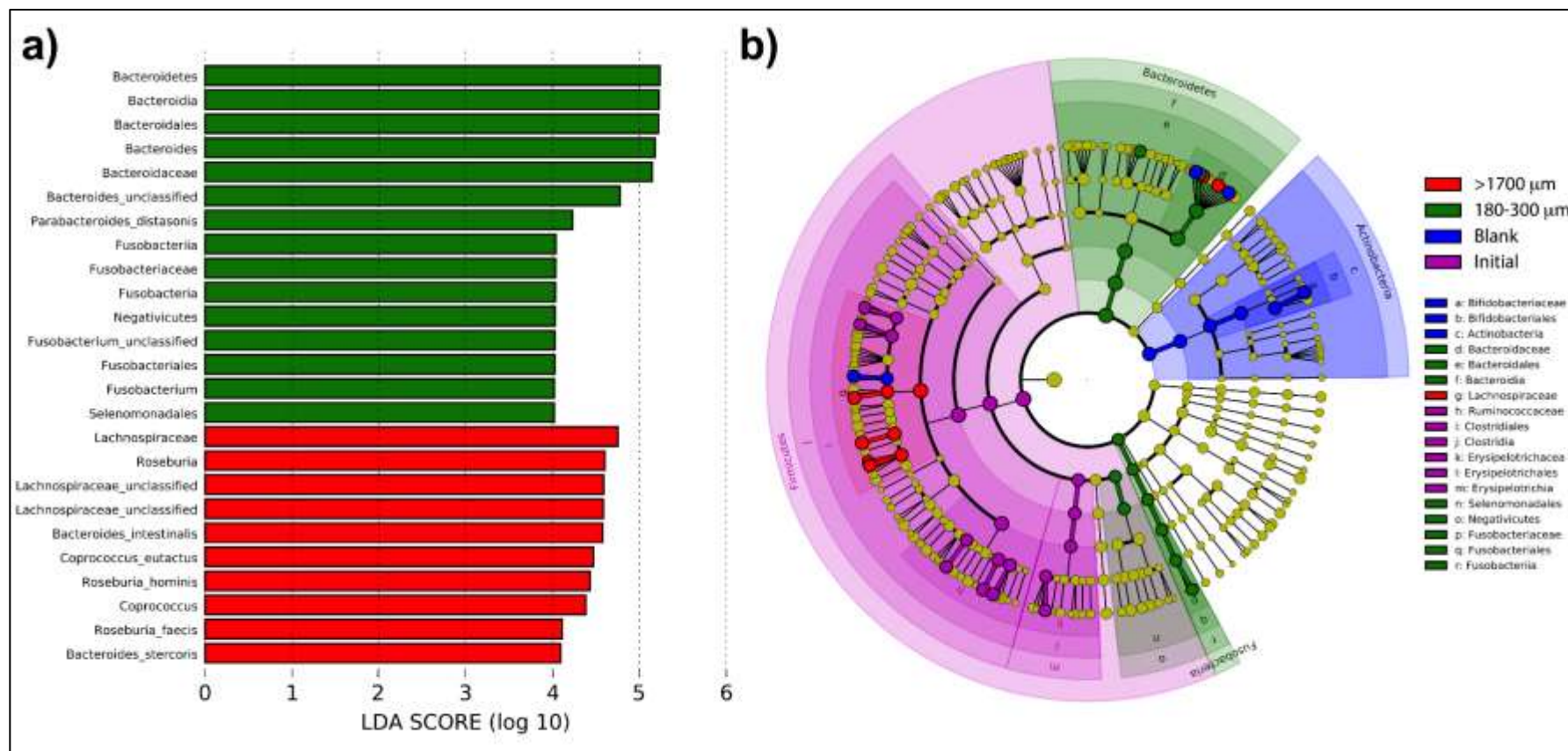
Figure 5: Linear discriminant analysis of taxa differentiating the finest and coarsest wheat bran size fractions.
Initial and blank communities were also included in the analysis to prevent misattribution of taxa to size fractions that were more highly represented in controls. **a)** Taxa with LDA scores > 4.0 in the finest (180 – 300 μm) and coarsest (> 1700 μm) size fractions at 48 h post-inoculation; linear discriminants of initial and blank conditions not shown. **b)** Cladogram depicting taxa that are overrepresented in the finest and coarsest bran fractions compared with abundances in the initial inoculum and substrate-free blank incubations.

Different wheat bran fractions possess distinct monosaccharide compositions

To identify potential mechanisms driving differences in microbial metabolism of wheat bran size fractions and its consequent impact upon community structure, we next investigated whether, in addition to differences in physical size, the variously-sized wheat bran fractions were chemically distinct. Accordingly, we measured the neutral sugar and starch contents of wheat bran fractions after they had been digested with salivary α-amylase, pepsin and pancreatin enzymes to mimic upper-gastrointestinal (GI) tract transit; this treatment removes the vast majority of accessible starch (**Fig. 6**). No significant differences in the proportions of rhamnose, mannose, and galactose were observed among the samples ($p < 0.05$) (**Fig. 6a**). The finest wheat bran particles contained the highest amount of glucose (47.92 %), and the glucose proportion of the brans decreased as the particle size increased (**Fig. 6a**). In wheat, the two main sources of glucose are starch and cellulose, so the majority of the glucose measured in the neutral sugar analysis likely belonged to one of these two polysaccharides. We then measured the total starch content of the samples to determine whether the observed glucose proportions correlated with particle starch content (**Fig. 6b**). Indeed, the finest bran particles displayed the greatest amount of starch (11.72 %) and, as particle size increased, starch content decreased (with the coarsest bran displaying less than 1% starch). These data strongly suggested that that the distinctions in the glucose proportions measured by neutral sugar analysis arose from differences in the starch content of the wheat bran fractions. However, these starches were not removed by treatment with upper-GI-simulating amylase treatment, suggesting that the starches measured are appropriately classified as resistant.

We also found significant differences in the proportions of xylose and arabinose among the size fractions ($p < 0.05$). We observed direct relationships between wheat bran particle size fraction and xylose and arabinose content, with the finest bran fraction displaying the lowest proportions

of xylose (22.66 %) and arabinose (24.06 %) (**Fig. 6a**). The relative proportions of these sugars increased with bran size fraction, with the coarsest bran possessing the highest proportions (37.39 %, and 38.68 % xylose and arabinose, respectively) (**Fig. 6a**). Xylose and arabinose are the building blocks of arabinoxylan polymers, with the former composing the backbone of the molecule and the latter forming the branching points (Rumpagaporn et al., 2015) . Therefore, the arabinose-to-xylose ratio is useful in estimating the branch density of the molecule. The arabinose to xylose ratio among bran size fractions were not significantly different ($p < 0.05$) (**Fig. 6c**), suggesting similar arabinoxylan structure among size fractions.

Figure 6: a) Neutral monosaccharide compositions of the wheat bran samples (%, mole basis). b) Total starch contents of the samples. c) Arabinose:xylose ratio of the samples.

a) Neutral monosaccharide compositions of the wheat bran samples (%, mole basis). Mean values of each constituents were compared across the samples, and those with the same letter are not significantly different (Tukey's test, $P < 0.05$). No letter was included in case the mean values are not statistically different (Tukey's test, $P < 0.05$). **b)** Total starch contents of the samples. Error bars represent the standard error of the mean of three separate replicates. Mean values with the same letter are not significantly different (Tukey's multiple comparisons test, $P < 0.05$). **c)** Arabinose:xylose ratio of the samples. Error bars represent the standard error of the mean of three separate replicates. No statistical differences were observed between the mean values (Tukey's multiple comparisons test, $P < 0.05$).

a)

0.18-0.30 mm
- 1.20%
- 24.06% E
- 22.66% B
- 1.27%
- 2.89% AB
- 47.92% A

0.30-0.50 mm
- 1.20%
- 31.32% D
- 32.40% B
- 2.86% A
- 1.30%
- 31.05% AB

0.50-0.80 mm
- 1.00%
- 33.40% CD
- 30.68% B
- 2.98% A
- 1.02%
- 30.92% AB

0.85-1.00 mm
- 1.33%
- 34.13% BCD
- 22.98% BC
- 1.44% B
- 0.81%
- 39.32% A

>1.70 mm
- 1.19%
- 38.68% A
- 17.89% C
- 3.08% A
- 1.78%
- 37.39% A

Legend:
- Rhamnose (blue)
- Arabinose (red)
- Xylose (green)
- Mannose (purple)
- Galactose (orange)
- Glucose (light green)

b) Starch content (%)
- 0.18-0.30 mm: A
- 0.3-0.5 mm: BC
- 0.5-0.8 mm: B
- 0.85-1.0 mm: C
- >1.7 mm: D

c) Arabino:Xylose Ratio
- 0.18-0.30 mm
- 0.3-0.5 mm
- 0.5-0.8 mm
- 0.85-1.0 mm
- >1.7 mm

Coarse bran fractions sequester starches from degradation by amylase that are enzyme-accessible in finer fractions

Though both involve amylase digestion, key differences exist between the upper-GI digestion protocol and starch content analysis that may explain the large amount of starch that seemed to evade upper-GI digestion of fine particles. The latter treatment includes a high-pH treatment that solubilizes otherwise-insoluble arabinoxylans, which may restrict enzymatic access to entrapped starch granules. Consequently, we reasoned that differences in particle size may strongly influence enzyme access to starches, which may manifest in the detected starch content via our method; we therefore hypothesized that coarse brans may contain an equivalent starch content to fine particles, but in a less-accessible form. To test this hypothesis, we further reduced the particle size of upper-GI-digested coarse bran particles (> 1700 μm) to < 500 μm using a cyclone mill, and then performed starch analysis again. We observed a twelve-fold increase (11.45 % vs. an original 0.87 %) in the detected starch content of coarse brans post-milling, supporting our hypothesis that the starch content among bran particles is similar, but enzyme access to these starches varies with size (**Fig. 7**). These results suggested that bran cell wall structures may exclude amylases from accessing entrapped starches, restricting access both to human amylases and to degradation by microbiota.
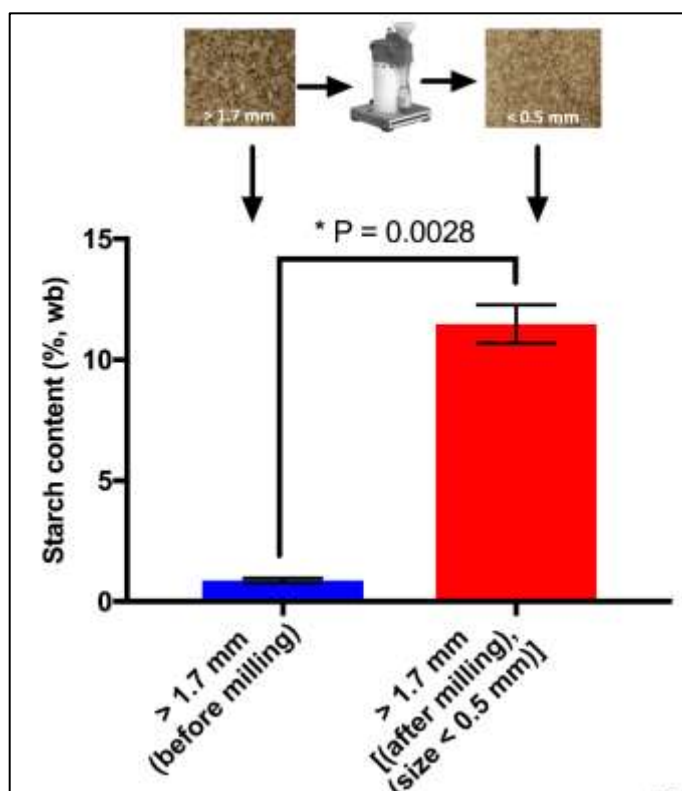
Figure 7: Total starch content measured on wheat bran sample having > 1700 μm particle size and that measured after reducing its size using cyclone mill with a 500 μm screen.

