EFFECT OF GLUCAN CHEMICAL STRUCTURE ON GUT MICROBIOTA COMPOSITION AND FUNCTION

by

Arianna D. Romero Marcia

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STATEMENT OF COMMITTEE APPROVAL

Dr. Steve Lindemann, Chair

Department of Food Science

Dr. Brad Reuhs

Department of Food Science

Dr. Cindy Nakatsu

Department of Agronomy

Approved by:

Dr. Arun K. Bhunia

Dedicated to my advisor, professor, and mentor Steve Lindemann for pushing me and believing in me. Thank you for patiently guiding and supporting me throughout this journey.

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ABSTRACT

It is well known that colonic microbiota is influenced by both intrinsic and extrinsic factors; out of all these, diet plays a major role. The traditional human diet has typically been high in overall dietary fiber intake, due its inherent presence in plant-derived foods. However, over the years, dietary patterns have transitioned into a low-fiber Westernized diet. This diet is increasingly implicated in colonic diseases. Dietary fiber consumption is known to increase microbial diversity, yet the mechanisms are still unclear. This is partially true because dietary fiber as a category is composed of a wide variety of structures, which may have divergent effects on the gut microbiome. The food industry has extracted, isolated, refined and purified non-digestible carbohydrates and, in some cases, modified them for improved function, which may influence their interaction with the gut microbiome. This study was developed in two phases: we first hypothesized that glucans produced by different processes were structurally distinct and that these fine structural differences in glucans would govern microbial responses to the polymers. To test this hypothesis, we first determined the structural characteristics of the glucans by gas chromatography and mass spectrometry, which revealed substantial structural differences among the glucans with respect to size and linkage patterns, consequently categorizing the glucans by structure (i.e., mixed linkage α-glucans, resistant maltodextrins, and polydextroses). The second study involved the *in vitro* fecal fermentation of these commercially available soluble glucans which are uniformly composed of glucose linked into different structural arrangements. We further hypothesized that each glucan would select for different microbiota and that there would be glucan-specific general responses across microbiomes. We were able to identify a variety of idiosyncratic metabolic patterns as well as differential organisms selecting for specific glucan structures. Although there were associations with glucan classes at the family level (e.g., *Bacteriodaceae* and *Lachnospiraceae* were discriminants of the resistant maltodextrins and polydextroses respectively), associations with glucans across individual species within these families varied. These findings suggest that microbiome responses to structurally distinct glucans depend upon both fine glucan structure and community context, and community metabolic phenotypes emerge from the interaction of the two. These findings are relevant to the food industry as they may enable optimization of synthesis to generate chemical structures that select for specific organisms and/or improve overall gut health.

CHAPTER 1. INTRODUCTION

1.1 Diet-microbiome interactions

It is well known that many factors influence the host's colonic microbiota, such as genetics, disease, use of medications, and demographics, and among which diet plays a major role (Cho & Blaser, 2012; Cuesta-Zuluaga et al., 2019; Hasan & Yang, 2019; Read & Holmes, 2017; Wen & Duffy, 2017). The interactions between diet and the microbiome go beyond dietary patterns and intake of food components to encompass gut microbial composition, diversity, and overall gut health. Human and animal studies (Carmody et al., 2015; Faith et al., 2011) have demonstrated that microbiomes can be modulated by dietary interventions, in some cases resulting in predictable outcomes; however, the extent to and mechanisms by which these alterations occur may vary tremendously among idiosyncratic individual microbiomes. The gastrointestinal ecosystem involves complex interactions and dynamics among the microbes and the host's lifestyle (Kolodziejczyk et al., 2019), in addition to diversity in microbiota and the mechanisms they utilize to degrade these foods, which are, in turn, diverse in chemical composition. Generally, diet is known to drive both negative and positive microbial responses but identifying mechanisms by which diet shapes the microbiome is challenging as it entails multiple, entangled intrinsic and extrinsic factors.

The main food components in almost every human diet are protein, lipids and carbohydrates (Fennema et al., 2017); the first two are largely absorbed in the small intestine, whereas certain undigestible carbohydrates reach the colon because humans lack the enzymes capable of depolymerizing them (Frayn, 2010). These undigested carbohydrates are then available for large populations of colonic microbes to ferment (Frayn, 2010). These carbohydrates are in some cases referred to as microbiota accessible carbohydrates (MACs), which are that subset of fibers that can be consumed by gut microbes as food (E. D. Sonnenburg & Sonnenburg, 2014). Many of these are found intrinsically in plant-based foods (typically as components of plant cell walls). However, modern industrial processing technology allows refinement of human-consumable carbohydrates (e.g. starches) from their native contexts and associated dietary fibers for inclusion as ingredients in food products. Products made with refined carbohydrates tend to have improved sensory profiles over whole-grain products, which has driven adoption of a high-fat, low-fiber diet in many affluent

nations, most commonly known as the Western diet (Vangay et al., 2018), which has long been thought to predispose people to colonic diseases (Legaki & Gazouli, 2016; Martini & Brandes, 1976) Recently, disturbance of the gut microbiome has been implicated as a mechanism of this increased risk (Bull & Plummer, 2014, p. 1). Thus, understanding how diet components, especially fibers, shape the gut microbiome's structure and function will be beneficial, both as a set of health-relevant biomarkers and as a therapeutic target (Kho & Lal, 2018).

1.2 The Gut

The human gastrointestinal tract is a series of ecosystems – frequently together termed the gut microbiome – that is inhabited by about 10^{13} commensal microbes (Sender et al., 2016). The microbial load increases as it reaches the colon (Kelly et al., 2007; Wang et al., 2005), where the combination of optimal conditions exist (reduced immune pressure, less oxygen, and slower transit). This ecosystem is increasingly understood to influence the health of multiple body systems. For example, recently relationships between gut microbiota and the brain have been uncovered, affecting mood, sleep, appetite regulation, among other things (Krueger & Opp, 2016; Sharon et al., 2016; Silva et al., 2020; Stamper et al., 2016). These commensal bacteria also exist as a barrier against colonization by opportunistic pathogens and help the host gain energy through degradation of the undigested food components, such as fibers. The gut microbiota encode enzymes capable of degrading different types of undigestible food components (Kaoutari et al., 2013; Ye et al., 2019) and produce end products such as acetate, butyrate and propionate, also known as short-chain fatty acids (SCFAs). These SCFAs are beneficial to the human, as they are a primary fuel for colonic epithelial cells and help maintain intestinal homeostasis (Parada Venegas et al., 2019). SCFAs can be both produced and consumed by the different taxa present in the microbiome (den Besten et al., 2013; Duncan et al., 2004), and multiple intermediates can be exchanged through cross-feeding interactions among members (Ríos-Covián et al., 2016) in addition to diverse other metabolic interactions. These interactions underlie microbial metabolic cooperation and competition, which can impact different taxa both negatively and positively (Coyte & Rakoff-Nahoum, 2019). The interactions among members are often context-dependent, as they vary across conditions and time, determining community structure as well as stability of the microbiome. External conditions, such as resource availability, can generate states were the abundance of a member can be either increased or depleted by competition with other members, or by production

of toxic compounds that exclude other members (Hoek et al., 2016). In most cases it is the most dominant members that seem to determine what types of interactions prevail overall. However, it is difficult to predict and measure interactions and outcomes in complex communities; in some communities, rare taxa may perform an important function required for overall community function. Both responses to perturbations from external stimuli and the cascading interactions among members drive dynamics in the microbiomes and influence microbiome stability (Song et al., 2015).

It has been recently suggested that a reduction in microbial diversity and the associated diversity of metabolic outputs increases the vulnerability of the host to chronic diseases (Hawrelak & Myers, 2004). Alteration in gut microbiota structure and function due to external factors, such as diet, may be mitigated and/or prevented by high diversity, which may impart greater resistance or resilience to stress (Konopka et al., 2015). With respect to the gut microbiome, diverse microbiota have been demonstrated to be more resistant to external perturbations, such as antibiotics or diet, yet these are still unlikely to return to their initial microbial state post-perturbation (Antonopoulos et al., 2009; Dethlefsen & Relman, 2011; Girvan et al., 2005). These data suggest that sustaining microbial diversity may be important to maintain gut homeostasis. A widely diverse community of microbiota is capable of fermenting complex carbohydrates and produce large amounts of SCFAs, providing many functional benefits to the host, whereas a low-diversity microbiota may not have the same magnitude of beneficial response (J. L. Sonnenburg & Bäckhed, 2016). Complex carbohydrates are incredibly diverse in chemical structures (with respect to size, sugar composition, and how those sugars are linked together). As gut microbiota vary in their capacity to degrade different structures, complex carbohydrate structure may influence microbial diversity, which, in turn, may influence the range of carbohydrates that can be degraded by the microbiota (Lindemann, 2020a).

1.3 Carbohydrates

Carbohydrates are one of the primary macronutrients and are considered the main source of energy for humans in the diet. Dietary carbohydrates include sugars, starches, and dietary fibers. In contrast to starches, dietary fiber is composed of a mixture of nondigestible components that can be both intrinsic in plants and/or synthetic, and may be added to foods for its bulking properties as well as other functional properties, depending on its solubility (*Dietary Fiber Intake of the U.S.*

Population, 2014). Carbohydrates are classified into different size categories, as the number of sugar units linked together increase: these categories are mono- (1 sugar), di- (2 sugar residues), oligo- (<10 residues) and polysaccharides (≥10 residues) (Cummings & Stephen, 2007; Kerry C Huber & James N. BeMiller, 2017). Polysaccharides constitute the largest fraction of the typical human's carbohydrate intake, including both starches and other components of plant cell walls. Because such carbohydrates are typically both very abundant components of the diet and the easiest to manipulate in foods due to their variability in composition and structure, they are key control points for adapting dietary patterns and food design to improve the human diet via the gut microbiome.

1.3.1 Dietary fiber

Dietary fiber is defined by the American Association of Cereal Chemists International (AACCI) as "the edible parts of plants and analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (American Association of Cereal Chemists, 2001) and by the Food and Drug Administration (FDA) as "non-digestible soluble and insoluble carbohydrates, isolated or synthetic, providing physiological effects that are beneficial to human health" (FDA, n.d.). The daily recommended intake of dietary fiber for the U.S. population is around 25 grams per day for women and 38 grams per day for men (*"Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids" at NAP.Edu*, 2005); however, this dietary intake is very infrequently met, despite efforts in the food industry to incorporate fiber in processed foods and public education campaigns (e.g. "Dietary Guidelines for Americans"). In 2015-2016 the average consumption of dietary fiber for both females and males over the age of 2 was 16.5 grams (NHANES, 2015), this deficit likely increases the incidence of certain diseases associated with low-fiber diets, such as obesity, type 2 diabetes, hypertension, and other cardiovascular diseases (Casiglia et al., 2013; Williams et al., 2017; B. Yao et al., 2014; Zhang et al., 2013).

Fibers are the largest constituents of plant cell walls, e.g. those of vegetables, fruits and most whole grains (Williams et al., 2019). If these food categories are being under-consumed, the full benefits of fiber will not be achieved. To close this "fiber gap," the food industry has effectively extracted, isolated, refined and purified non-digestible carbohydrates and, in some cases, modified

them for improved function. Some of the non-digestible carbohydrates that have shown to have a physiological effect to human health are psyllium husk, beta-glucan, pectin, galactooligosaccharides (GOS), inulin and fructooligosaccharides, resistant starch, arabinoxylan, among others; these can be found in natural and/or synthetic forms (FDA, 2018). These fibers have been tested in several clinical, *in vitro*, and *in vivo* studies to evaluate their effect, with respect to physiological response (e.g. calcium absorption, appetite and blood glucose regulation), or in their effect on the structure and function of gut microbiota (Holscher, 2017; Schroeder et al., 2013; Whisner et al., 2016). These non-digestible carbohydrates are fermented in the gut, producing SCFAs, which are then absorbed in the bloodstream and there are thought to mediate at least some of the positive impact (Wong et al., 2006). Not all fibers are easily degraded by microbiota, as they are composed of a mixture of different sugars, with multiple types of glycosidic linkages which increase the complexity of each fiber (Sharma et al., 2018). In some cases, it is difficult to pin-point the impact of these complex fibers on gut microbiota, as there are several structural properties, both physical and chemical, that may be exerting some type of effect on the composition, abundance, and metabolic function of gut microbes.

1.4 Thesis Summary

The goal of this thesis is to better understand how the chemical structures of commercially available glucans with potential prebiotic properties impact the structure and function of gut microbiota. I tested the hypothesis that the chemical structure of a glycan – independent of its composition of glycosyl residues – governs microbial responses to the polymer. Variation in glucan chemical structure and composition served as the independent variable in fermentation experiments using fecal microbiota from three individuals. Fibers generated from natural sources vary in both the sugar composition of residues and their fine structure (Tuncil et al., 2018), making it challenging to deconvolute the effect of each variable. In contrast, because these glucans are homogenous in composition, chemical structural arrangement serves as the sole source of variation. This allowed me to more easily determine how these structural arrangements and chemical characteristics change the microbial abundances and metabolite production (SCFAs).