Error bars represent the standard error of the mean of three separate replicates. Statistical comparisons were made using a two-tailed Student's t-test.

Discussion

Consumption of specific dietary fibers has been shown to exert significant selective effects on bacterial taxa in the colon, at multiple taxonomic levels, due to variations in genetically encoded fiber-utilization abilities of different species (De Filippo et al., 2010; Sonnenburg et al., 2010; Koropatkin et al., 2012; Desai et al., 2016). These selective effects suggest that modulation of the gut microbiota's structure or function through variation in the type or quantity of dietary fibers consumed may be an effective strategy for countering the rising burden of chronic disease, which is increasingly recognized to be connected to the gut microbiome (Rook et al., 2013; Pallister and Spector, 2016). However, design of such strategies requires a mechanistic understanding of the

interactions between dietary fiber types, (along with their associated physical and chemical structural variables) and the colonic microbiota, as subtle variations in carbohydrate structure are known to dramatically influence microbial responses to the substrate (Hamaker and Tuncil, 2014; Martens et al., 2014; Barratt et al., 2017). Many studies have been conducted to determine how variations in the chemical structures of dietary fibers impact the responses of individual bacterial species (Tuncil et al., 2017a) as well as overall colonic microbiota composition (Hughes et al., 2008; Martinez et al., 2010; Rumpagaporn et al., 2015). In contrast, studies focusing on interactions between physical structural variables (e.g., particle size) of insoluble fibers and colonic microbiota have heretofore been relatively sparing.

Here, we show for the first time that wheat bran particle size fraction strongly influences both the metabolism and composition of gut microbiota in *in vitro* fermentations. Alterations in community structure were evident at the family level, with coarser size fractions favoring members of *Lachnospiraceae* and finer fractions generally favoring members of *Bacteroidaceae*. Within these trends at higher taxonomic levels, however, differential responses to size fractions revealed competition at the species or strain level within genus *Bacteroides*. In some cases, organismal relative abundances displayed nearly-linear relationships with fraction size; for example, the relative abundance of *Bacteroides* OTUs OTU3 (similar to *B. dorei*) generally decreases as size increases. In contrast, *Bacteroides* OTU6 and *Coprococcus* OTU8, and unclassified *Lachnospiraceae* OTUs OTU12 and OTU15 abundances increase nearly linearly with size.

Even though members of *Bacteroides* members are typically regarded as generalists that can digest a broad range of plant cell-wall polysaccharides (Dodd et al., 2011; Martens et al., 2011; Koropatkin et al., 2012; El Kaoutari et al., 2013), they seem to be better adapted to the degradation of soluble fibers compared to insoluble substrates, including wheat bran particles (Leitch et al.,

2007; Flint et al., 2008; Walker et al., 2008). Only one cellulolytic *Bacteriodes* sp., *B. cellulosilyticus*, has been described to date (Robert et al., 2007). In contrast, members of *Lachnospiraceae* are widely reported to be fibrolytic and, along with the members of neighboring family *Ruminococcaceae*, play an important role in degradation of plant cell wall polysaccharides, including cellulose (Flint et al., 2012). These microbial taxa are known to ferment cell wall polysaccharides and starches to butyrate (Louis and Flint, 2009; Reichardt et al., 2014). As members of *Bacteroides* are known to be more commonly propiogenic than butyrogenic (Louis et al., 2007), the abundance of *Bacteroides* in association with fine particle fractions may explain elevated propionate molar ratios in these cultures.

One potential explanation for these relationships is that differential starch availability due to size fraction early in microbial succession on finer and coarser particles governed organismal abundances at the end of the experiment. Relatively large abundances of *Bacteroides* species consuming finer particles may be driven by relatively easy, early access to entrapped starches in these size fractions, as this genus has been reported to contain efficient starch degraders (Martens et al., 2011; Cameron et al., 2014). Relatively little degradation of cell wall polysaccharides may be required to gain access to entrapped starches in small bran particles compared with larger ones. This may arise either due to differences in starch content among fractions or in particle surface area-to-volume ratios that govern rates at which starches are made available; as we demonstrated differences in neutral sugar content (but similar starch content of the coarsest particle fraction to finer fractions after milling) among particles, the present study cannot discern which is more likely true. However, it is noteworthy that, though butyrate production from the coarse particle fraction at 48 h post-inoculation was similar to that of fine particles at earlier time points, the microbial community structure associated with coarse particles did not approach that of smaller particles

over time. This suggests that the kinetics of starch release may be as or more important for controlling microbial succession on wheat bran particles than total starch content.

Another interesting finding from this study is that the coarsest bran was as butyrogenic as FOS, and this butyrate formation occurred slowly. This further suggests that coarser wheat bran particles may deliver more butyrate into more distal regions of the colon than finer ones. Thus, coarse wheat bran particles could have possibly important practical implications as the majority of chronic colonic diseases, such as ulcerative colitis, inflammatory bowel diseases, and colorectal cancer occur in the more distal regions of the colon (Bufill, 1990; Cummings, 1997; Rose et al., 2007) and butyrate has been implicated as important for mitigating these disease conditions (Segain et al., 2000; Louis et al., 2014).

Though previous studies have detected differences in SCFA production from different wheat bran size fractions, our study holistically links size-dependent metabolic outcomes with underlying microbiota structures. Our results agree with one previous study investigating SCFA production from fine and coarse wheat bran size fractions (Stewart and Slavin, 2009). Stewart and Slavin reported that fermentation of coarse wheat bran particles (average particle size is 1239 µm) by fecal microbiota resulted in higher butyrate production compared to fine wheat bran fractions (average particle size is 551µm) 24 h post-inoculation (Stewart and Slavin, 2009). However, in the experiment of Dziedzic and coworkers, fine wheat bran (average particle size ~ 90 µm) produced more butyrate than coarse bran (average particle size ~ 500 µm), though the incubation time was not reported (Dziedzic et al., 2016). In addition to differences in the particle sizes investigated, disagreement in the results may also stem from distinct fermenting bacteria; Dziedzic and coworkers use cultivation-based methods and report only the abundances of *Enterococcus*, *Bifidobacterium*, *Escherichia coli*, and *Lactobacillus*. It is unclear whether other species were

present in the inoculum, as the organisms were described as fecal isolates. In a more recent *in vivo* study, where mice were fed a Western-simulating diet supplemented with either coarse (particle size: 1690 µm) or fine (particle size: 150 µm), similar in size range to that examined in the present study, wheat bran indicated no significant changes in the SCFA levels in the cecum of the mice; however, coarse (but not fine) bran fractions promoted fat excretion and increases in the abundance of genus *Akkermansia* (Suriano et al., 2017). Though these data hint that different bran size fractions may exert distinct physiological impacts *in vivo*, previous studies have not investigated this effect at high size resolution. Near-linear relationships between microbial metabolism and community structure in the present study suggest that increasing size resolution may help tease out *in vivo* relationships between bran fraction size and microbiota and host physiological outcomes, as the number of potentially-confounding variables is much greater *in vivo*.

Importantly, this study reveals differences in the chemical structures of wheat bran size fractions (**Fig. 6**), even though these brans were fractionated by directly sieving the wheat bran obtained from the same initial sample (i.e., from the same container). This observation has two important implications. The first is that variation in chemical structures might be the driving force for the formation of different particle sizes during the milling process. This leads naturally to the second implication, that these chemically-distinct size fractions do not perform identically in their interaction with the gut microbiota. Taken together, these data suggest that future studies involving the interaction of wheat brans (and possibly other insoluble fiber sources) with the gut microbiota both *in vitro* and *in vivo* should measure and report physical characteristics – such as particle size – of the bran sources employed. Unreported differences in size fractions may otherwise drive inconclusive or confusing results due to differences in physical structure, chemical composition, and/or fermentation by the microbiota.

In a broader sense, our data support the idea that physical structuring of insoluble particles may lead to significant differences in spatial patterning of substrates at relatively-fine spatial scales (on the order of hundreds of microns), which in turn leads to alternate microbial succession as substrates for some organisms are made available due to the metabolism of others. As such, this suggests that bran particles may function ecologically as patchy habitats in which non-homogenous patterning of resources (Silver et al., 2000) drives microbial metabolism and community structure outcomes. However, it further suggests that differences in these spatial patterns give rise to temporal patterns in resource availability, from which distinct succession and trophic networks arise through interactions among organisms in particle-degrading consortia (Datta et al., 2016). We submit that future studies should 1) focus on isolation of bran particle size as a variable distinct from chemical composition (if possible), 2) advance mechanistic understanding of bran resource distribution and fermentation by gut microbiota, and 3) reconstruct the interaction networks that result from bran particle fermentation to determine the extent to which spatial patterning drives the structure and function of microbial food webs.

CHAPTER 3: MAIZE BRAN PARTICLE SIZE STUDY

Summary:

Differences in the chemical and physical properties of dietary fiber are increasingly known to exert effects on their fermentation by gut microbiota. Here we demonstrate that maize bran size fractions show metabolic output and microbial community differences similar to those we previously observed for wheat brans. We fermented maize bran particles varying in size *in vitro*, measuring their metabolic fate (i.e. short-chain fatty acids, SCFAs) and resulting community structure (via 16S rRNA amplicon sequencing). Metabolically, acetate, propionate, and butyrate (as well as the total amounts of all three) were size-dependent. 16S rRNA sequencing revealed that the size-dependent SCFAs production was linked to the differential microbial communities at genus and some at species level. These differences could also be linked back to the chemical differences (monosaccharides composition, starch and protein content) between the particle sizes. These results further suggest that the physical properties like bran size should be considered as a variable while designing fermentation studies.

Introduction

The human gut houses trillions of microorganisms and their physiological importance in human health is becoming increasingly understood. The microbial associates that live in and on the human body are collectively referred to as its microbiota (Clemente, Ursell, Parfrey, & Knight, 2012). The human gut is thought to be populated with 100 trillion microbial cells which code for approximately 100-fold more unique genes as the human genome (Ley et al., 2006), composing microbial communities important enough to be referred to as a 'forgotten organ' (O'Hara & Shanahan, 2006). Highly-important environmental factors influencing gut microbiome

populations are diet, host health status (including differences between healthy states, such as pregnancy), xenobiotic exposure, and antibiotic intake (Björkholm et al., 2009; Dethlefsen, Huse, Sogin, & Relman, 2008; Koren et al., 2012; Turnbaugh et al., 2009). Out of the above factors, diet is a very significant driver of the population sizes of different gut-dwelling microbial species (Ley et al., 2008).

Colonic microbes ferment carbohydrates and proteins that escape human digestion in the small intestine (Koropatkin, Cameron, & Martens, 2012), metabolizing dietary fibers and resistant starches into short-chain fatty acids (SCFAs) that are largely absorbed by the host (Ohira, Tsutsui, & Fujioka, 2017). The main terminal SCFAs in humans produced by fermentation in the gut are acetate, propionate and butyrate (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). Dietary fiber intake is strongly linked with maintenance of microbial diversity; in mice fed a low-MAC (microbiota-accessible carbohydrates) diet drastically reduces the diversity of microbiota over a few generations (Sonnenburg et al., 2016) compared with high-MAC-fed controls. Variation in overall dietary fiber intake is also linked to changes in the population and abundances of gut specific microbiota due to their different fiber-metabolizing capacities (Sonnenburg et al., 2010) However, it is increasingly obvious that it is not just overall quantity of dietary fiber that is important for structuring the gut microbiome; consumption of different dietary fiber sources is associated with growth in specific microbial populations in the gut (Dominianni et al., 2015). Therefore, use of different dietary fibers has gained attention as a potential mechanism for strategically altering the gut microbiota.

Widespread consumption of the high-sugar/high-fat and low-fiber 'Western' diet is thought to be linked to changes in the genetic composition and metabolic activity of our gut microbes compared with more-traditional diets (Turnbaugh et al., 2009). In Western countries,

approximately 50% of dietary fiber intake is cereal-derived (mostly from wheat, maize, oat, barley, and rye) (Gebruers et al., 2008; Lambo et al., 2005). However, one often-overlooked correlate of the overall reduction in dietary fiber intake with westernization of diet is a reduction in particle sizes of grains. Specifically, milling methods changed from stone milling to mechanized steel roller mills and automated sifting devices (Teague, 1952). In the former case, flours included the entire content of cereal grain (i.e., germ, bran and endosperm fractions) unless the flour was sieved (Teague, 1952). In the latter case, refined flour is comprised mainly of endosperm of very small particles due to the removal of germ and bran during the milling process. Brans are typically milled separately to small particle sizes and added back to refined flour to produce whole grain flour (Teague, 1952). The reduction in the particle size of the grains consumed is a potentially-critical but overlooked dietary change occurring in the process of westernization (Cordain et al., 2005).

The effects of particle size on microbial fermentation of dietary fibers are not well understood. Previous studies have focused on comparing coarse and fine wheat brans to evaluate size effects on transit time and laxation (Brodribb & Groves, 1978; Jenkins et al., 1999; Kirwan, Smith, McConnell, Mitchell, & Eastwood, 1974). Brodribb and coworkers measured stool weight after consumption of coarse and fine wheat brans and found larger increases with coarse bran than fine (Brodribb & Groves, 1978). They presented three possible reasons for this effect: first, that coarser brans have higher water-holding capacity; second, that coarser brans may be less digestible by gut bacteria; and third, that larger fiber particles might trap more gas produced by gut bacteria, thus increasing stool bulk (Walker, 1947). With respect to microbial fermentation, Stewart and Slavin found that coarse (average size=1239μm) and fine (average size=551μm) wheat brans elicited different short-chain fatty acid (SCFA) production (Stewart & Slavin, 2009).

Recently, we reported that different size fractions of wheat bran particles selected for different microbial communities and fermented to different metabolic outcomes. (Tuncil, Thakkar, Marcia, Hamaker, & Lindemann, 2018). The selective effects of bran particles were observed at very fine taxonomic levels, even within operational taxonomic units (OTUs, computational analogs of species) identified from the same genera, and were likely responsive to differences in chemical composition among differently-sized particles. To determine whether size effects were constrained to wheat bran particles or whether size-dependence was a more general properties of bran fermentation by gut microbiota, we milled maize brans into similar size ranges and performed the same fermentation experiment.

Our results from the present study strongly suggest that there is clear bran size-dependence in SCFA production and microbial community structure in maize brans as in wheat brans. These data suggest that size-dependence is likely to apply generally to colonic fermentation of bran particles by gut microbiota. Further, it supports the hypothesis that particle size may be manipulated by milling to foster targeted impacts upon gut microbiome structure and function.

Materials and Methods

Maize brans

Maize bran particles were a gift from Bunge Milling (St Louis, MO, USA). To obtain size fractions of maize bran, particles were sieved using a sieving machine (Portable Sieve Shaker Model RX-24, both sieving machine and screens were from W. S. Tyler Combustion Engineering, Inc., Mentor, OH.) The biggest size fraction obtained on sieving was 500 to 850 micron. This maize bran was further milled using a cyclone mill (FOSS North America, Eden Prairie, MN, USA) to obtain smaller particle sizes to ensure that all the size fractions were from the same parent

source of maize bran. We obtained 4 sizes of maize bran particles : 1) 180-250 μm 2) 250-300 μm 3) 300-500 μm and 4) 500-850 μm using sieving machines and screens both from W.S. Tyler Combustion Engineering, Inc., Mentor, OH. These size fractions were used for further experiments.
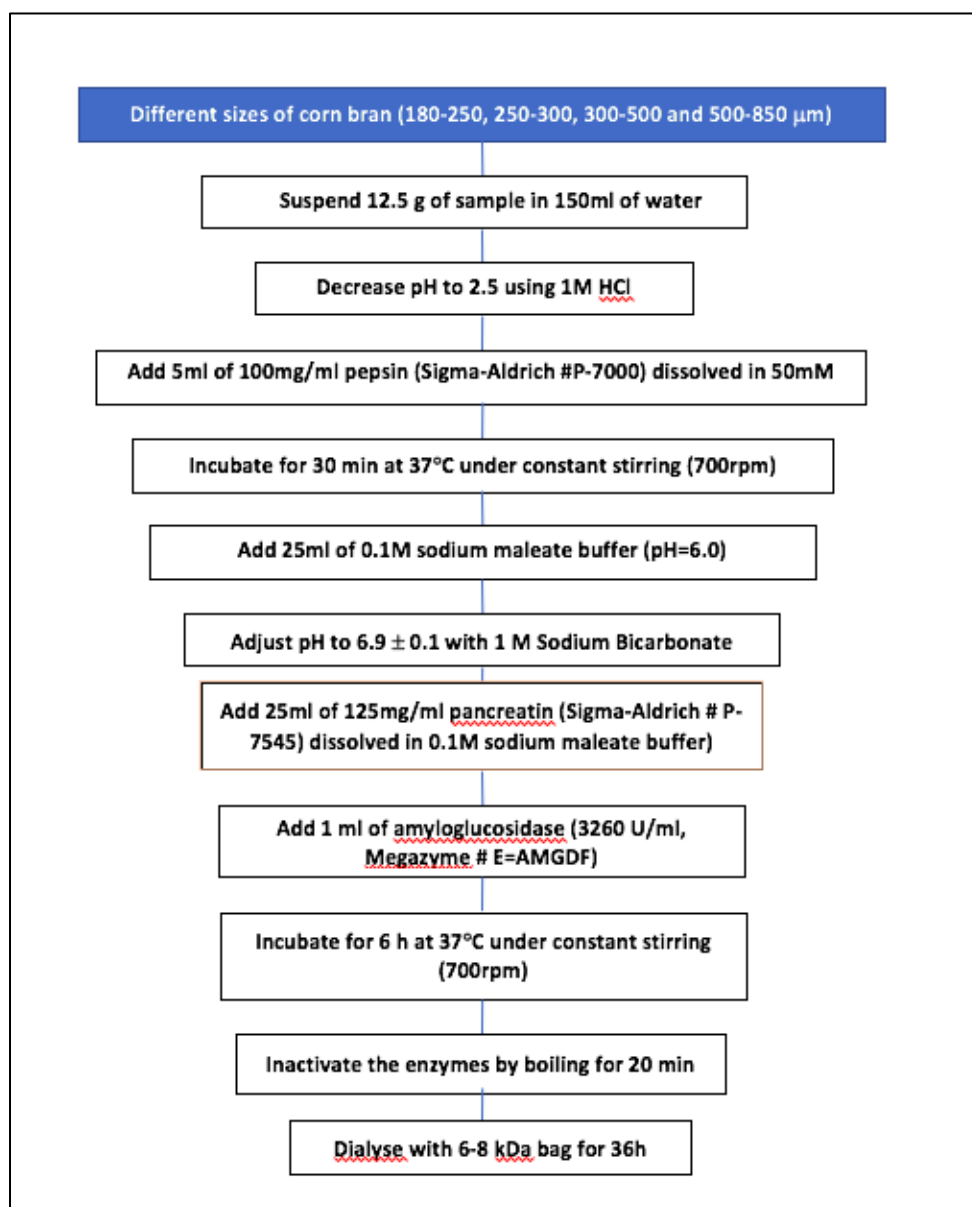

Figure 8: Flow chart of upper GI tract digestion method used for maize bran of different particle sizes

This method was adopted from Mishra and Monro (Mishra & Monro, 2009a) and modified by Yang et al. (Yang, Keshavarzian, & Rose, 2013) method.

Upper gastrointestinal digestion of brans

We simulated passage through the upper gastrointestinal (GI) tract of the different sizes of maize bran particles obtained after milling and sieving through *in vitro* digestion as previously described (Yang et al., 2013) as shown in **Figure 8**. . We chose this method for simulated digestion because we recently determined that this method is generally more effective at removal of starch content for maize (Tuncil, Thakkar, Arioglu-Tuncil, Hamaker, & Lindemann, 2018). During the upper GI digestion, the maize bran particles were treated with pepsin, pancreatin and amyloglucosidase for 30min, 6h and 6h respectively. This was followed by dialysis for 36h and freeze drying prior to *in vitro* fermentation.