The first step in testing my hypothesis was to determine that glucans produced by different industrial processes were, indeed, structurally distinct. To accomplish this, I tested monosaccharide composition of the polymers and measured their linkage patterns and molecular

weight profiles. These results confirmed the glucose content and revealed that different commercial glucans varied in structure, indicating they were suitable substrates for *in vitro* fermentation studies.

Having demonstrated the feasibility of my study, I then went on to more broadly test the hypothesis that glucan fine structure differentially impacts the structure and function of gut microbiota. The chemical diversity exhibited among glucans further allowed us to test whether or not individuals' microbiota would respond in similar ways. This work involved a series of individual batch anaerobic cultivations using three separate fecal inocula prepared from the microbiota of healthy donors. Each of these donors' microbiota were inoculated into cultures in which 11 different glucans were the sole carbon source. We hypothesized that individual microbes would not only have selective preferences for one substrate over another, but also that there would be glucan-specific general responses across the different donors' microbiomes. As described in Chapter 3, I observed both idiosyncratic and common responses of microbiota in response to the identical glucans as carbon sources, suggesting both that hard-wired competitive (and possibly cooperative) relationships exist, but also that context is important in determining the outcome of microbial competition for these substrates.

CHAPTER 2. GLUCAN CHEMICAL CHARACTERIZATION

2.1 Introduction

Glucans are polymers solely composed of glucose, and may be derived from multiple sources such as plants, fungi, yeast, and bacteria (Synytsya & Novak, 2014). Despite their uniformity in the sugar residues that constitute them, glucans differ in the position of glycosidic bonds, branching structure and branch density, and alpha or beta isomers (Goodridge et al., 2009), demonstrating considerable structural variability. Molecular weight can also differ based on the source, type of extraction, and purification method. The glucans used in the food industry are, in most cases, starch derivatives, commonly isolated from plants; mainly amylose and amylopectin. These starches are hydrolyzed into smaller, low molecular weight polymers with multiple glycosidic bonds formed by the chemical and physical processes employed (James N. BeMiller, 2019a). Such glucans are typically included in products for their food functionality (e.g. utility as bulking agents, gel-forming capacity, or solubility) and lately these have been more significantly considered for their fermentability and dietary purposes (James N. BeMiller, 2019d). Glucans generated largely with gut microbiome impacts in mind include mixed linkage α-glucans, resistant maltodextrins, β-glucans, and polydextroses.

Starch is a polymer composed of amylose, a linear chain of glucose molecules linked by α-(1,4) glycosidic bonds and amylopectin, a more branched glucose polymer which also contains α-(1,6) glycosidic bonds (James N. BeMiller, 2019c). Starchy polysaccharides are digested by enzyme activity; α-amylase and α-glucosidases break the polymer at α-(1,4) linkages, converting it into smaller molecules, such as maltotriose, maltose, and glucose (Molnar & Gair, 2019). Once transported into the epithelial cells, glucose is respired for energy and transported throughout the body through the circulatory system. A fraction of native starches is resistant to digestion by human enzymes and are known as resistant starch (RS). Starches may be inaccessible due to physical barriers (RS type 1), resistant granule structures (RS2), retrogradation (RS3), or chemical modification by physical, chemical, and/or enzymatic methods (James N. BeMiller, 2019c; Sajilata et al., 2006). These starches largely escape small intestinal degradation and reach the colon largely intact. In addition, another important category of food polysaccharides are the non-starch polysaccharides (NSP), which are also dietary fibers (cellulose, gums, pectins, etc.) and can be

fermented in the colon. A partial hydrolysis of polysaccharides (for example, starch) generates oligosaccharides and short polysaccharides; those that cannot be digested by human enzymes may be fermented in the large intestine and may behave as a prebiotic (Davani-Davari et al., 2019). Prebiotics are defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Davani-Davari et al., 2019; Gibson et al., 2010).

Resistant maltodextrins/dextrins are a class of soluble fibers comprised of digestible and non-digestible linkages. These are made by heating of starch (typically derived from corn, tapioca or potato), which depolymerizes the amylose/amylopectin structure into smaller oligosaccharides and, in cases where acids are added, introduces α- and β-(1,2), (1,3), and (1,6) glycosidic bonds by transglycosylation. These linkages are indigestible to the human enzymes found in the small intestinal brush border (Śliżewska et al., 2012), protecting much of these molecules from human degradation. The difference between resistant dextrins and maltodextrins is that the dextrinization process uses amylase to remove the majority of the digestible linkages, making resistant dextrins more indigestible than maltodextrins (James N. BeMiller, 2019d). Resistant wheat/corn dextrin products are abundant on the market and are the major components of commercially available products such as Fibersol, PROMITOR, and Nutriose. These fibers have been previously studied for their effect on animal and human microbiota. In humans consuming a dosage of 15 g/day over a 3 week period, increases in the abundance of members of genus *Bifidobacterium* (Fastinger et al., 2008) were observed, whereas at a dosage of 21 g/day the abundance of the *Porphyromonadaceae* family was shown to increase, especially in the genus *Parabacteroides* (Holscher et al., 2015). This increase in abundance of this genus also coincides in a different study with an increase in calcium absorption (Whisner et al., 2016). The increase in bifidobacteria with the consumption of resistant maltodextrins appears to be a re-occurring result, as its effect has been studied at different dosages with similar outcomes (Costabile et al., 2016a; Maathuis et al., 2009). Resistant maltodextrins/dextrins are well-tolerated fibers with solubility and sensory profiles that support their inclusion in food products.

β-glucans are non-starch polysaccharides that can be naturally present (3-7%) in cereal brans from oats, barley, and rye. They can be extracted with hot water, and in some cases, using an alkaline solution. β-glucan is composed of a linear, mixed-linkage chain of D-glucose by (1,4), (1,3); chain length and branching will depend on the source (James N. BeMiller, 2019d). β-glucans

are soluble, viscosity-inducing fibers which are fermentable by the gut microbiota. Most studies, including clinical trials, with β-glucans have shown to increase the abundance of lactobacilli and bifidobacteria (Lam & Chi-Keung Cheung, 2013), these findings depend on the source of the β-glucan. In another *in vitro* study *Roseburia* and *Prevotella* were the most enriched genera with this fiber type (Fehlbaum et al., 2018). Therefore, β-glucans appear to act as a prebiotic in that they enhance the growth of these organisms across the dosages applied in each study.

Polydextroses (PDX) are synthetic polymers composed of many dextrose (D-glucose) monomers. They are produced by the mixture of D-glucose monomers, sorbitol and citric acid, the structural arrangement is somewhat random, generating a highly branched polymer (James N. BeMiller, 2019a). This random cross-linking generates all types of glycosidic bonds, but predominantly (1,6) linkages. These polymers are highly soluble, non-viscosity-inducing glucans with a low caloric value and that are commonly used in the food industry for their functional properties (e.g., sweetener replacer, bulking agent, humectant) and considered as dietary fiber as they are fermentable in the large intestine. Consumption of 21 g/day of PDX in a bar format has been shown to enhance the abundance of *Faecalibacterium prausnitzii* (Hooda et al., 2012). Similar results were observed in a different study, where consumption of 8 g/day increased the numbers of *Clostridium* clusters I, II and IV and *R. intestinalis*, as detected by qPCR analysis, whereas levels of *Bacteroides* and *Bifidobacterium* remained unchanged (Costabile et al., 2012). Other studies have reported significantly higher concentrations of *Lactobacillus* and *Bifidobacterium* with a dosage of 4-12 g/day of PDX (Jie et al., 2000), this result agrees with a different study of dietary supplementation with 2% polydextrose, supporting the growth of bifidobacterial species (Probert et al., 2004). In contrast, a study in pigs with a dosage of 30 g/day increased the abundance of lactobacilli but not of bifidobacteria (Fava et al., 2007). These studies show a range of significant, likely positive influences on the microbiota both *in vivo* and *in vitro*.

Commercially available glucans were provided by several industrial partners with the purpose of understanding the fermentative patterns and changes in gut microbiota in response to subtle chemical structural variants. Despite known differences in processes among industrial producers of resistant glucans, it was not initially clear that these substrates were sufficiently different structurally from each other and sufficiently free of contamination by free sugars to test my hypothesis. Consequently, I undertook a pilot study to investigate the physical and chemical properties of these glucans. The first part of this study was to determine their chemical

characteristics and to identify differences among products. To this end, I evaluated the monosaccharide composition, degree of polymerization (DP), free sugar content, and glycosidic linkages of the glucans. As these glucans were used in their final ingredient format, there was no previous sample preparation.

The glucans used in this study were resistant maltodextrins, mixed linkage α-glucans, and polydextroses. These glucans have been industrially processed to resist digestion by human enzymes through the insertion of unnatural (for starch) glycosidic bonds besides α-1,4 and α-1,6, which are the most common in starch. These structural modifications make these starch- and sugar-derived glucans (otherwise highly metabolizable by humans) into dietary fibers with prebiotic features.

I determined the structural properties of these glucans using gas chromatography and mass spectrometry techniques. To quantify the monosaccharide composition, I used the alditol acetate method in which glucans were derivatized and their alditol acetates measured. This analysis involved hydrolysis of soluble polysaccharides with trifluoroacetic acid (TFA) and detection of derivatives by GC-MS (Pettolino et al., 2012). In addition, to measure molecular size, I used matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight (TOF) mass spectrometry. MALDI-TOF is a technique that can be used for analysis of a homologous series of oligosaccharides (James N. BeMiller, 2017), this method can assist in the determination of degrees of polymerization (DP).

The chemical characterization of these glucans allowed me to deduce fine structural characteristics of these glucans, which revealed substantial structural differences among them both with respect to size and linkage patterns. Understanding these chemical differences is critical to interpreting the distinct patterns of their fermentation outputs.

2.2 Methods

2.2.1 Glucan moisture content

The moisture content of the glucans was measured with a Rapid Moisture Analyzer HE73 (Mettler Toledo, Columbus, OH). First, the empty aluminum pan was weighed, then 3 ± 0.5 grams of sample weighed and heated with a halogen lamp. Glucans were analyzed in triplicate and weight loss was recorded continuously until it reached a final weight, which was expressed as a moisture

content percentage. Glucan M was in syrup form, and thus was analyzed using a convection oven. Five grams of syrup were weighed in 3 separate pans and heated at 105℃ for 24 hours. For glucan M moisture content was determined by the following formula MC% = (Wet Weight – Dry Weight/ Wet Weight) *100.

2.2.2 Free sugar analysis

Glucans were analyzed for the content of glucose, sucrose, and fructose with collaboration of the Laboratory of Renewable Resources Engineering (LORRE), located at the Potter Engineering Center, with the use of a high-pressure liquid chromatograph (HPLC) equipped with a Bio-Rad HPX-87P column. External standards were prepared with concentrations of 4%, 2%,1%, 0.5%, 0.025% and 0.0125% and used to quantitate the free sugars. Glucans were diluted in deionized water (1% solution) and filter sterilized using a 0.22 µm filter and injected in duplicate (10 µl) with a run time of 35 minutes. Sucrose was identified at 10.76 minutes, glucose at 12.79 minutes, and fructose 16.64 minutes' retention time.

2.2.3 Monosaccharide composition

Glucan neutral monosaccharide composition was determined as alditol acetates (AA) with a TFA hydrolysis. First, I weighed 1 ± 0.5 mg of sample, followed by hydrolysis using trifluoroacetic acid (TFA) at 121°C for soluble samples and then reduction using $NaBD_4$ and acetylation using acetic anhydride with multiple dichloromethane (DCM) and multiple ddH_2O washes described by Pettolino (Pettolino et al., 2012), the obtained residue was dissolved in acetone and volatized by GC-MS. Samples were analyzed on a GC-MS with a 7890A GC and a 5975C inert MSD with a Triple-Axis detector (Agilent Technologies, Inc., Santa Clara, CA) using a capillary column (SP2330; SUPELCO, Bellefonte, PA). Helium was used as a carrier gas and the GC-MS run conditions were as follows: injection volume of 2 µl with a split ratio of 1:2; injector temperature at 240 ℃; detector temperature at 300 ℃; the gradient temperature program set was 160 ℃ for 6min, then 4 ℃ /min to 220 ℃ for 4min, then 3 ℃ /min to 240 ℃ for 5min, and then 11 ℃ /min to 255 ℃ for 5min.

2.2.4 Degrees of Polymerization

Glucan degrees of polymerization (i.e., the number of glycosyl residues linked together in the oligosaccharide chain) were determined by MALDI-TOF. Samples were prepared by mixing the glucan with deionized water, making a 2% filter-sterilized solution. An alpha-cyano-4-hydroxycinnamic acid matrix mixed with a saturated salt solution (NaCl dissolved in methanol) was deposited in the MALDI plate (0.5 µl) and allowed to dry at room temperature. Afterwards, 0.5 µl of each sample was added to the spot and placed in the MALDI-TOF. MALDI spectra were acquired using a MALDI-TOF Applied Biosystems 4700 Reflector Spec (Applied Biosystems, Framingham, MA). Full-scan mass spectra ranging from m/z 500 to 5000 were acquired in the positive mode.

2.3 Results and Discussion

2.3.1 Moisture content

In general, glucan samples contained a low moisture content. As expected, glucan M had the highest moisture content, as it was delivered as a syrup. Measuring moisture content was necessary to calculate the appropriate carbohydrate loading for the fecal *in vitro* fermentations in Ch. 3, to ensure that each of the cultures received equivalent amounts of substrate.

Table 2.1 Average percentage of moisture content (MC) of the studied glucans

GLUCAN CATEGORIES	GLUCAN	REP 1	REP 2	REP 3	AVERAGE MC (%)
Mixed linked α-glucan	A	7.63	8.03	8.27	7.98
	B	8.03	7.86	8.33	8.07
	C	7.08	6.91	6.99	6.99
Polydextrose	E	3.94	3.86	3.92	3.91
	H	3.37	3.49	3.39	3.42
Resistant maltodextrin	D	4.16	4.15	4.05	4.12
	G	4.15	4.61	4.63	4.46
	J	5.63	5.60	5.49	5.57
	K	3.56	3.61	3.75	3.64
	L	4.26	4.25	4.16	4.22
	M	19.43	19.96	19.76	19.72

2.3.2 Free sugar analysis

HPLC analysis was able to successfully separate and quantify the amount of total free sugars in each glucan, dissolved in water at a concentration of 2 g/L (after adjustment for moisture content). The samples contained a very low amount a total free sugar (less than 5% for all glucans). The fructan control group F contained 6% of total free sugars (Table 2). In some cases, free sugars were below the limit of detection (Table 3).

Table 2.2 Glucan injection quantifying the amount of glucose, fructose, and sucrose determined by HPLC

		INJECTION 1			INJECTION 2			AVERAGE g/L		
Control		**Glucose**	**Fructose**	**Sucrose**	**Glucose**	**Fructose**	**Sucrose**	**Glucose**	**Fructose**	**Sucrose**
	F	-	0.059	1.036	0.033	0.035	1.205	0.033	0.047	1.1205
Mixed linked α-glucan	**A**	0.1670	-	0.0530	0.16	-	-	0.1635	-	0.053
	B	-	0.029	-	BELOW DETECTION LIMIT			-	0.029	-
	C	0.005	0.003	-	0.008	0.041	-	0.0065	0.022	-
Polydextroses	**E**	0.309	0.026	0.049	0.327	0.027	0.049	0.318	0.0265	0.049
	H	0.6860	0.0360	0.1970	0.607	0.001	0.14	0.6465	0.036	0.197
Resistant maltodextrins	**D**	0.0490	0.1650	0.0540	0.041	0.15	-	0.045	0.165	0.054
	G	0.026	0.032	-	0.026	0.013	-	0.026	0.0225	-
	J	0.494	0.045	0.181	0.509	0.041	0.186	0.5015	0.043	0.1835
	K	0.1390	-	-	0.148	-	-	0.1435	-	-
	L	0.0360	0.0110	-	0.041	0.017	-	0.0385	0.014	-
	M	0.2530	-	-	0.248	0.013	-	0.2505	0.013	-

Table 2.3 Average percentage of individual glucose, fructose and sucrose and total free sugars in each glucan determined by high pressure liquid chromatography (HPLC)

		AVERAGE (BOTH INJECTIONS)			
		Glucose	**Fructose**	**Sucrose**	**Total**
Control	F	0.17%	0.24%	5.67%	6.07%
Mixed linked α-glucan	A	0.89%	-	0.29%	1.18%
	B	-	0.16%	-	0.16%
	C	0.03%	0.12%	0.00%	0.15%
Polydextrose	E	1.62%	0.14%	0.25%	2.01%
	H	3.37%	0.19%	1.03%	4.59%
Resistant maltodextrins	D	0.23%	0.86%	0.28%	1.37%
	G	0.13%	0.12%	-	0.25%
	J	2.62%	0.22%	0.96%	3.80%
	K	0.76%	-	-	0.76%
	L	0.20%	0.07%	-	0.27%
	M	1.56%	0.08%	-	1.64%

2.3.3 Monosaccharide composition

Because mixed linkage α-glucans and resistant maltodextrins are derived from starch and polydextrose is synthesized from glucose, their main glycosyl residue composition and linkage profile should be dominated by glucose, if pure. Indeed, I measured these commercially available glucans to have glucose relative abundances of > 97%, using the alditol acetates (AA) protocol by GC-MS. These monosaccharides were identified by comparing them to an external standard, which was composed of a mixture of rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose. Trace galactose and mannose may have arisen from impurities in the starch fractions or glucose sources employed in dextrinization or polymerization reactions. Diversity in glucan production processes also likely influenced the amounts of contaminating disaccharides. Acid-hydrolyzed glucans and enzyme-hydrolyzed glucans differ in the distribution of disaccharides present (Lloyd & Nelson, 1984). The presence of alternate monosaccharides was in sufficiently low percentages, especially for the polydextroses and resistant maltodextrins (< 5%, except for glucan G ~10%), that we deemed that these impurities were unlikely to substantially influence fermentation by gut microbiota.

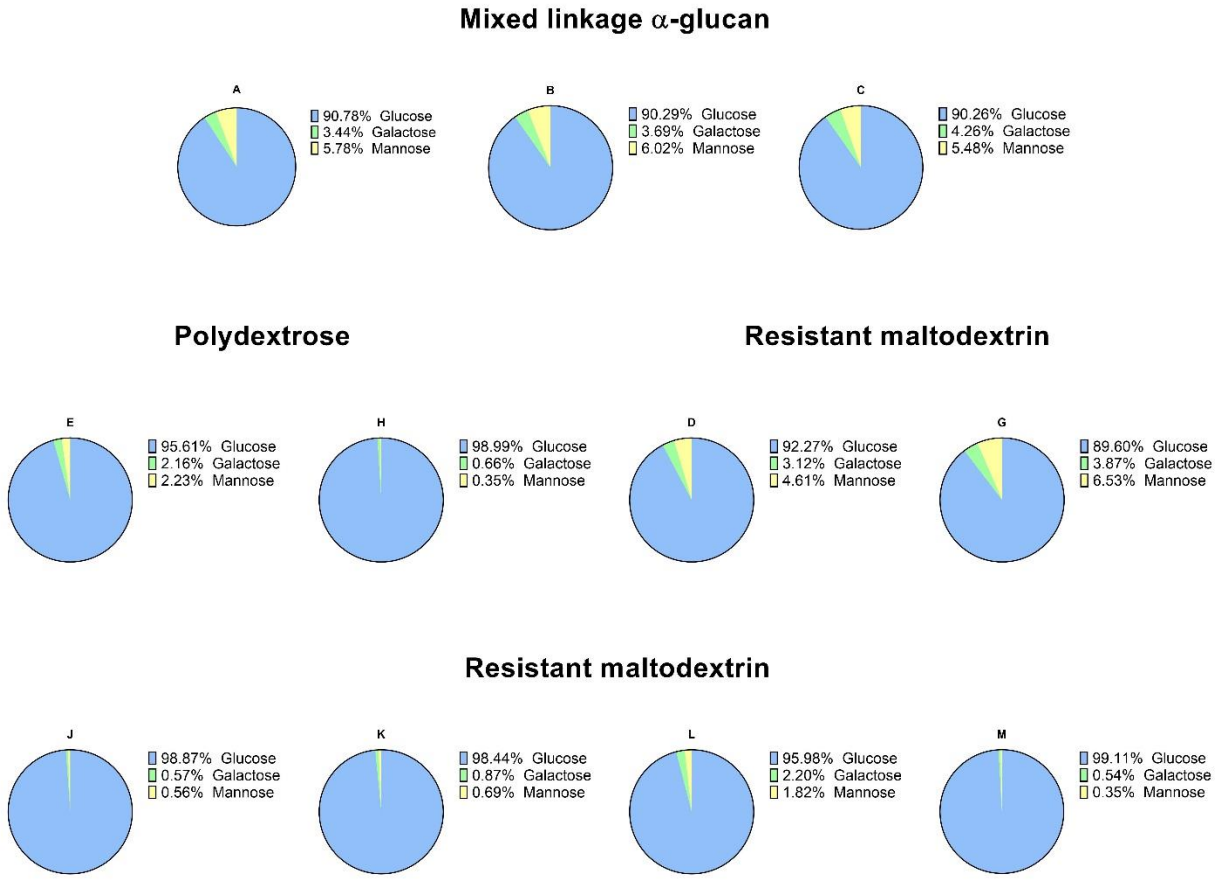

Figure 2.1 Total monosaccharide composition of each glucan identified by alditol acetates, indicating glucose as the major component in all glucan categories.

2.3.4 Degrees of Polymerization

I also determined the size distribution of the different glucans using MALDI-TOF to separate oligosaccharides with different degree of polymerization. MALDI-TOF spectra showed that glucans were composed of oligosaccharides with DPs ranging from 3-10, determined by comparison with a mixture of maltose (DP 2), maltotriose (DP 3), maltotetraose (DP 4) and maltopentaose (DP 5). Polydextroses had the smallest DP out of all three glucan categories, both mixed linkage α-glucans and resistant maltodextrins have an average DP ranging from 9-10, except for glucan D with DP 4. The molar mass of glucose is 180 g/mol, which should be the difference between peaks, however in this case the difference is 162, due to dehydration (H2O is 18 g/mol). This method not only is responsive to mass but also reflects polymer linearity; the more branched glucans have fewer isomers (which result in smaller intermediate peaks). Polydextroses and

resistant maltodextrins exhibited increased branching compared with the mixed linkage α-glucans which have more peak dispersity. These results revealed that the glucans in study are in fact low-molecular weight oligosaccharides, which certainly depends on the various manufacturing processes. DP is relevant in the food industry, as it correlates to functional properties such as gelling, viscosity, and dextrose equivalent (DE) (J. N. BeMiller, 2003). A higher DP (larger molecular weight) a higher viscosity and lower DE i.e., glucose has a DE 100% lower molecular weight vs. native starch has a DE of 0 with a higher molecular weight. All these are properties may have a potential impact on gut health via differences in interactions with microbiota.

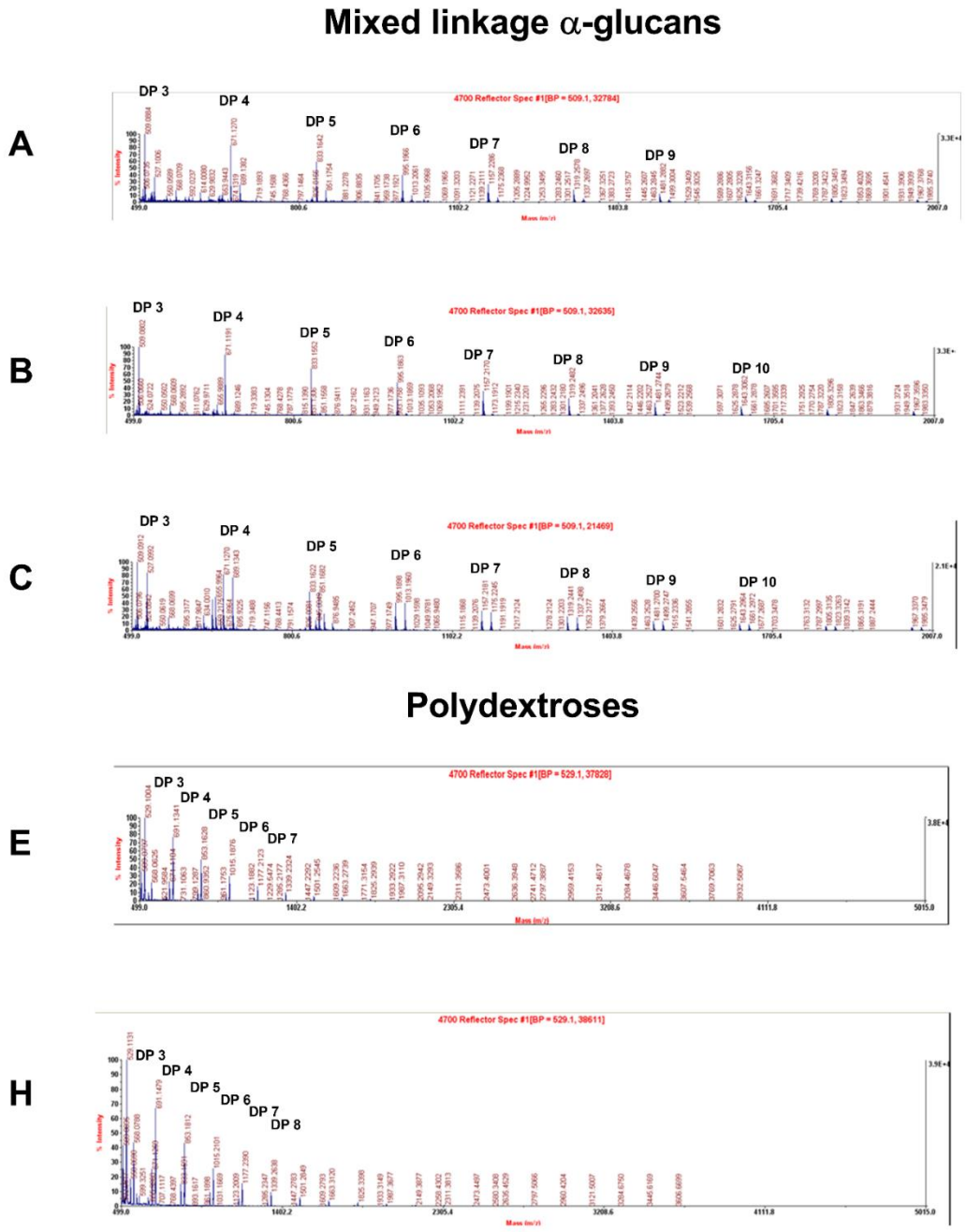

Figure 2.2 MALDI-TOF spectra of each glucan within the mixed linkage α-glucans and polydextroses depicting the mass-to-charge ratio (m/z) ranging from 500 to 5000.