Neutral monosaccharide composition analysis

We analyzed neutral monosaccharides from different maize bran particle sizes post upper GI tract digestion using gas chromatography coupled with mass spectroscopy as described previously (Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). Helium was used as carrier gas.

Total starch content analysis

After upper-GI tract digestion, total starch content of the different sizes of maize bran were analyzed. The total starch content were analyzed spectrophotometrically using the total starch assay kit (K-TSTA; Megazyme International, Wicklow, Ireland) according to the manufacturer's instructions.

***In vitro* fermentation**

We performed *in vitro* anaerobic fermentations of maize bran particles of distinct sizes using fecal microbiota as inocula within an anaerobic chamber (Coy Laboratory Products Inc.,

Grass Lake, MI). Different sizes of maize bran samples 1) 180-250 μm 2) 250-300 μm 3) 300-500

μm and 4) 500-850 μm were weighed (44 ± 1 mg) and placed in Balch tubes. (Chemglass Life

Sciences, Vineland, NJ). The anaerobic chamber was supplied with the following gas mix: 90%

N_2, 5% CO_2 and 5% H_2. We transferred the tubes containing maize bran particles, FOS and blank

in the anaerobic chambers and equilibrated them with the chamber atmosphere. For all

experiments, we used the previously-described phosphate-buffered gut mineral medium

containing trace elements (Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). The media was placed

in the anaerobic chamber overnight to remove oxygen and resazurin was used as the oxygen

indicator.

On the day of innoculation, we added 4 ml of gut mineral medium to all the tubes with

different sizes of maize bran, the blank tubes and a fermentation positive control containing

fructooligosachharides (FOS; Sigma-Aldrich, St. Louis, MO). We collected and pooled fecal

samples from 3 healthy donors (2 male, 27 and 32 years old, respectively; 1 female, 31 years old,

all three were omnivores) and pooled as previously described (Tuncil, Thakkar, Marcia, et al.,

2018). Human stool collection and use protocols were reviewed and approved by Purdue

University's Institutional Review Board (IRB Protocol #1701018645). Pooling of fecal samples

provides a diverse pool of species that is not limited by an individual's idiosyncratic gut

microbiome (Tuncil, Thakkar, Marcia, et al., 2018). A past study has shown that pooling of fecal

microbiota shows similar microbial responses compared to similar experiments using individual

donors (Aguirre, Ramiro-Garcia, Koenen, & Venema, 2014). The donors had been following their

habitual diets and had not taken any antibiotics at least 12 weeks before the study began. To prevent

the loss of bacterial viability, the fecal samples were collected and sealed as quickly as possible in

50 mL Falcon tubes, kept on ice, and rapidly transfered to the anaerobic chamber. We inoculated

the bran particles and media with fecal slurry within 2 hours of receiving the fecal microbiota from donors. We mixed the fecal samples and gut mineral media in the ratio 1:10 (w/v), and then filtered through 4 layers of cheese cloth. After filtration, the we pooled the fecal slurries from individual donors in equal ratios (Aguirre et al., 2014). We added 0.4ml of the pooled fecal slurry mix to each Balch tube, closed the balch tubes with butyl rubber stoppers, sealed them with aluminium seals(both from Chemglass Life Sciences), and incubated them at 37°C in a shaking incubator (Innova 42, New Brunswick Scientific, Edison, NJ) at 150 rpm at an approximately 45° angle. Fermentations was performed in triplicate.

Gas, pH, and SCFA measurements

At 6, 12, 24, 36, and 48 h time points after inoculation, we measured total gas production from fermentation as overpressure using a graduated syringe and passing a needle through the rubber stopper prior to unsealing the tubes. For the pH measurement, the supernatant was transferred to a separate 15ml Falcon tube and measured using a pH meter. We collected two aliquots from each tube, one for SCFA (0.4 ml) and other for DNA extraction (1 ml) and stored the aliquots at -80°C . An internal standard (157.5μl of 4-methyl valeric acid, 1.47ml of 85% phosphoric acid, 39mg of copper sulfate pentahydrate in a total volume of 25ml) was immediately added to the SCFA aliquots samples before vortexing.

We measured SCFAs as previously described (Tuncil, Thakkar, Marcia, et al., 2018). Briefly, 4 μl of the supernatent were analyzed on fused silica capillary column (Nukon™, SUPELCO No: 40369-03A, Bellefonte, PA) using a gas chromatograph (GC-FID 7890A, Agilent Technologies Inc.) (Tuncil et al., 2017). We used 4- methylvaleric acid (Fisher Scientific) as an

internal standard and acetate, propionate and butyrate (Fisher Scientific, Hanpton, NH, USA) to generate standard curves.

DNA extraction

FastDNA SPIN® kit for Feces (product code: 116570200) was used for DNA extraction. The protocol was followed the same as described in the user's manual (MP Biomedical, Santa Ana, USA) with the following modifications: as a first step before following the protocol, we thawed the DNA, centrifuged the samples at 13,000 rpm for 10 mins, and discarded the supernatant. We then added 825 µl of phosphate buffer as in the first step of protocol to resuspend the pellet by pipetting it multiple times. We followed the user's manual for the rest of the protocol.

16S rRNA sequencing

We amplified the V4-V5 region of 16S rRNA gene by PCR using the universal bacterial primers: 515-FB (GTGYCAGCMGCCGCGGTAA) and 926-R (CCGYCAATTYMTTTRAGTTT) as previously described (Tuncil, Thakkar, Marcia, et al., 2018). The PCR cycle paramters were : 1) initial denaturation: 95 °C for 5 minutes, 2) denaturation: 98 °C for 20 seconds, annealing: 60 °C for 15 seconds, extension: 72 °C for 30 seconds for each of 22 cycles, and 3) sinal extension: 72 °C for 10 minutes. After this, the samples were held at 4 °C. From this amplified product we cleaned up the leftover unattached primers, primer-dimers and dNTPs using the AxyPrep Mag PCR Cleanup Kit (Corning, Inc. Corning, NY), which was followed by barcoding the PCR product using the TruSeq dual indexing approach for 5 cycles and a final clean up step to remove excess primers and primer dimers, if any, all as previously described in detail (Tuncil, Thakkar, Marcia, et al., 2018). We quantified the cleaned,

barcoded amplicons using Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA), and grouped and pooled amplicons according to similarities in concentration. The Purdue Genomics Core Facility performed quality control for pools by running 1 μl of each pool on an Agilent Bioanalyzer with a high ensitivity chip (Agilent, Santa Clara, CA) and then quantified the the pool loading via the KAPA Library Quantitation Kit for Ilumina Platforms. Sequencing was then performed using an Ilumina MiSeq run with 2 x 250 cycles and V2 chemistry (Ilumina, Inc., San Diego, CA).

Sequence processing and community analysis

Sequences were processed as previously described (Tuncil, Thakkar, Marcia, et al., 2018). Briefly, we used mothur v. 1.39.5 according to the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP [accessed 12/1/2017]) with the following modifications: sequences were screened with a maximum length of 411 and maximum homopolymer length of 9. For classification, we used the mothur-formatted version of the RDP training set v. 16 to which species eptihets had been added and classified sequences at a bootstrap cutoff of 95%. We removed sequences classified as chloroplasts, mitochondria, or within *Eukarya*. Groups were subsampled to 2462 reads to normalize sampling effort across samples. Ecological α-diversity metrics were calculated using the nseqs, coverage, sobs, chao, simpsoneven, invsimpson, and shannon calculators in mothur. β-diversity metrics were generated using the braycurtis, thetayc, and jclass calculators and plotted using the pcoa calculator in mothur. Linear discriminant analysis was performed using LEfSe v. 1.6 at an LDA cutoff of 3.5.

Statistical data analysis

We used GraphPad Prism version 8.0.1 (GraphPad Software, Inc. La Jolla, CA) for statistical analysis. ANOVA (Analysis of Variance) at $α = 0.05$ was performed to identify if there

were any significant differences amongst treatments. The ANOVA test was followed by Tukey's multiple comparison test at α = 0.05 for SCFA analysis and neutral monosaccharide, starch and protein composition analysis to identify if means differed significantly between treatments. The PCoA plot was generated using R, version 3.5.1, using the vegan package.