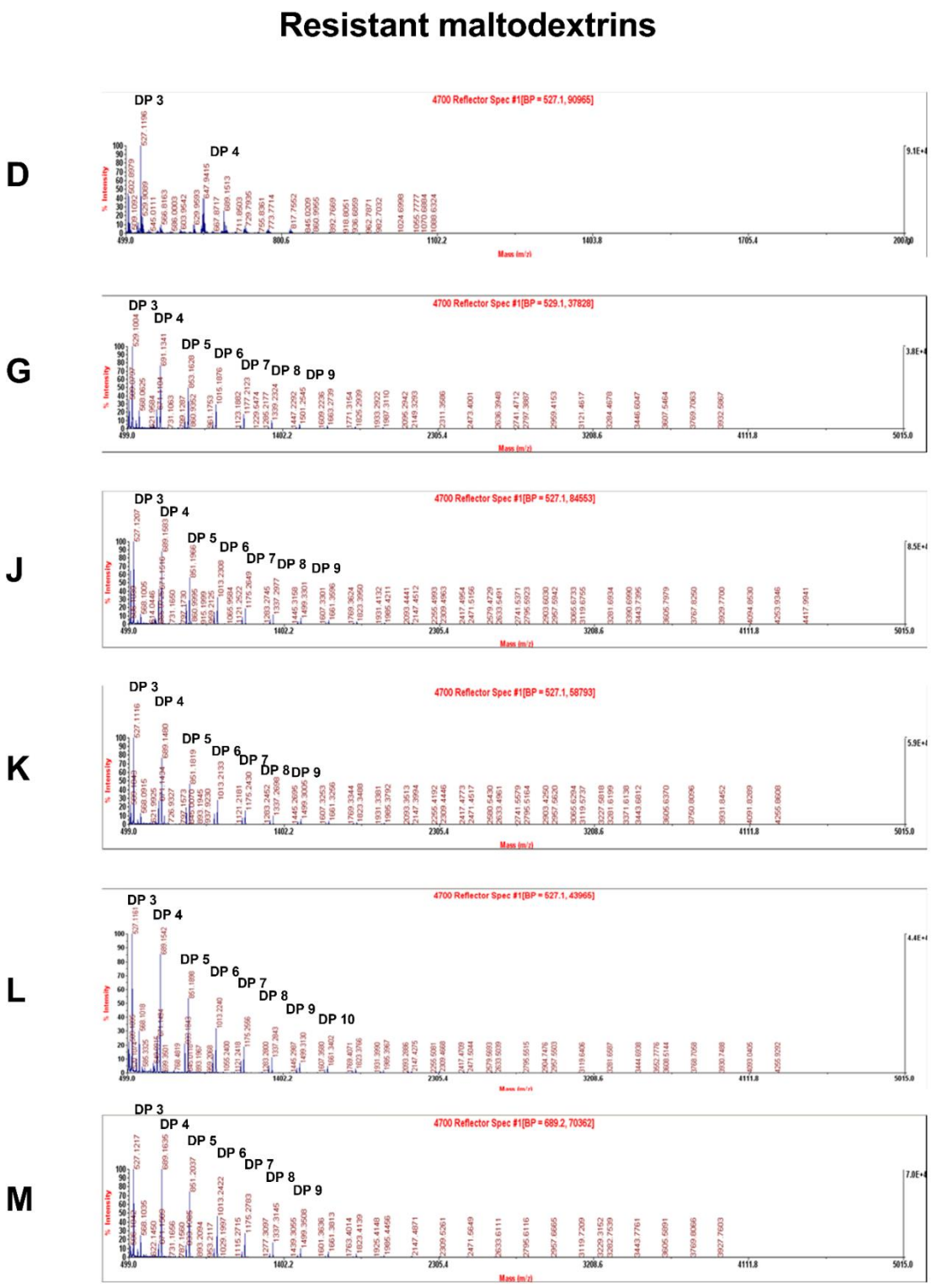

Figure 2.3 MALDI-TOF spectra of each glucan within the resistant maltodextrins depicting the mass-to-charge ratio (m/z) ranging from 500 to 5000.

2.4 Conclusions

Chemical characterization of the glucans employed in this study revealed that the samples provided met the standards of purity (with minimal free sugar contamination), homogeneity (with respect to glucose content) and size required for substrates in this and future fermentation studies. The identification of glucans' chemical and structural arrangement is important as it unveils the true composition of these, facilitating the understanding of changes in gut microbiota attributed to glucan consumption, as there are fewer sources of compositional variants (e.g., different mono or disaccharides with its own set of structures) that could also influence the microbiota. Glucans had very little residual free sugar contamination. Although sugar composition was 97% similar across glucans, they were distinct in the glycosidic linkage composition and degrees of polymerization. Glucans appeared to have structural differences, allowing me to categorize them into three categories: mixed linkage α-glucans, polydextroses and resistant maltodextrins. The most notable difference between the glucan categories lies in the glycosidic linkage structures. The mixed linkage α-glucans were >80% composed of non-branched linkages, whereas the polydextroses and resistant maltodextrins were more similar in the type of single and multiple branched linkages containing a higher percentage (>16% single branched for both categories) compared to the mixed linkage α-glucans and differing in the amount of multiple branched linkages (4% and 2% respectively). Although glucans within each category did not have significant differences in the amount and type of linkages, there were more distinguishable differences among glucan categories. Mass spectrometry analysis revealed these glucans were low molecular weight, confirming the categorization of glucans based on degrees of polymerization. I recommend that these structures be studied at a finer level, using Nuclear Magnetic Resonance (NMR), to identify structural characteristics such as molecular weight and anomeric configuration. These results are relevant to the food industry as they aim to improve and optimize processes to obtain these glucans, which are incorporated in finished foods as dietary fiber with prebiotic features. It is widely known that industry has developed multiple methods to extract and isolate fiber, however the end goal of these processes is to modify the physico-chemical properties of these fibers to improve functionality (e.g., water-holding capacity, gelling, binding, bulking, color etc.). Processing involves incorporation or removal of molecular components that affect the biochemical composition and textural characteristics of the fibers and, hence, the food. There are multiple patents to obtain resistant glucans with well described parameters (temperatures, times, volumes) employed to

obtain them. However, these processes generate a wide range of structural variants in their current forms. Modification of the synthesis parameters can be targeted to have a specific final percentage of α-1,4 and α-1,6 linkages derived from the starting digestible material. My results reveal that manufacturing processes have a great impact in the modification/depolymerization of starch and final structure of starch- (and glucose-) derived resistant glucans. I hypothesize that these molecules will behave differently in their interaction with gut microbiota. If true, the data presented in this chapter further suggest that processes for manufacturing of these glucans may be purposefully modified to target desired structural and compositional changes in the gut microbiome, with the goal of improving health. These results underscore the necessity to understand the chemical and structural composition of fibers at fine detail that could predictably shape the colonic microbiota to improve gut health.

CHAPTER 3. FINE CARBOHYDRATE STRUCTURE GOVERNS THE STRUCTURE AND FUNCTION OF HUMAN GUT MICROBIOTA INDEPENDENTLY OF VARIATION IN GLYCOSYL RESIDUE COMPOSITION

3.1 Abstract

Increased dietary fiber consumption has been shown to increase human gut microbial diversity, but the mechanisms driving this effect remain unclear. One possible explanation is that microbes are able to divide metabolic labor in consumption of complex carbohydrates, which are composed of diverse glycosidic linkages that require specific cognate enzymes for degradation. However, as naturally derived fibers vary in both sugar composition and linkage structure, it is challenging to separate out the impact of each of these variables. We hypothesized that fine differences in carbohydrate linkage structure would govern microbial community structure and function independently of variation in glycosyl residue composition. To test this hypothesis, we fermented commercially available soluble resistant glucans, which are uniformly composed of glucose linked in different structural arrangements, *in vitro* with fecal inocula from each of three individuals. We measured metabolic outputs (pH, gas, and short-chain fatty acid production) and community structure via 16S rRNA amplicon sequencing. We determined that community metabolic outputs from identical glucans were highly individual, emerging from divergent initial microbiome structures. However, specific operational taxonomic units responded similarly in growth responses across individuals' microbiota, though in context-dependent ways; these data suggested that certain taxa were more efficient in competing for some structures than others. Together, these data support the hypothesis that variation in linkage structure, independent of sugar composition, governs compositional and functional responses of microbiota.

3.2 Introduction

Although diet is increasingly understood to play a major role in modulating the gut microbiome (Klingbeil & de La Serre, 2018; Rinninella et al., 2019), the mechanisms by which this occurs are still unclear. Human diets vary widely across populations (Gupta et al., 2017; Yatsunenko et al., 2012), and even within a single individual over relatively short periods. Thus, the diversity and idiosyncrasy of diets makes it challenging to identify how the individual

components thereof influence gut microbiome structure and function in predictable ways across individuals. One class of food components known to significantly shape the gut microbiome's structure and function, and, thereby, human health, is fermentable dietary fibers, which are resistant to hydrolysis by human enzymes but are degraded by colonic microbes, increasing and maintaining diversity of the gut microbiome (J. L. Sonnenburg & Bäckhed, 2016). The task of linking fiber polysaccharides with predictable gut microbiome responses is made more difficult in that complex carbohydrates vary in both composition (i.e., the ratio of sugars that compose the polymer) and in structure (i.e., how those sugars are linked via glycosidic bonds). Specifically, polysaccharides can vary across multiple structural dimensions, including monosaccharide composition, anomeric configurations, glycosidic linkages, linear chain lengths, and branch chain compositions (James N. BeMiller, 2019b). This diversity generates a wide diversity of possible higher-order polymer structures (Hamaker & Tuncil, 2014); for example, a pentasaccharide is estimated to have over 1.5 billion possible structural forms (Pérez & Tvaroška, 2014). Because glycans are so heterogeneous and these structural properties (i.e. composition and structure) covary in most naturally derived fiber polysaccharides, it is very challenging to separate the effects of composition and structure on gut microbiota using fibers extracted directly from plants.

The gut microbiome has recently been shown to be very sensitive to even subtle structural differences in both insoluble (Deehan et al., 2020) and soluble (Tuncil et al., 2020) plant-derived fibers; this has supported the hypothesis that discrete fiber structures may target certain microbial taxa with wide variation in specificity (Cantu-Jungles & Hamaker, 2020). Because the enzymes required for degradation of complex carbohydrates are highly specific to their cognate glycosidic linkages, and possibly to higher-order structures (Déjean et al., 2019; dos Santos et al., 2015), there exists the potential for organisms to specialize in hydrolyzing specific bonds and/or consuming specific parts of the molecule (Hibbing et al., 2010). If true, this niche partitioning may allow cooperative consumption of a polysaccharide, in which organisms avoid competition through division of metabolic labor (T. Yao et al., 2020) and, thereby, increase the microbial diversity that can be sustained.

Here, I aimed to test the related hypotheses that 1) complex carbohydrates identical in composition (i.e., composed solely of glucose) but varying in structure (i.e. with different structural parameters) would select for distinct microbiota, and 2) that the same microbes would be selected by the same structures across different individuals' microbiota. To test this hypothesis,

I used commercially available glucans, generated either by modification of starches (maltodextrins) or polymerization of glucose (polydextroses) by heating or enzymatic catalysis to be at least partially resistant to degradation by human enzymes (together, here collectively termed resistant glucans). Resistant glucans are made by multiple companies with varying sugar and starch sources and processes, and are commonly added to food products in order to increase dietary fiber content to improve health outcomes (Boler et al., 2011; Costabile et al., 2016b; Hooda et al., 2012; Maathuis et al., 2009; Whisner et al., 2016). I performed *in vitro* fermentation of 11 distinct resistant glucan products using fecal microbiota from 3 fecal donors (individually) and measured community structure and metabolic function (pH, gas, and short-chain fatty acid (SCFA) production) over time. These results suggest that, although different initial microbiota respond divergently at the whole-community level with respect to metabolism and overall community structure, specific taxa respond strongly to certain glucan structures across all individuals' microbiota. These data suggest that the fine structure of a carbohydrate alone, independent of differences in composition, can target certain microbial taxa and alter community structure in similar ways across individuals.

3.3 Methods

3.3.1 Fibers

The glucans and one fructan used in this study were gifts from several companies (Tate & Lyle, Hoffman Estates, IL; Ingredion, Westchester, IL; Archer Daniels Midland, Chicago, IL; Samyang, Seoul, South Korea). As reported in Ch. 2, these glucans ranged in moisture content from 2-8% (dry weight), which was measured by a Mettler Toledo Moisture Analyzer HE53 (115V) (Columbus, OH), except for glucan M in a syrup format with higher moisture measured by drying in a conventional oven Masses added for all analytical procedures and microbial cultures was adjusted for moisture content to ensure equivalent carbohydrate loading.

3.3.2 Carbohydrate linkage analysis

The glycosidic linkage composition of the glucans was determined via the partially methylated alditol acetate approach and detected using gas chromatography (model 7890A, Agilent Technologies Inc.) with a fused capillary column (SP-2330, Supelco Analytical) coupled

with a mass spectrometer (model 5975C, Agilent Technologies Inc) as previously described by Pettolino *et al*. Briefly, reactions started with 0.5-1mg of sample and were methylated with CH_3I, hydrolyzed with 2M TFA at 121°C. Myo-inositol (20 ul of 2.5 mg per ml 2M TFA) was added as an internal standard. Prior to the reduction of the samples with acetic acid, they were treated with 2M NH_4OH and 1M freshly prepared $NaBD_4$ in 2M NH_4OH. Acetylation was performed with the addition of acetic anhydrate, followed by dichloromethane (DCM) and multiple ddH_2O washes, and the residue was dissolved in acetone. The GC-MS conditions were as follows: injector volume, 1 µl; injector temperature, 240 ºC; detector temperature, 300 ºC; carrier gas, helium: 1.9 meter/second; split ratio, 100:1 and the temperature program, 100 ºC for 2 min, 8 ºC/min to 240 ºC for 20 min.

3.3.3 Fecal sample collection

Feces from three healthy donors were collected as initial microbiota; these donors had no use of antibiotics for the past six months. All donors were male with an omnivorous diet and reported distinct dietary patterns. Donor 1 was a 25-year-old with a BMI of 31.7 and reported frequent consumption of high-fat and spicy foods. Donor 2 was a 22-year-old with a BMI of 24.9 and reported a diet with elevated consumption of fruits and vegetables. Donor 3 was a 22-year-old male with a BMI of 25.4 and reported a diet with high dairy consumption. Involvement of human subjects in this study was reviewed and approved by Purdue University under the IRB Protocol #: 1701018645.

3.3.4 *In vitro* fecal fermentation

For each donor's microbiota, collected samples were immediately placed into ice post-collection and rapidly transferred to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI), where they were processed. The basal media (phosphate buffer) contained NaCl, KCl, urea as the sole nitrogen source, Na_2SO_4, resazurin, and Na_2HPO_4 and were autoclaved together; heat sensitive compounds such as $CaCl_2$, $MgCl_2$, 1000X P1 metal solution, and cysteine hydrochloride were added post-cooling. Once inside the chamber, fecal samples were mixed with anoxic 40 mM sodium phosphate buffer with pH 6.95 in a 1:20 ratio (w/v) and filtrated through four layers of cheese cloth to make a slurry. Glucans were dissolved in sterile water, passed through a 0.22 µm

Millipore syringe filter and mixed 1:1 with anoxic 2X phosphate buffer to a concentration of 1.25% w/v. This solution was then mixed with the fecal slurry to a 4:1 ratio into 25 ml Balch tubes for each time point in triplicate, with a total carbohydrate concentration of 1% w/v. Tubes were closed with butyl rubber stoppers and aluminum seals and incubated outside the chamber in an incubator at 37℃ shaking at 150 rpm. Culture tubes were taken out of the incubator at each time point (4, 8, 12, 24, 36 and 48 hours) and pH and gas measurements were taken. Gas was measured by overpressure with an 18 ga needle attached to a 10 mL glass syringe; tubes were unsealed and two aliquots were taken out and stored at -80℃ for further SCFA analysis and DNA extraction. pH was measured using a Mettler Toledo pH meter.

3.3.5 Short Chain Fatty Acid Analysis (SCFA analysis)

Short chain fatty acids were analyzed by gas chromatography with a flame ionization detector (GC-FID 7890 A Agilent Technologies Inc.) with a fused silica capillary column (Nukon SUPELCO, Bellefonte, PA) under the following conditions: front detector temperature at 230℃, oven temperature at 100℃ and helium as carrier gas. Frozen samples were thawed and centrifuged for 10 mins at 13,000 rpm. 400 µL of supernatants were mixed with 100 µL of an internal standard (4-methylvaleric acid in 6% v/v phosphoric acid and copper sulfate pentahydrate). The external standards used were acetic, propionic, butyric, isobutyric, and isovaleric acids mixed in equimolar proportions.

3.3.6 DNA extraction

DNA was extracted using the FastDNA SPIN extraction kit (MPBiomedicals, Santa Ana, CA). The aliquots taken at 48-hour were thawed, centrifuged, and the pellet was re-suspended, the process was followed as the FastPrep 24 protocol indicates with the following modifications: bead beating time (twice for 40 s at 6.0 m/s), and triplicating shaking time of the sample and binding matrix (shake 15 min instead of 5 min). The extracted genomic DNA was stored at -20℃ until further sequencing analysis.

3.3.7 16S rRNA gene sequencing

DNA was quantified using the BioTek Take3 EPOCH plate reader (Winooski, VT) and normalized to 100 ng/ul. The V4–V5 region of the 16S rRNA gene was amplified by PCR using the universal bacterial primers: 515-FB forward (GTGYCAGCMGCCGCGGTAA) and 926-R reverse (CCGYCAATTYMTTTRAGTTT) and KAPA HiFi Hot Start ReadyMix
the following thermal cycler conditions: initial denaturation, 95℃ for 5 min, 20 cycles of denaturation (98℃, 20 s), annealing (60℃ for 15 s), and extension (72℃ for 30 s), and a final extension (72℃, 10 min) followed by infinite hold at 4°C. PCR products were cleaned and prepared as previously described by Tuncil et al. (Tuncil et al., 2018), briefly, after the first amplification, unincorporated primers and nucleotides were removed using 1.2X the PCR volume of the AxyPrep Mag PCR Clean-up beads according to the protocol, then quantitated and normalized with TruSeq indexed primers (IDT, Coralville, IA) followed by a second cleanup using a bead volume of 1.8X the PCR volume. Post-cleanup DNA was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. DNA with similar concentrations were then pooled into 11 pools for sequencing. Pools were examined for size using a BioAnalyzer (Agilent, Santa Clara, CA) and quantified using the KAPA Library Quantification Kit (Roche Diagnostics) prior to sequencing on a 2x250 cycle Illumina MiSeq run at the Purdue Genomics Core Facility.

3.3.8 Sequence processing

Sequences were processed with mothur (v.1.39.3) following the MiSeq SOP. Sequences were screened for a max. 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 across positions 13862 to 27654. Sequences were classified based on the Ribosomal Database Project reference training set with an 80% cutoff. Sequences identified as chloroplast, mitochondria, archaea, or eukaryota and those with unknown classifications were removed from further processing. OTU classifications at the species levels are reported as percentage of reads based on a 97% similarity. Ecological metrics were calculated within mothur: α-diversity metrics were calculated using the simpsoneven, chao, invsimpson and sobs calculators and β-diversity metrics were calculated using the jaccard and thetayc calculators as implemented in mothur.

Distance matrices for β-diversity metrics based on the pcoa command in mothur were plotted for visualization using R. Abundance changes were $\log_2$-transformed for the most-abundant 33 OTUs.

3.3.9 Statistical Analyses

All analyses were performed in triplicate. SCFA data are presented as means and standard deviation. Tukey HSD at $\alpha = 0.05$ was used to compare mean differences. Analysis of molecular variance (AMOVA) tests command in mothur were also computed between glucan classes to determine whether centroids were significantly distinct. Alpha-diversity was determined by One-way ANOVA $p < 0.0001$ with Kruskal-Wallis test for multiple comparisons. Linear discriminant effect size (LEfSe) analysis was conducted using LEfSe v. 3.12 for glucans and glucan types. Results

3.3.10 Resistant glucans produced by different methods varied significantly in structure

Glucans provided for this study came from different commercial sources and were categorized into three different groups based upon their source and structural characteristics. Significant differences in glucan structure were revealed by carbohydrate linkage analysis (Fig. 3.1). These glucans were categorized as mixed linkage α-glucans (A-C), resistant maltodextrins (D, G and J-M) and polydextroses (E and H). These glucans are completely or semi-synthetic, non-digestible carbohydrates that meet the FDA dietary fiber definition (FDA, 2018). Resistant maltodextrins and mixed linkage α-glucans are generated from starch hydrolysis products, and hence are composed of human-digestible linkages (α-1,4, α-1,6) as well as bonds that cannot by hydrolyzed by human enzymes, such as α-1,2 and α-1,3 linkages. In contrast, polydextrose is polymerized from monomeric glucose and is composed of random α- and β-1,2, -1,3, -1,4 and -1,6 linkages. Interestingly, even within categories, glucans differed in the type and amount of multiply branched glycosyl residues and spanned a range of chemical complexity (defined by the number of distinct carbohydrate linkages). Of the set, the mixed-linkage α-glucans A-C can be classified as relatively simpler polymers (with less modification of the native starch structure and lacking evidence of multiply linked branches), compared to glucans E, H, D, G and J-M. These more complex glucans revealed a higher diversity of carbohydrate linkages, both with respect to distinct linkage types and abundances of multiply branched glycosyl residues. The three mixed-

linkage α-glucans spanned a complexity gradient, with glucan A the most complex and C resembling, in linkage profile, native starch structure. However, it is important to note that anomeric configuration of these linkages is not indicated via the partially methylated alditol acetate method; thus, a fraction of the 4- and 6-linked glucose in these glucans may occur in beta linkages, which are also indigestible by human enzymes.

3.3.11 Community metabolic outputs from identical glucans diverges across distinct microbiota compositions.

I tested multiple glucans with different configurations to determine how similar or different metabolic outputs would be in fermentation by fecal microbiota from each of three different healthy donors of the most common short-chain fatty acids (SCFAs; acetate, butyrate and propionate) were measured at intervals over a 48-hr. time course (Fig. 3.3).

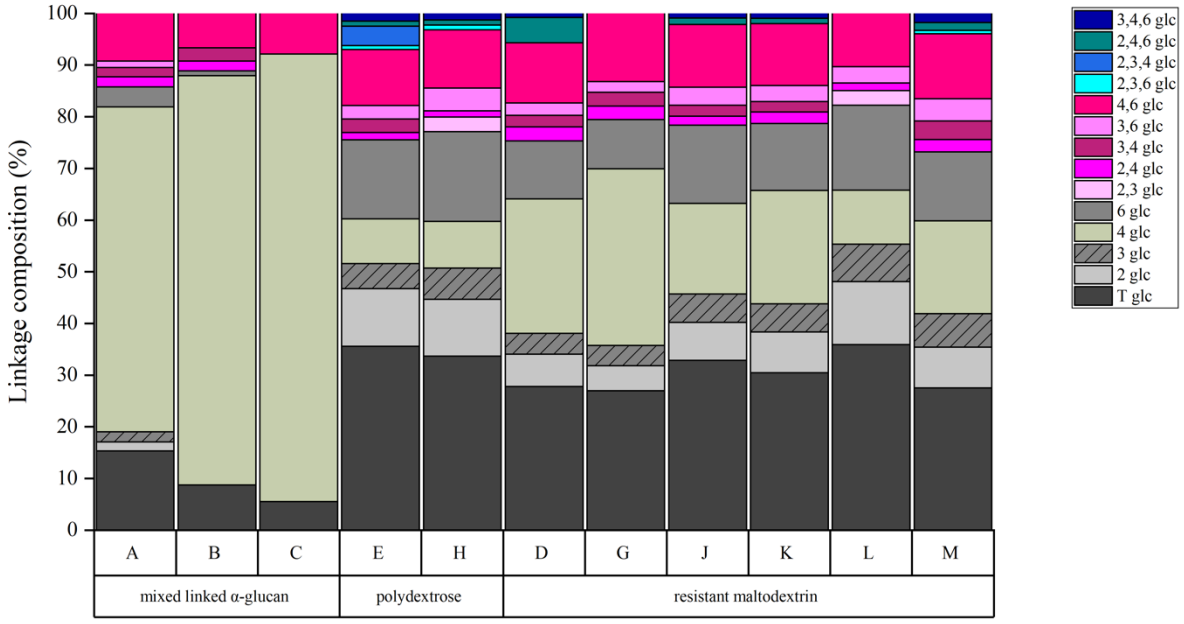

Figure 3.1 Glycosidic linkage composition of glucans determined by PMAA with GC-MS.