Results

SCFA production from maize bran fermentation is particle size-dependent

To test our hypothesis that the fermentation of maize bran is particle size-dependent, we performed *in vitro* fermentation with different sizes of maize bran. After *in vitro* fermentation, we measured the gas production, pH, and short chain fatty acid (SCFA) composition at 0, 6, 12, 24, 36, and 48 h. **Figure 9** shows significant differences among size fractions in acetate, propionate, butyrate, and total SCFAs produced at different time points; acetate, propionate, and butyrate were produced in a roughly 75:13:12 ratio across all size fractions. Fast-fermenting FOS was used as a positive control. The smallest size fraction (180-250μm) was much more extensively fermented, eliciting the highest amount of all three SCFAs (acetate, propionate and butyrate) at all time points. The other three sizes (250-300 μm, 300-500μm, 500-850μm) produced less than half the amount of the smallest size for all the SCFAs. Even given the much smaller differences in SCFA production among the three larger sizes, acetate, propionate, and butyrate were all significantly higher in the 250-300 μm size cultures; the 300-500μm and 500-850μm fractions were only slightly fermented, being insignificantly different from one another and only approximately double concentrations observed in no-carbon controls. These data strongly suggested that the fate of particles was different among size fractions,most notably due to greater rates and extents of fermentation by microbiota for the smallest fractions.
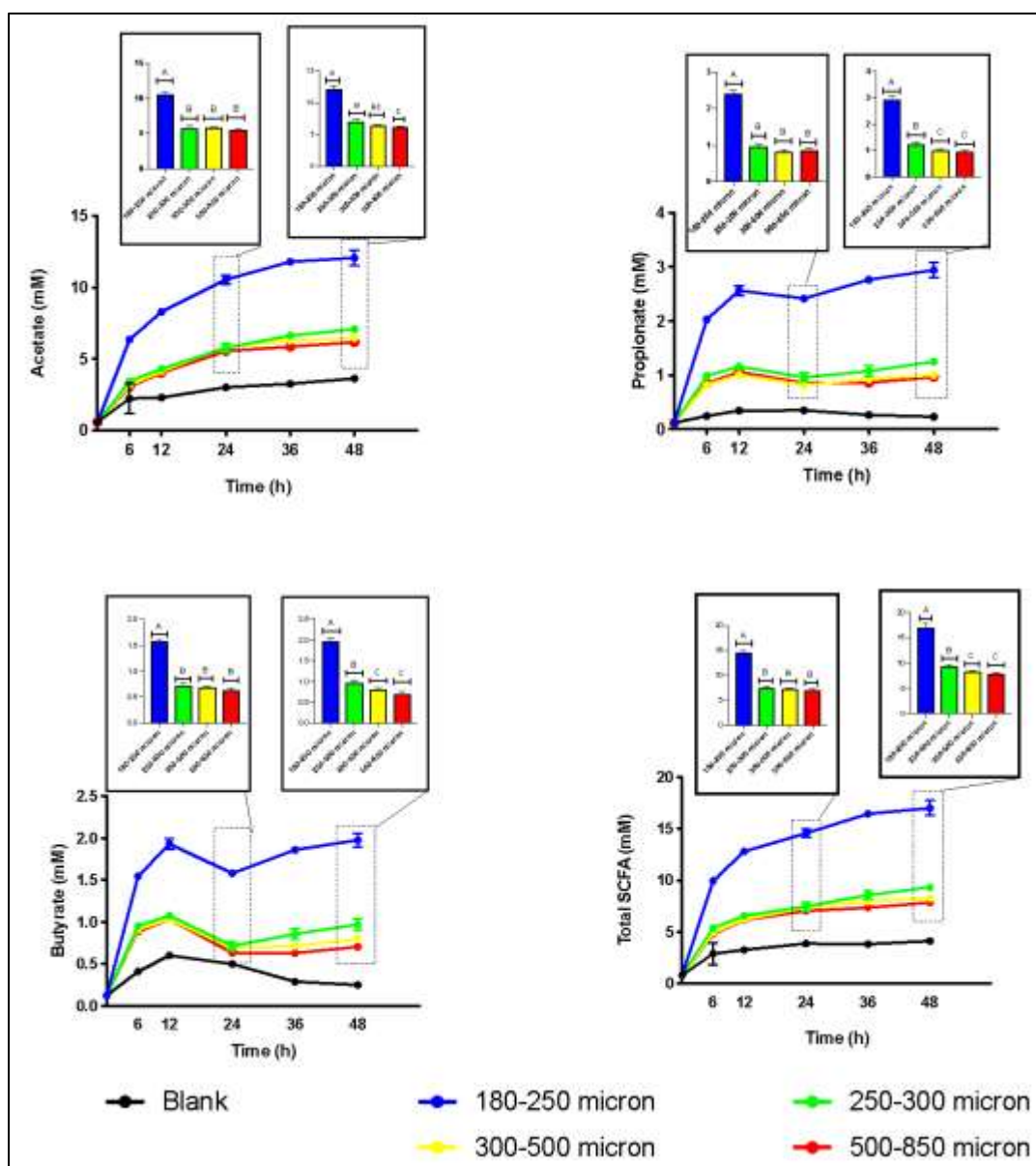
Figure 9: Short-chain fatty acid (SCFA) production by maize bran particles of different sizes over fermentation.

   The x-axis represents time in hours and y-axis represents concentration of SCFA in mM. Blanks contain no carbon source. Bar graphs represent the concentrations of SCFAs at 24h and 48h. Total SCFA displays the sum of all three acetate, propionate, butyrate. Error bars represent the standard error of mean of the triplicates. In the bar graph, the values with same letters are not significantly different using Tukey's test and $p < 0.05$.

Different particle sizes of maize bran selected for distinct microbial community structures

   Particle size also significantly influenced the community structure of fermenting

microbiota. **Figure 10** shows changes in microbial community structure over 12 h, 24 h and 48 h

of *in vitro* fermentation. After 12 h, separations among communities consuming different particle sizes were small; however, by 24 h, the smallest particle size (180-250 μm) began to separate in community structure from the other three bigger sizes, which clustered together. After 24 h, differences emerged among the communities fermenting bran particles larger than 250 μm. We observed apparent separation between the 180-250 μm fraction, the 250-300 μm fraction, and the 500-850 μm fraction after 48 h; however, two replicates of the 300-500 μm fraction more resembled the 250-300 μm fraction and one clustered with the 500-850 μm size. These data suggested divergent microbial community structures develop in fermentation of differently-sized maize bran particles.
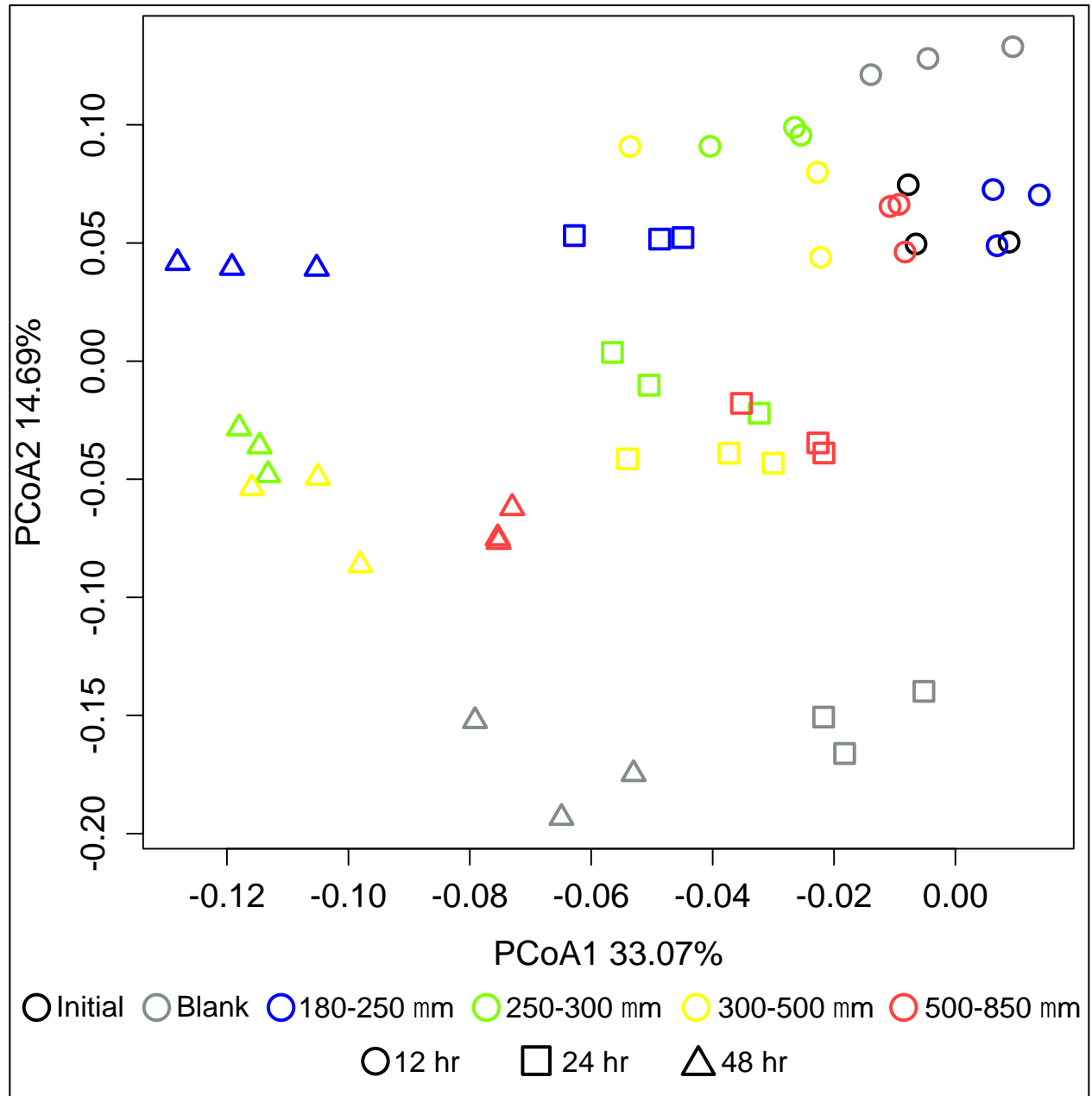
Figure 10: Differences in microbial community structure between maize bran fermenting pooled fecal microbiota from three healthy donors.

Principal component analysis(PCoA) of maize bran size fractions were determined by 16S rRNA amplicon sequencing, and β-diversity metrics were based upon Bray-Curtis dissimilarity calculated using from OTU relative abundances after 12 h, 24 h and 48 h of *in vitro* fermentation. Blank represents a no-carbon control.

Microbial preferences for maize bran particle sizes occurred at fine taxonomic resolution

As in our previous study of fermentation of wheat bran particle size fractions (Tuncil, Thakkar, Marcia, et al., 2018), in the case of maize bran we also observed that OTU abundances related to maize bran particle sizes **(Fig. 11)**. We used LEfSe to identify statistically-significant linear discriminants of three size fractions: small (180-250 μm), medium (250-300 μm), and large (500-850 μm) **(Fig 12)**. We omitted the 300-500 μm group from the analysis, as its replicates were not clearly separated from the smaller and larger size categories **(Fig. 11).** Relative abundances of reads classified taxonomically corresponded to particle size; small particles generally favored members of families *Ruminococcaceae* and *Porphyromonadaceae* (specifically, genus *Parabacteroides*), and medium particles for family *Bacteroidaceae*. However, as we observed for wheat brans, relationships of taxa to maize bran particle sizes frequently occurred at fine taxonomic levels (**Fig. 5**; Tuncil, Thakkar, Marcia, et al., 2018). For example, though members of family *Bacteroidaceae* as a whole were linear discriminants of medium-sized particles (typified by *Bacteroides ovatus*), *B. uniformis* was enriched on small particles and members *Bacteriodes* unclassified at the species level were enriched on large particles. Linear discriminants for large particles also included *Coprococcus eutactus* and members of *Coriobacteraceae* unclassified at the genus level.

We also observed species-level preferences for maize bran particle size at the level of individual OTUs. OTUs classified within *Ruminococcaceae* were divided amongst preferences for small and large particles; OTU5 (*Ruminococcus bromii*), OTU33 *(Ruminococcus callidus)*, OTU35 and OTU49 (both *Ruminococcaceae* sp.) were linear discriminants for small particles, whereas OTU32 (*Ruminococcaceae* sp.) were enriched on large particles. Similarly, OTUs within family *Lachnospriaceae* showed differential size preferences; OTU37 (*Lachnospiraceae* sp.)

preferred smaller particles, whereas OTU22 (*Lachnospiraceae* sp.) and OTU47 (*Roseburia* sp.) were most successful on medium-size particles. As observed for higher taxonomic classifications, OTUs within *Parabacteroides* also were linear discriminants of small particles and *B. eggerthii* of medium particles. Taken together, these data suggest that preferences for different maize particle size are properties of individual species or strains.

Figure 11: Relative abundances of top 50 OTUs arising in fermentation of maize bran particles diverging in size.