As expected, we observed significant gas and acid production from all glucans, although the magnitude varied across glucan classes and individual donor microbiota. Although all glucans were strongly fermented, terminating at pHs near or below 5, the mixed linkage α-glucans group fermented more rapidly (as evidenced by a more rapid pH drop and more rapid evolution of gas),

especially glucan C. Whether these glucans fermented strongly to acid or gas was donor-dependent and, as expected, acid and gas production were generally inversely related. The decrease in pH was especially marked in fermentation of glucans A-C by donor 3 microbiota; conversely, donor 2 microbiota produced significantly more gas from the same mixed linkage α-glucans, excepting donor 1 microbiota's strong response to glucan A. In contrast, fermentations of polydextroses and resistant maltodextrins decreased in pH much less rapidly for all donors. Interestingly, gas and acid production rates were microbiota- and glucan-specific, suggesting initial microbiome structures were differently poised for specific glucans and their corresponding metabolic outputs.

Despite similarity in overall gas and acid production for most glucans, patterns of SCFA production from different structures varied substantially across donors' microbiota (Fig. 3.3). Although same-glucan patterns of acetate production were similar across all the donors' microbiota, the magnitude of acetogenesis varied substantially across individuals; however, within-donor acetate production from the mixed linkage α-glucans was stronger than for any other glucan. In contrast, patterns of propiogenesis and butyrogenesis were glucan- and donor-specific. Donor 1 microbiota were generally the most butyrogenic on all substrates except the mixed linkage α-glucans; on these substrates, donor 2 microbiota were more strongly propiogenic than butyrogenic. Conversely, donor 2 microbiota were by far the most propiogenic on polydextroses and resistant maltodextrins but were very strongly butyrogenic and less propiogenic in consumption of mixed linkage α-glucans. Within glucan categories, SCFA production from distinct glucans varied, especially for donor 3 microbiota. Generally, the SCFA output of donor 3 microbiota was lower than either of the other two, especially on mixed linkage α-glucans and for acetate and propionate; however, on some substrates (i.e., polydextrose E and resistant maltodextrin G) butyrate production from donor 3 microbiota was equivalent to those of other donors and substantially lower on others (i.e. resistant maltodextrin J).

Despite these idiosyncrasies, we observed some general tendencies in fermentation of different glucan classes. Across donors, the resistant maltodextrin category produced the most butyrate, whereas the mixed linkage α-glucans produced the least butyrate and propionate but the most acetate ($p < 0.05$). Together, these data suggested that, although distinct glucan structures were differently metabolized by different microbiota, which produced different metabolic outputs at equivalent total carbohydrate loadings, the eventual fate of glucan metabolism emerged largely from initial microbial community structure.

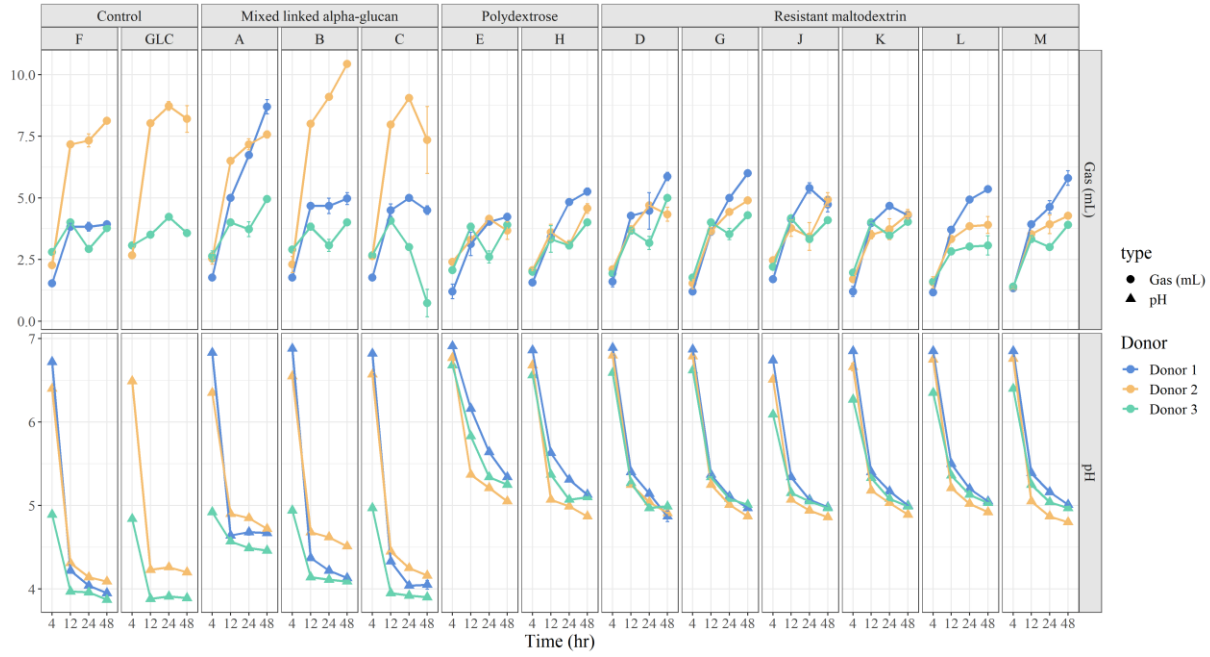

Figure 3.2 Gas (mL) and acid (pH) productions measured at 4-, 12-, 24-, and 48 hr time points for all glucans. Error bars represent standard deviation of the mean of three separate replicates.

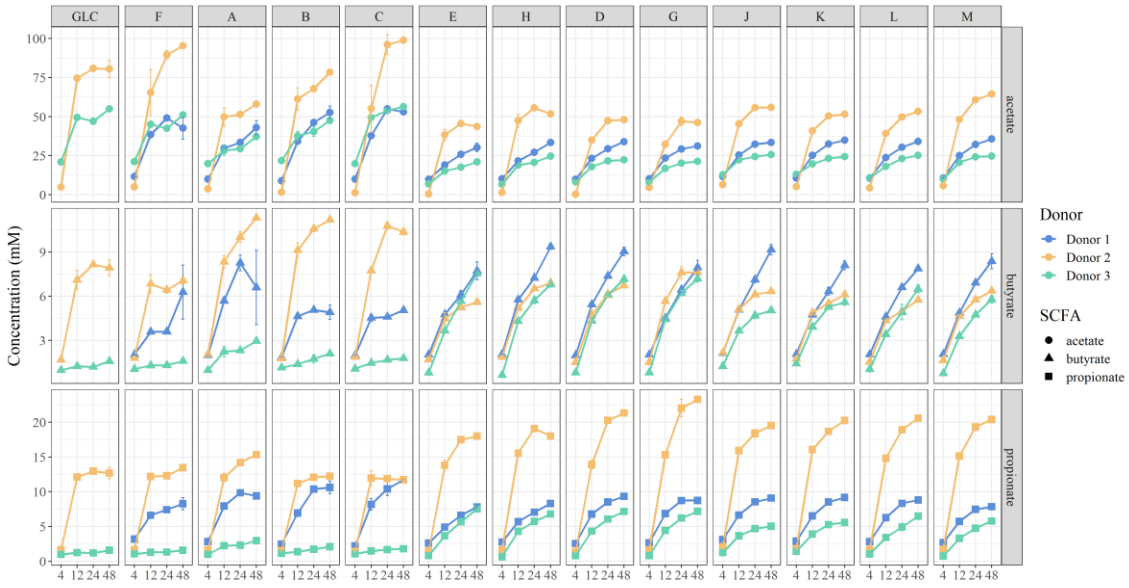

Figure 3.3 SCFA production at 4,12, 24- and 48-hours post-inoculation measured by gas chromatography. Error bars represent the average mean of three separate replicates.

3.3.12 Glucan structure affects the community structure, but not diversity, of fermenting microbiota

Changes were assessed in microbial abundances after 48 hr of fermentation using 16S rRNA gene amplicon sequencing. Differences in α-diversity among glucan fermentations were strongly driven by initial differences in donors' fecal microbiota. With Good's coverage above 97% for all donors' fecal samples, donors varied in overall α-diversity and both the richness (species observed) and evenness (Simpson's evenness) components. Most notably, donor 1 microbiota displayed significantly lower overall diversity compared with the other two donors ($p < 0.0001$) as determined using the inverse Simpson index, and donor 2 and donor 3 microbiota were not significantly different ($p = 0.09$). However, donor 2's initial community was significantly higher in richness compared to the other donors ($p < 0.0001$). Donor 1's initial community was significantly less even when compared with the other two donors ($p < 0.0001$), however donor 2 and donor 3 did not significantly differ in evenness (Fig. 3.4). By comparison, differences among glucans in post-fermentation α-diversity were minor. From the fecal inoculum, decreases in the inverse Simpson index and its richness and evenness components were observed for fructan controls and mixed linkage α-glucan cultures with donor 1 microbiota and for resistant maltodextrin D, G, and L cultures with donor 2 microbiota ($p < 0.05$) (Fig. 3.4). No significant differences were observed among glucans or with respect to the fecal inoculum for donor 3 microbiota.

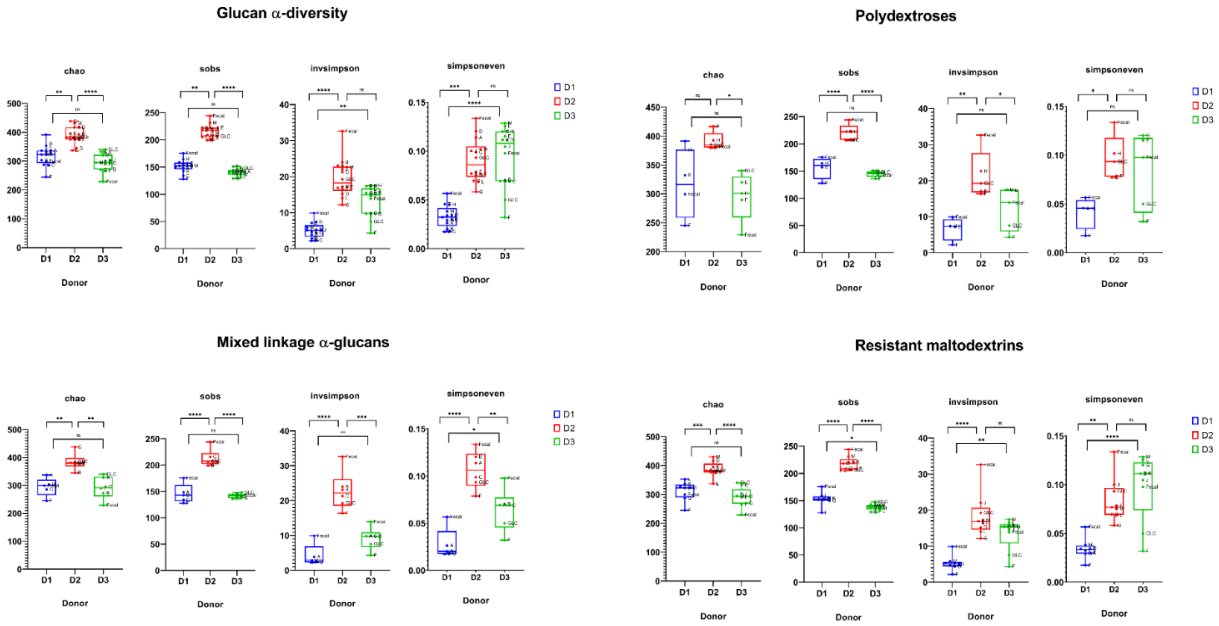

Figure 3.4 Comparison of alpha diversity metrics for community richness, evenness, and diversity among donors and within glucan categories, the initial fecal inoculum, and control groups containing glucose (GLC) and a fructan (F).

Although all initial donor microbiomes were dominated by members of phylum *Firmicutes* (53%, 63%, and 54% for donors 1,2, and 3, respectively), they displayed very different initial microbiota structures. Each had at least one dominant OTU from phyla other than *Firmicutes*; OTU00001 (*Prevotella copri*, 37%) and OTU00006 (*Faecalibacterium prausnitzii*, 20%) dominated the donor 1 community, OTU00003 and OTU00004 (both *Bacteroides* spp.) constituted ~26% of the reads from the donor 2 community, and OTU00002 (*Bifidobacterium* spp., 25%) and OTU00006 (*F. prausnitzii*, 16%) were dominant in the donor 3 community. These initial community structures governed relative abundances in fermentations and, therefore, donor identity was much more influential than glucan structure in clustering based upon β-diversity. Consequently, although centroids of clusters based upon glucan class were not significantly different across all donors, significant differences were observed within donors (AMOVA, $p < 0.001$; Table 4).

Table 3.1 Analysis of Molecular Variance (AMOVA) showing separation of glucan categories for each donor (D1, D2, D3), calculated by mothur. *Indicates statistically significant differences between the glucan categories.

	Initial			**Control**			**Mixed linkage α-glucan**			**Resistant maltodextrin**			**Polydextrose**		
	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
Initial				0.024	<0.001*	0.036	0.003	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.019	0.008	0.004
Control	0.024	<0.001*	0.036				0.014	<0.001*	0.012	<0.001*	<0.001*	<0.001*	0.005	<0.001*	<0.001*
Mixed linkage α-glucan	0.003	<0.001*	<0.001*	0.014	<0.001*	0.012				<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Resistant maltodextrin	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*				<0.001*	0.232	<0.001*
Polydextrose	0.019	0.008	0.004	0.005	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.232	<0.001*			

However, some similarities in overall community composition responses were observed across donors. When considered at the level of the individual donors, clusters separated based upon glucan category for each (Fig 3.5). Interestingly, as observed with respect to metabolic outputs, community structures of microbiota fermenting resistant maltodextrins and polydextroses were more similar to one another than those fermenting mixed linkage α-glucans (which were, in turn, more similar to fructan and glucan controls).