OTU abundances were log$_2$-transformed for display in the heatmap. Blank represents a no-carbon substrate control, and fructooligosaccharide (FOS) is a fast-fermenting positive control
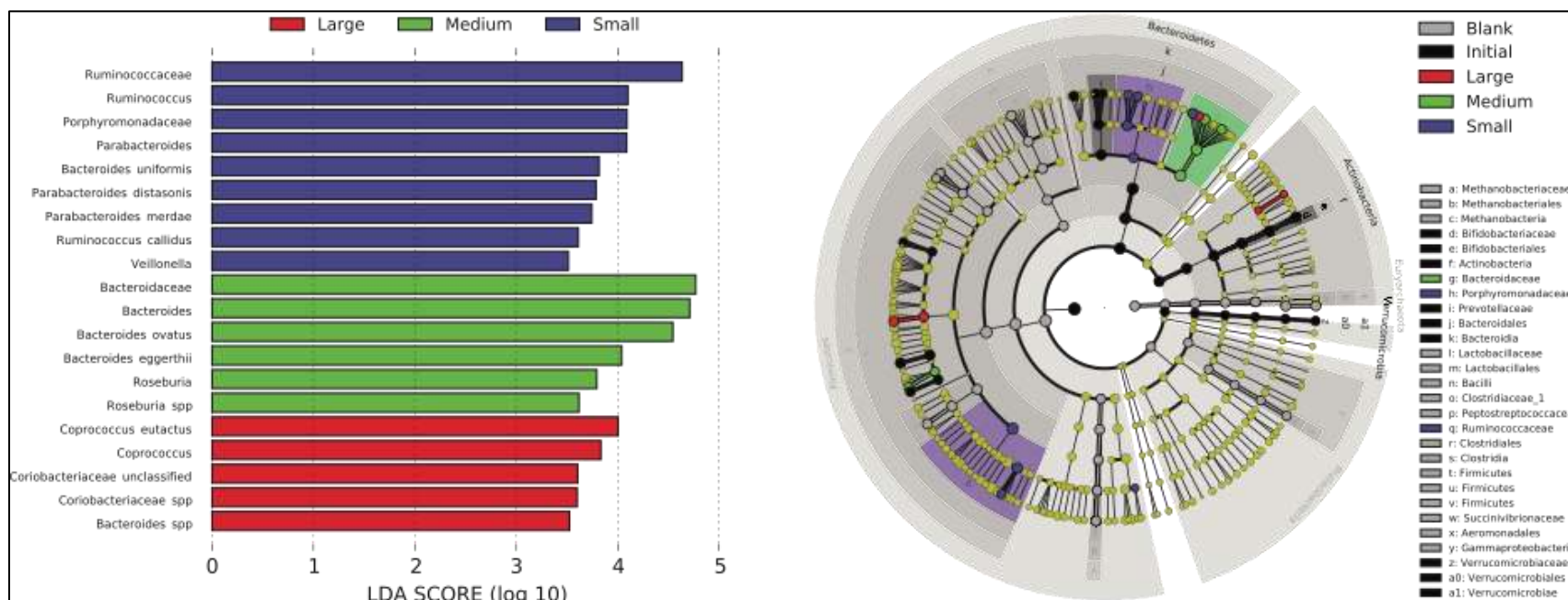
Figure 12: Linear discriminant analysis of taxa representing the small, medium, and large sizes of maize bran. We also included initial and blank communities in the analysis in order to avoid misrepresentation of taxa to size fractions that were overrepresented in controls. **a)** Taxa with LDA scores > 3.5 in the Small (180 – 250 μm), Medium (250-300 μm) and Large (500-850 μm) size fractions at 48 after inoculation; we did not show linear discriminants of initial and blank conditions. **b)** Cladogram showing taxa that are overrepresented in the small, medium, and large maize bran fractions comparing to abundances in the initial inoculum and blank (without substrate).

Different maize bran particle sizes have different chemical compositions

To attempt to identify mechanisms driving maize bran particle size effects on microbial community structure and function, we examined the chemical compositions of each size fraction to identify if there were differences among different maize bran particle sizes. We measured neutral monosachharide, starch, and protein contents in each of the maize bran particle sizes after the bran particles had been treated by *in vitro* upper-GI digestion (see Methods).

Figure 13 shows the neutral monosaccharide composition of different sizes of maize bran particles. The smallest size (180-250 micron) has significantly higher ($p < 0.05$) amount of glucose compared to the bigger size fractions. Mannose and galactose were also in significantly ($p < 0.05$) higher amounts in the 180-250 micron size range compared to larger sizes. In contrast, arabinose and xylose were in significantly smaller amounts ($p < 0.05$) in the smallest maize bran particle size compared to larger sizes. There were no significant differences observed in any of the five sugars (glucose, mannose, galactose, arabinose and xylose) across the larger three particle sizes of maize bran. Arabinose and xylose are the building blocks of the arabinoxylan polymer, with xylose as the backbone and arabinose forming the branching points (Rumpagaporn et al., 2015), suggesting relatively lower arabinoxylan content in the smallest size fraction. Additionally, the arabinose:xylose ratio was subtly higher in the smallest particle size range. This, combined with statistically-significant elevations in galactose and mannose content, suggest that arabinoxylans within the smallest size fraction may be sigificantly more branched than in the largest particle sizes.
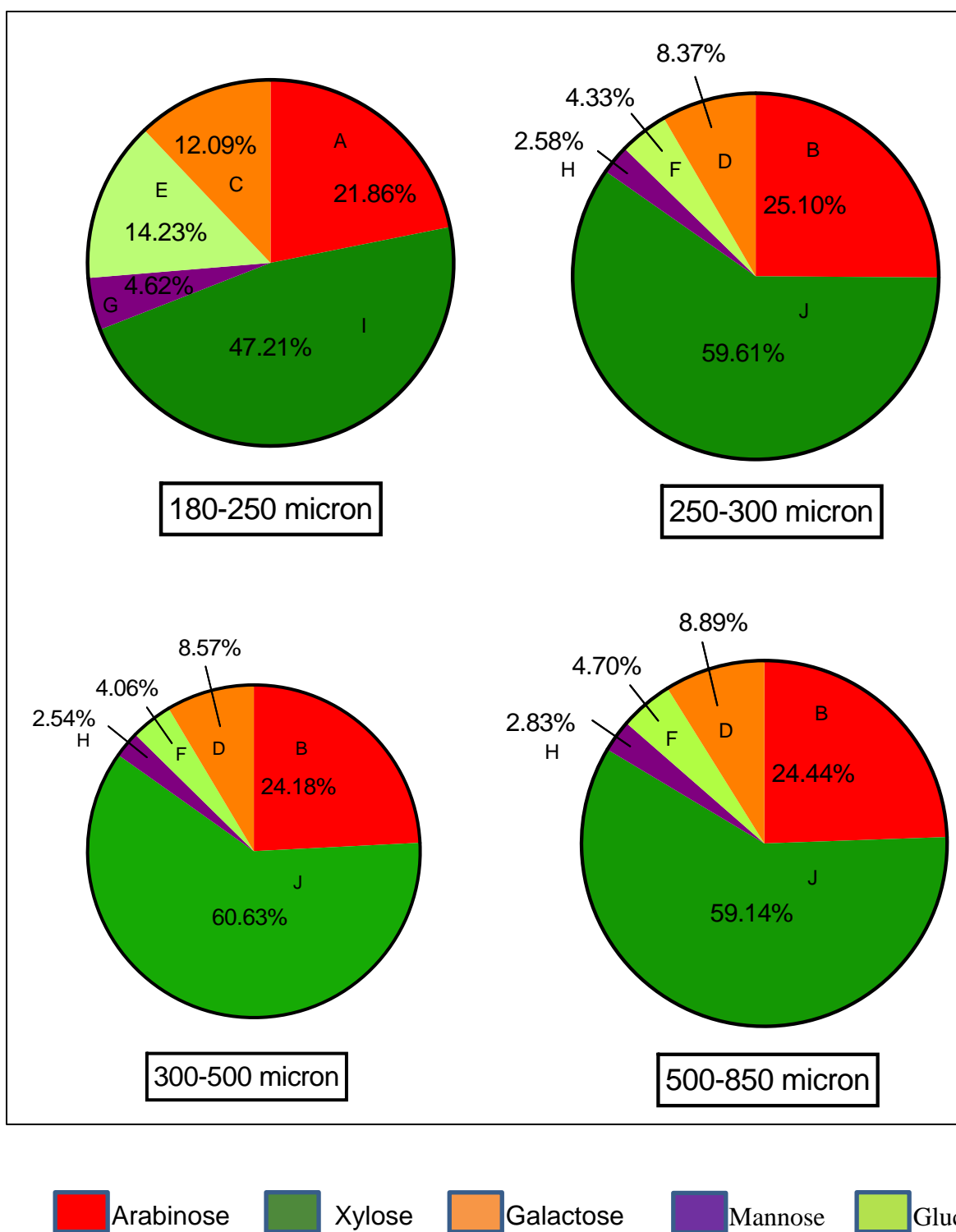
Figure 13: Neutral monosaccharide composition of maize bran particles.
Values are presented on a mole percent basis. We compared mean values of all five monosaccharides across all the sizes of maize bran. The different letters for each type of sugars indicate the significant differences in the mean value by Tukey's test and $p < 0.05$.