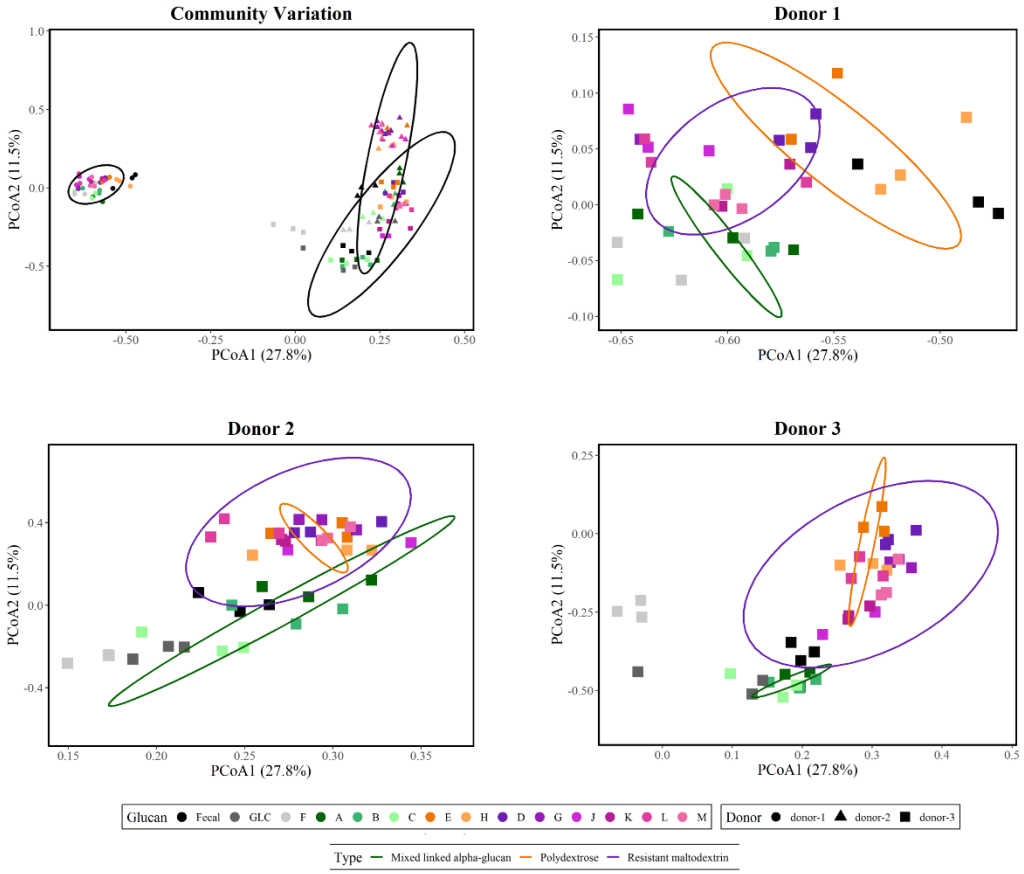

Figure 3.5 Principal coordinate analysis (PCoA) plot displaying differences in α-diversity, as calculated using the Yue and Clayton theta metric. Ellipses drawn based on 99% confidence level.

3.3.13 Glucan structural variants selected for specific OTU's in a context-dependent manner

Although changes in overall community structure were observed, these changes were relatively minor over the 48 hr fermentation. Relative abundances of OTUs in post-fermentation communities strongly resembled the initial community structures, structuring significantly more strongly by donor than by glucan (Fig. 3.6). However, some broad patterns of OTU responses to glucans across donors could be discerned. Notably, mixed-linkage α-glucans supported large populations of OTU00002 (*Bifidobacterium* spp.) across donors, although the population sizes were linked with α-glucan complexity (increasing in abundance with decreasing complexity) only with donor 2 microbiota. Additionally, members of order *Bacteroidales* (here, genera *Bacteroides*, *Prevotella*, and *Parabacteroides*) and family *Lachnospiraceae* (here, genera *Anaerostipes*,

Roseburia, *Fusicatenibacter*, and *Blautia*) were very abundant and diverse in fermentations of polydextroses and/or resistant maltodextrins; however, the most abundant OTUs within these group varied by donor, largely based upon initial population sizes. Members of family *Ruminococcaceae* generally decreased in abundance from the inoculum irrespective of substrate. In general, however, community compositional responses to glucans appeared context-dependent and, largely, idiosyncratic to donors.

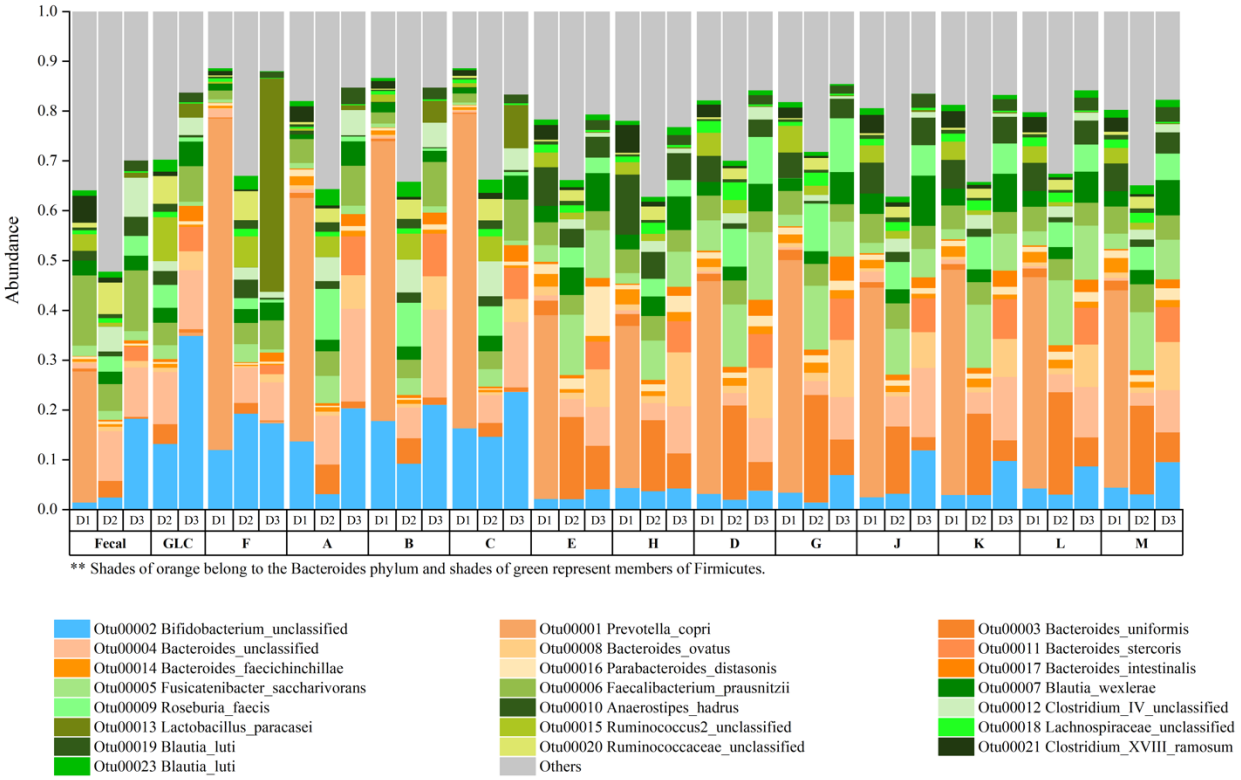

Figure 3.6 Relative abundances of dominant OTUs (≥ 1%) after 48 hours of fermentation of distinct glucan structures. by inoculum donor and glucan. D1, D2, and D3 represent donor 1, donor 2, and donor 3 inocula, respectively. Letters A-M indicate different glucans (described in Fig. 3.1), except that GLC indicates the glucose positive control and F indicates the fructan positive control.

Despite the context-dependent responses in community structure, linear discriminant analysis revealed significant associations of taxa with distinct glucan structures across donors' initial community structures (Fig. 3.7). When glucans were considered together in their classes, members of *Bacteriodaceae* were linear discriminants of resistant maltodextrins, whereas

members of families *Porphyromonadaceae*, *Erysipelotrichaceae*, *Veillonellaceae*, and *Lachnospiraceae* were discriminants of polydextroses (LDA ≥ 3.5). However, within families *Bacteroidaceae* and *Lachnospiraceae*, many genera and species exhibited preferences for the other classes of glucans; for example, *Bacteroides ovatus* and *Bacteroides uniformis* were discriminants of polydextroses, whereas *Fusicatenibacter saccharivorans* and *Roseburia faecis* were discriminants of resistant maltodextrins (Fig. 3.8). I also observed significant preferences of different taxa for distinct glucans within categories; for example, although most members of genus *Bacteroides* (including *B. ovatus* and *B. uniformis*) were most abundant in fermentations of resistant maltodextrin G, *B. faecichinchillae* was overrepresented in fermentations of resistant maltodextrin M (Fig. 3.9). Similarly, resistant maltodextrin D favored *Fusicatenibacter saccharivorans*. The two polydextroses also favored different taxa; polydextrose E was preferential to *Parabacteriodes distasonis* and *Blautia wexlerae*, and *Anaerostipes hadrus* and *Clostridium ramosum* were discriminants of polydextrose H. Together, these data suggested that organisms responded distinctly to resistant glucans with subtle variations in structure. Interestingly, there were no discriminants of the mixed linkage α-glucans; this result may be explained by less specific consumption of these glucans by certain OTUs (Cantu-Jungles & Hamaker, 2020) and, therefore, less significant community structure variations from the initial inoculum or positive controls.

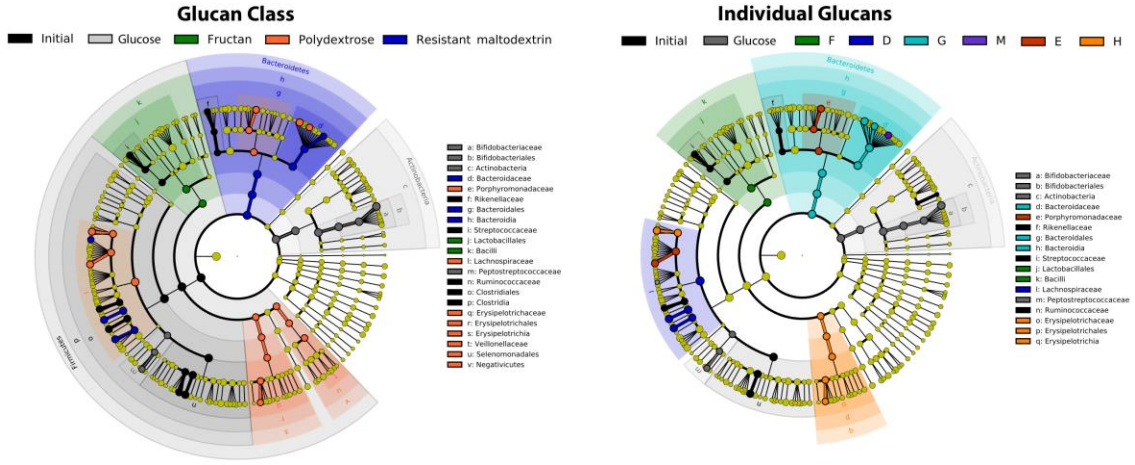

Figure 3.7 Linear discriminant analysis of bacterial taxa differentiating glucans and glucan classes (LDA ≥3.5). Nodes represent different taxonomic levels increasing in taxonomic resolution (species on the outer ring).