Cellulose and starch are the main sources of glucose, thus we tested total starch content (**Figure 14a**) to determine whether this explained the higher glucose content in the smallest size fraction. As shown in Figure 7a, we measured significantly more starch (about triple) from the smallest particle size, even after *in vitro* upper-GI digestion; differences among the other bran sizes were not significant. This aligns with the smallest maize particles displaying the highest glucose content as shown by the neutral monosaccharide composition results in **Fig. 13** suggesting that the elevated glucose content (with respect to larger particles) is derived from starch and not cellulose. In addition to increases in available starch content, the smallest particle size range contained approximately double the protein of the larger size range (**Figure 14b**). As with starch, we observed no significant difference among the other three bigger maize bran sizes for protein content. The starch assay we employed an enzymatic mechanism, so there could be a potential link between the protein content of the bran and the *in vitro* fermentation rate by fecal microbiota.
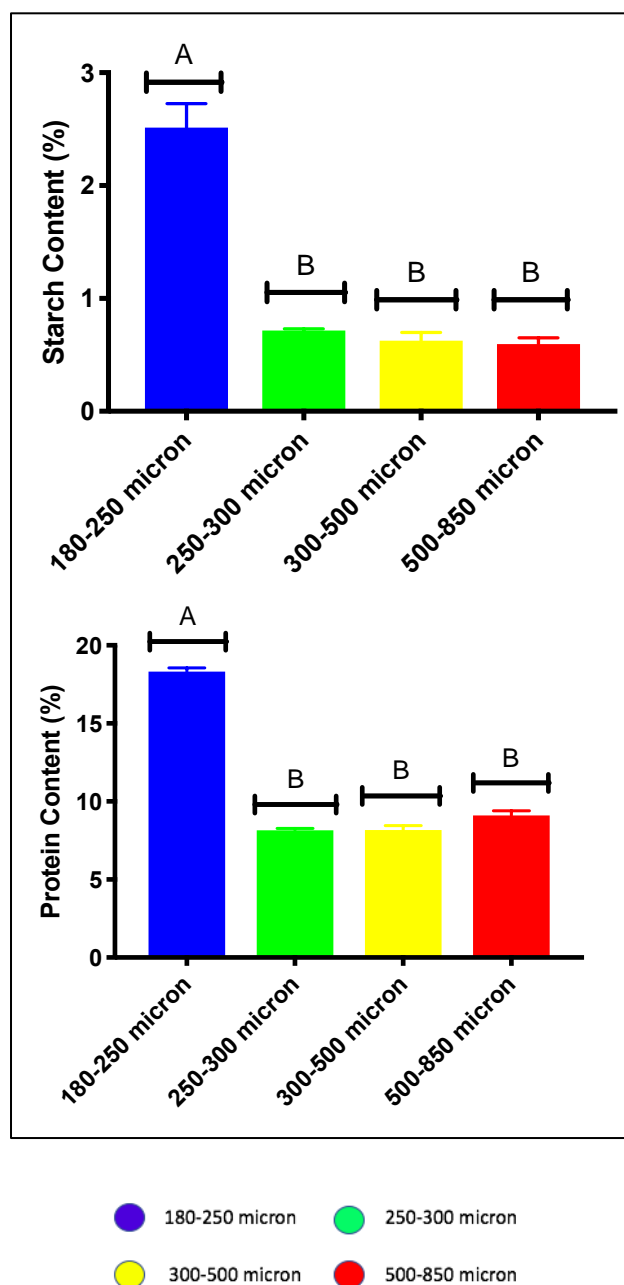
Figure 14: (a): Total starch content of different sizes of maize bran post-upper gastrointestinal tract digestion. (b): Total protein content of different sizes of maize bran post-upper gastrointestinal tract digestion.

The error bars represent standard error of mean of triplicates. There is no significant difference between the mean values with the same letter at $p < 0.05$ using Tukey's test. The error bars represent standard error of mean of triplicates. There is no significant difference between the mean values with the same letter at $p < 0.05$ using Tukey's test.

Discussion

Microbial utilization of dietary fiber is thought to depend on many interrelated structural variables like plant source, sugar type, linkage types, chain length, particle size, anomers, epimers and association with other compounds (Hamaker & Tuncil, 2014a). Amongst these variables, few studies have demonstrated differential gut microbial responses to distinct particle sizes of dietary fibers. A prior study from our laboratory revealed that variously-sized wheat bran fractions resulted in different metabolic outcomes, particularly short-chain fatty acid (SCFA) production. 16S rRNA sequencing also revealed that distinct microbial communities arose in fermentation of differently-sized wheat bran fractions(Tuncil, Thakkar, Marcia, et al., 2018). In this study, we tested our hypothesis that size-dependent fermentation by gut microbiota transcended wheat brans by performing a similar *in vitro* fermentation using identically-sized maize bran particles, so far as they could be recapitulated. As in that study, we found that maize bran size preferences were occured at fine taxonomic resolution, especially for members of genus *Bacteroides*, which differed in their size preferences. Although not broadly selective for members of *Parabacteroides* as observed in this study, we previously observed increases in *Parabacteroides distasonis* in the smallest fractions of wheat brans. These data suggest that, although there were bran-specific variations, similar microbial responses to particle size occur across brans.

Furthermore, although the differences in metabolic outcome were more significant for wheat brans, we also observed some size-dependence in SCFA production from different maize bran sizes. This was most obvious for the smallest particle size range, but fermentation of the 250-300 µm range also resulted in elevated production of all three SCFAs acetate, propionate, and butyrate. Unlike size-dependent fermentation of wheat brans, however, maize brans tended to ferment to roughly the same SCFA molar ratios, but different total abundances of SCFAs. This

could be explained by the relatively lower fermentability of maize brans compared with wheat brans, as we observed previously (Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). Accordingly, relative abundance changes over time in fermentation of maize brans were substantially smaller than for wheat brans, suggesting though organisms displayed differential growth, this growth was smaller in magnitude. Despite relatively lower metabolism of 300-500 μm maize brans compared with other cereals of identical size ranges that we reported in that study, some similarities in microbial responses between that study and the present one were obvious. For example, similarly to the present study, OTUs within *Ruminococcaceae* and *B. ovatus* showed substantial growth on these medium-sized maize bran particles

One key difference between this study and our previous work is that, in this case, we milled down the largest size fraction of maize bran into varying sizes to attempt to reduce chemical composition variation among fractions. Regardless, as we observed previously for wheat bran fractions, the smallest particle size, 180-250 μm, retained substantially more starch and produced the most SCFAs compared with larger particles. The lack of removal of these starches by *in vitro* digestion suggests that they may be classified type I resistant starches. However, it should be noted that the enzymatic method for quantifying starches may be sensitive to starch accessibility, and therefore physical barriers preventing enzyme access to starches in larger particles may influence the result. However, the glucose content (as determined by acid hydrolysis) is consistent with larger maize particles containing less starch *in toto*. These increased starch contents may preferentially select for members of *Parabacteroides*, which have been shown to be increased in abundance in human feeding trials with resistant starches (Upadhyaya et al., 2016). Yet it should be noted that particle size effects in this study were not solely dependent upon starch and protein content; medium size particles (250-300 μm) did not substantially differ in their monosaccharide,

protein, or starch content from larger fractions, yet the microbial community response was different. This suggests that there may be other size-dependent resource constraints that influence interaciton with gut microbiota.

We also measured protein content and found that the smallest particles also exhibited substantially higher amounts of protein; as this work used a complete combustion method, no accesibility effects would be involved. These data, combined with glucose and starch abundance data, suggest very substantial overall differences in composition of the smallest bran particles compared with larger fractions. The three larger fractions, on the other hand, have higher arabinose and xylose contents, which are components of the arabinoxylan polymer (Rumpagaporn et al., 2015) and suggest relatively higher relative arabinoxylan content in the larger bran particles. Thus, microbial genotypes that possess genes for hydrolyzing these complex, branched arabinoxylan structures might be selected on the bigger particle sizes of maize bran on this basis.Taken together, these data, in concert with our previous work on wheat bran particle sizes, suggest that there are preferential break points within brans that are labile to cyclone milling and that potentially release small sections of endosperm or aleurone adherent to brans as particles that fall within the smallest size range we measured. The high-arabinoxylan portions may be relatively harder to break using these methods. Whether similar preferential break points exist when using other milling methods and whether they partition brans equivalently should be examined in future work to determine whether the choice of milling method influences microbial responses to generated particles standardized across particle size.

Here, we show that different maize bran particle sizes elicit different microbial community structures and metabolic outcomes in *in vitro* fermentation. As similar responses were previously observed for wheat brans (Tuncil, Thakkar, Marcia, et al., 2018), we therefore suggest that

differences in bran physical structures arising from milling and processing may be a general feature influencing their fermentation by gut microbiota. These data further suggest that particle size is a variable that may be tunable. This shows that the gut microbial communities have varied preference for insoluble fibers based on their sizes. This suggests that not only chemical variations, but also physical variations,like particle size, should be accounted for in the field of fiber microbiota interaction studies.

CHAPTER 4: OVERALL DISCUSSION, CONCLUSION AND FUTURE WORK

Discussion

The benefits of dietary fiber to the host and metabolite production by microbial fermentation depend both on the fiber type and the resident microbiota of the host (Hamaker & Tuncil, 2014; Yang & Rose, 2014). Along with serving as energy source for the host, metabolites like SCFAs have health benefits including anti-carcinogenic and anti-inflammatory properties (Canani et al., 2011). Recently, reducing gut transit rate and increasing fermentation in the distal colon has gained attention due to several health benefits. These changes could potentially support the growth of some slower-growing bacterial populations, in turn increasing microbial diversity. Higher fermentation and SCFA production in the distal colon reduces the risk of colon cancer and other inflammatory bowel diseases. (Lewis & Heaton, 1997). Therefore, manipulation of fermentation properties based upon physical structuring of dietary fiber sources has the potential to influence human health at whole-population scales.

In my thesis, I hypothesized that the physical property of cereal bran particle size can be used as a tool for modulating populations of gut microbiota and controlling SCFA production. In the recent past, the food industry has moved towards the use of increasingly-finer grain products due to ease of processing, formulation, and sensory aspects. The use of refined grains has led to the reduction in the fiber consumption as the outermost layer of bran which is rich in dietary fiber is now removed during the process of milling (Steffen et al., 2003). Studies have shown that whole grains have many health benefits over refined grains that are only starchy endosperm. (Gani, Sm, & Fa, 2012; Okarter & Liu, 2010). As refined starches are easily gelatinized through cooking processes, reduction in particle size alone is unlikely to significantly influence the overall amount

or types of dietary fiber content (Schakel, Schauer, Himes, Harnack, & Van Heel, 2008) But for whole-grain products, reduction in particle size due to advanced milling methods has the potential to influence how components that largely retain their structure upon cooking (i.e. cereal brans) interact with the microbiota, among other host physiological processes.(Wahlqvist, 2016). I began this work by investigating whether differently-sized bran particles interacted distinctly with fecal microbiota under *in vitro* conditions, where conditions could be carefully controlled and outputs measured. Here, my work shows that microbiome responses and metabolic outputs of cereal bran particles, both wheat and maize, depend upon physical size and reveals that size governs how bran particles of both cereals will ferment. Consequently, particle size may influence microbial fermentation *in vivo*, and therefore the health effects of bran or whole grain consumption.

Transit through the upper gastrointestinal tract may significantly impact the chemical composition and microbial response in both bran- and particle size-dependent ways. To prepare for this work, we investigated two different methods for mimicking the upper gastrointestinal tract digestion have been widely used in the field of fiber microbiome interaction research on digestion of cereal bran particles. We compared the two methods, the first using pepsin, pancreatin and amyloglucosidase at higher treatment times and concentrations (Mishra & Monro, 2009b; Yang et al., 2013), compared to a second method , which uses alpha amylase, pepsin and pancreatin (Lebet, Arrigoni, & Amadò, 1998). We found that the first method was more effective in removal of starch and protein content, but that the effects of *in vitro* digestion methods were bran specific (Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). These results suggest that gastrointestinal passage may differentially impact the fate of brans in the colon. However, it also suggests the possibility that different *in vitro* digestion methods may, in part, influence our microbiome results. In the first wheat bran particle size study, we used the Lebet et al. protocol for mimicking upper

gastrointestinal tract digestion method, but soon after discovered that the Yang, et al protocol was more effective for starch removal from 300-500 μm maize bran particles, though less efficient for protein removal (Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). Therefore, I switched to this method for the maize bran particle size experiment to be more efficient for starch removal. However, I did not evaluate efficiency of starch and protein removal across all particle size ranges; the data presented in my thesis suggests that particle size may itself influence the efficiency of upper GI transit-mimicking *in vitro* digestion methods and should be rigorously evaluated in future work.

Comparison of microbial fermentation of maize and wheat bran in both our studies, we saw that overall fermentation in terms of all three SCFA production: acetate, propionate and butyrate was higher in the case of wheat bran fractions. The relatively low fermentability of maize brans has been well documented in previous studies by our lab and by others (Mongeau, Yiu, & Brassard, 1991; Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). This may arise due to the previously-demonstrated low fermentability of the maize arabinoxylan (Rumpagaporn et al., 2015). However, as wheat brans are much more commonly consumed in the Western world, another possible reason for this is that Western gut microbiota have been selected for wheat bran preference due to exposure rate. Species-level preferences for bran type and particle size intimate that it is possible that selective effects for one member of a genus or species, and therefore increases to that species' relative abundance, might strongly influence the fermentation behavior of microbial populations.

There is strong evidence from this study and others that wheat bran is highly fermentable compared to maize bran under *in vitro* conditions due to differences in their structural properties of their arabinoxylans. One study investigated the complexity of the glucuroarabinoxylan polymer in maize and sorghum brans and found that xylose is highly substituted in maize brans (Huisman,

Schols, & Voragen, 2000). The monosaccharide composition from our first study with wheat bran particle size shows that the xylose in arabinoxylan polymer from wheat brans is highly-branched with arabinose, making the arabinose to xylose ratio close to 1. In maize brans, the xylose-to-arabinose ratio is close to 2.5, suggesting much higher overall substitution of arabinose on the xylose backbone. Previous studies have shown that fine structure of arabinoxylan governs fermentation by gut microbiota (Mendis, Leclerc, & Simsek, 2016). This could also be potentially due to higher substitution by other functional groups in maize brans. Overall, higher chemical complexity in terms of substitution of the xylose backbone polymer in maize brans or increased complexity of the branching structures where they occur (i.e. greater length of side chains, greater linkage diversity) compared to that of wheat bran could be a potential reason for lower fermentability of maize brans.

In the structure of a cereal kernel, the endosperm is the site of starch synthesis in amyloplasts (James, Denyer, & Myers, 2003). Starches are not known to be associated with aleurone or pericarp cells.(Onipe, Jideani, & Beswa, 2015) For our study, we obtained pre-milled wheat and maize bran from the milling companies. The starch detected by the enzymatic detection method in our bran fractions could be a potential contaminant of the milling process. During the milling process, the first step after tempering is the separation of bran and endosperm and, potentially, some part of the endosperm could have been separated with or adhered to the bran and further processed along with it. This suggests that the starch could potentially be attached to bran as part of endosperm. Separation of adherent endosperm cells from brans by milling could be a potential reason for the higher starch content in the smallest size fractions of both wheat and maize bran.

One key limitation of my work is that, though the initial pool of microbiota were diverse in that they were collected from several individuals, the initial microbiota are representative of no one individual's gut microbiome. Thus, there could be substantial differences among the responses of individuals' microbiota to different particle sizes that we could not have observed in our experimental design. In these two wheat and maize bran particle size studies, we have used fecal slurries from three healthy donors and pooled them equally by weight as the microbial inoculum. This approach is valid with respect to overall applicability to microbiomes; for example, the metabolic output (SCFAs production) the pooled microbiota from four individual donors showed an SCFA output roughly average of each of the donors' individual microbiota (Aguirre et al., 2014). However, this study did not include detailed donor information, and it is possible that the donors have similar dietary patterns and harbor similar microbiota. Thus, though here we present the responses of a broad initial pool of microbiota and therefore likely averaging out inter-individual differences, it should be noted that some individuals' microbiota are expected to not behave in the same way with respect to bran particle size. Though the level of size resolution was not as high as our study, some evidence suggests individualized responses to wheat bran particles from different donors' microbiota (Paepe, Kerckhof, Verspreet, Courtin, & Wiele, 2017). Hence, I recommend that future studies rigorously investigate the extent to which individuals respond differently to fine differences in bran particle size.

Another important consideration is that, although I have proposed differences in the composition of available carbohydrates in bran particles as the main mechanisms by which size influences microbial fermentation, other chemical differences may significantly influence particle size-dependent fermentation. The particle size of wheat bran is known to have a significant effect on the amounts of available carotenoids and anthocyanins. For example, the smaller fraction (~200

μm) of wheat brans displayed significantly higher pigment content compared to that of the unmilled whole bran, although the antioxidant capacity and activity was higher in whole unmilled bran (Brewer, Kubola, Siriamornpun, Herald, & Shi, 2014; Rosa, Barron, Gaiani, Dufour, & Micard, 2013). Future research should rigorously quantify differences in phytochemicals among bran sizes to determine whether distinctions in these molecules might be partially or wholly responsible for the size-dependent fermentation.

Taken together, our data suggest that different bran sizes employed in food formulations may have differential health impacts. Therefore, interactions between bran size, microbiome responses, and health effects need to be more carefully examined, as the health claims made could be linked to the size of the bran fraction. Furthermore, prior research on brans that were relatively insensitive to size may be somewhat confounded by this variable. Moreover, during consumption of foods made with cereal bran, mastication will further reduce particle size to some extent. This step, which is the first step of digestion, in concert with enzymatic hydrolysis and physical process of peristalsis in the stomach may reduce the size of bran particles further. One study simulated human gastric passage and mainly focused on the physical changes that the food (rice and apple) would go through during the process of peristalsis, showing that even though there is significant amount of particle size reduction when it passes from the pylorus to duodenum, some particles of larger sizes are still retained (Kong & Singh, 2010). However, foods made with smaller particle sizes to begin with will only get further smaller during the process of mastication and digestion. Increased information on the degree to which bran particles are reduced in size by human GI passage will greatly enhance our ability to study relevant size interactions with gut microbiota.

Sensory science and consumer preference also need to be considered for broad-scale application of particle size as a variable to provide health benefit. The size of the bran particles

may also require potential alterations to processing and influence the end-product sensory, quality, and acceptability attributes. One study showed that bread loaves made with bran size fractions from four different varieties of bran had different loaf volumes; the volume increased linearly with size of bran fraction and sensory analysis deemed the loaf with largest bran (600 micron) fraction least acceptable in terms of quality (Zhang & Moore, 1999). Thus, sensory aspects may be the most significant barrier to use of larger bran particle size in food matrices; healthier food will not help if it is not eaten.

Our data suggest that smaller particle sizes in case of both wheat and maize bran produced higher gas amounts, and SCFAs acetate and propionate. However, butyrate production is higher in the larger size fractions from 24h to 48h of fermentation in case of wheat bran. In terms of gaining health benefits from overall SCFA production from wheat and maize bran, I would suggest consumption of brans of mixed size fractions that include all three small, medium, and coarse sizes. This would be beneficial as smaller and medium particle size will increase propionate and acetate production and larger fractions will contribute to increased butyrate production. Additionally, given species-level preferences for different bran size fractions, use of multiple size fractions may help to maintain overall diversity by providing distinct niches for closely-related organisms. Overall, with mixed particle sizes the gas production is expected to be less than produced in fermentation of small fractions alone and higher than in larger fractions. This may aid in reducing the discomfort that could potentially be caused by consumption of small fractions only.

For wheat bran, we see increased butyrate production between 24h and 48h with larger fraction (>1700µm) and hence this suggests that coarse wheat bran fraction is more butyrogenic at later time points during *in vitro* fermentation. If this is also seen in *in vivo* conditions, more butyrate production will be seen in the distal part of the colon. There is high interest to increase

butyrate production in the distal colon as it may reduce the incidence of diseases that predominantly arise there (e.g. colitis) (Wong et al., 2006). However, it should be noted that particle size influences transit time, which may alter the time available for microbial fermentation of particles and, consequently, over which health-benefitting SCFAs can be produced (Ferguson, Tasman-Jones, Englyst, & Harris, 2000). Thus, for the specific goal of increasing butyrate production in the distal colon, the use of larger wheat bran fractions should be more thoroughly investigated.

Conclusion

Studies have shown gut microbiota populations are malleable by inducing changes in the dietary intake (Claesson et al., 2012; Maslowski & Mackay, 2010). Particularly dietary fibers have shown a high potential to alter the gut microbiota community structure and function. Our first study focused on testing the effect of different particle sizes of wheat bran fractions obtained by sieving, and we saw clear size-based preferences for different fractions by the gut microbial community at the genus and, in some cases, at the species level. Concomitantly, microbial metabolic activities (i.e., SCFA production) were size-dependent.

In the second study, to reduce the compositional variability amongst the sizes of different bran particles, we sieved the maize bran particles and obtained the largest size fraction. After that, we milled down the largest bran size using a cyclone mill and obtained size fractions by sieving before fermentation. Recapitulation of similar size-dependent behavior of maize bran particles as we previously observed for wheat suggests the following two conclusions: first, particle-size effects in terms of variant bacterial preferences were also observed in case of maize bran, hence we can conclude that the effect is not limited to wheat bran only. Second, the reduction of particle size by the milling the biggest size fraction generates fractions with less-variant chemical

composition and muted differences in chemical composition amongst sizes, but did not eliminate size-dependent microbial responses. From the above studies, we can conclude that gut microbial species have differential preferences for bran particles based on their physical size. This may be due to variations in the chemical composition including starch content (resistant to *in vitro* digestion) and neutral monosaccharide composition among distinct particle sizes. However, size preferences of microbiota occur at very fine taxonomic levels (even differently manifest among different species of a genus) across both bran types tested. Overall, the use of targeted bran particle sizes by food industry has significant potential to be used as a tool to modulate the gut microbiome structure and function.

Future work

Though interesting, the *in vitro* data shown in this study requires significant further *in vivo* experimentation to test whether similar particle size trends are seen. I initially recommend mouse studies, using conventional and, potentially, humanized mice, as the next step to determining whether bran particle size effects are consequential to health. In the future, human feeding trials should also be considered to test the hypothesis. Bran particles of different sizes can be incorporated in muffins or cookies and tested across individuals with different initial microbiota. Other future work can include testing of other grains' bran particle size effects (sorghum bran, oat bran, etc.) to determine whether the particle size effect is translatable to brans other than wheat and maize. In terms of industrial applications, for ingredient and microbiome companies this could open a new research area to make claims that modulation of bran size can alter gut microbiota populations and can also potentially increase the production of certain metabolites. Research work in the area of testing of particle sizes of whole grain flour is also a potential future area to explore.

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