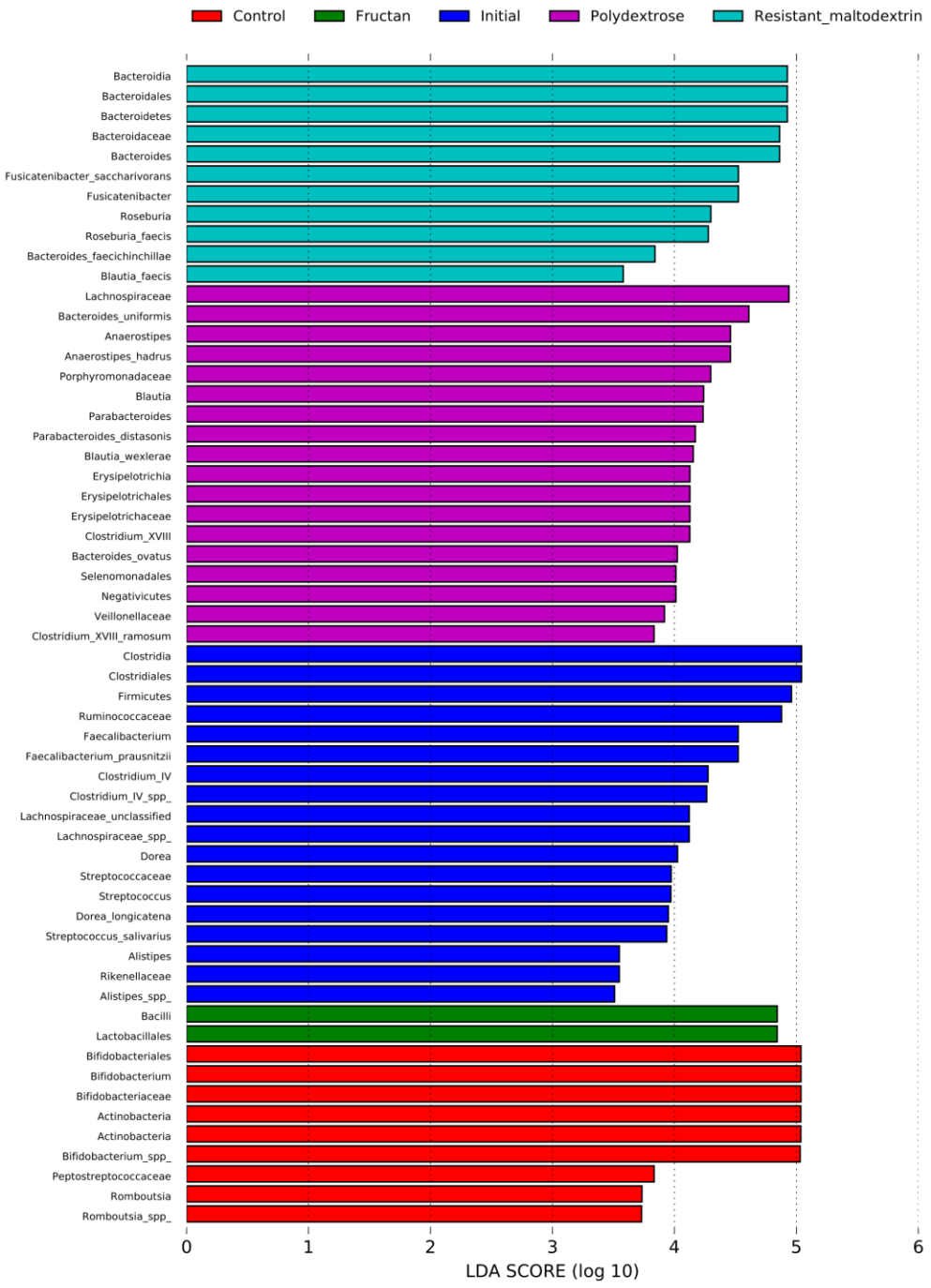

Figure 3.8 Linear Discriminant Analysis (LDA > 3.5) depicting the differential taxa selecting for the glucan categories.

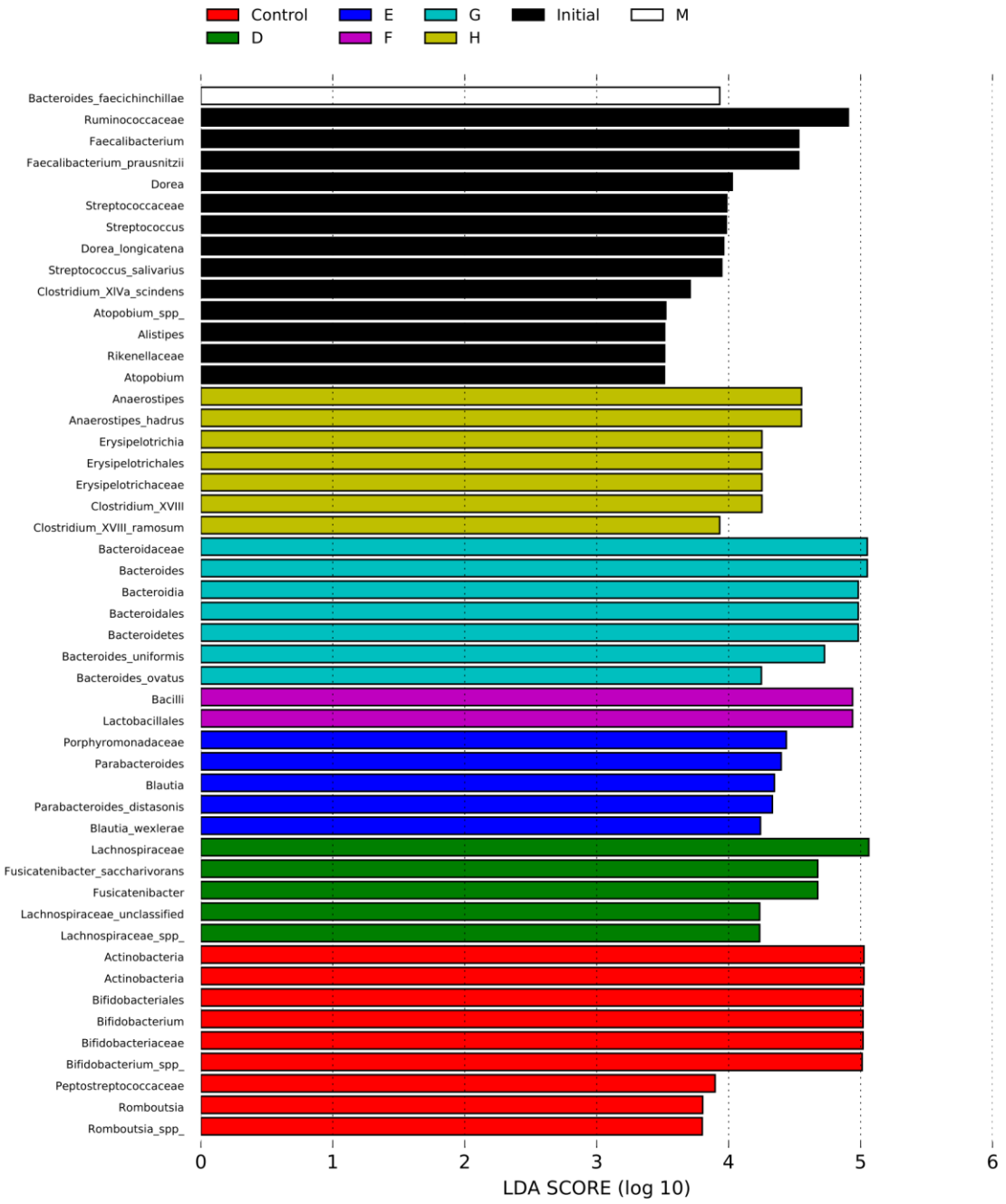

Figure 3.9 Linear Discriminant Analysis (LDA > 3.5) depicting differential taxa for distinct glucans within the glucan categories.

To determine whether OTU-level specificities for distinct glucans were present but obscured by idiosyncratic donor responses, OTUs were compared by their change in abundance over fermentation in all three donor contexts. In this analysis, organisms growing faster than average display increases in relative abundance, while those growing slower than average will be seen to decrease in abundance. This revealed both similarity and context-dependence in OTU responses to different glucan structures and, further, differences in growth patterns in different OTUs classified within the same genus (Fig. 3.8). For some OTUs, such as OTU00016 (*Parabacteroides*), responses were glucan-specific and similar across all donors (for this OTU, most notably consistently stronger responses to polydextrose E (increasing 3.7-, 3.5, and 20-fold for donors 1, 2, and 3, respectively) than H, but similar preferences for the different resistant maltodextrins). However, for other species, responses to distinct glucans were strongly influenced by donor of origin. For example, although OTUs within genus *Bacteroides* responded with generally above-average growth on polydextroses and resistant maltodextrins across donors, they responded to mixed linkage α-glucans with above average growth rates uniformly in the context of donor 3's microbiota and solely to glucan A in donor 1 and donor 2 communities. Further, different OTUs within *Bacteroides* displayed very different growth patterns on the resistant maltodextrins and polydextroses; for example, in donor 1 and 3 microbiota, OTU00003 responded more strongly to polydextroses than resistant maltodextrins, but vice versa in donor 2 microbiota. OTU00008 showed a similar pattern to OTU00003 for donors 1 and 2, but in donor 3 it was similarly responsive to all polydextroses and resistant maltodextrins. Similar OTU-level differences in glucan response based upon community context were observed within genus *Blautia* for all donors. Additionally, analogous dynamics occurred where a single dominant OTU was identified in a genus; OTU00005 (*Fusicatenibacter*) responded strongly to polydextrose H but not to polydextrose E or resistant maltodextrins in donor 1 communities, but grew at an above-average rate across all glucans in context of donors 2 and 3. OTU00010 (*Anaerostipes* sp.) responded strongly to polydextrose H and resistant maltodextrins J-M in donor 1 communities, only to polydextroses (especially H) in donor 2 communities, and did not record above-average growth on any glucan in donor 3 microbiota. OTU00027 (*Clostridium_XVIII*) responded strongly to resistant maltodextrins and polydextroses in donor 1's and 3's microbiota, however it did not exhibit above-average growth for resistant maltodextrin D and G in donor 2's. Together, these data suggest that

organismal preferences for distinct glucan structures exist, but that actual species' responses depend upon interaction with other community members (both positive and negative).

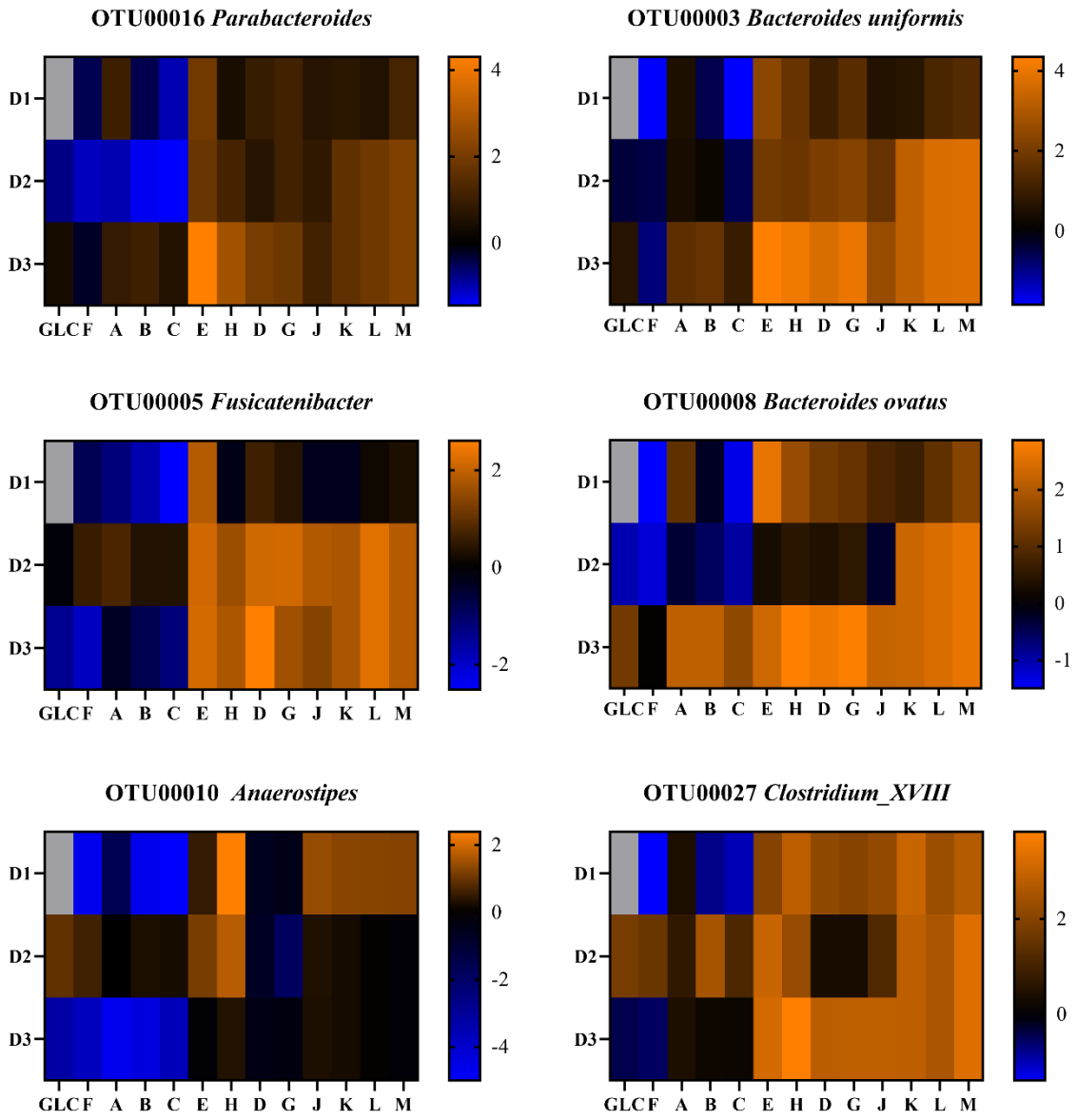

Figure 3.10 Log_2-transformed fold change of OTU abundances after glucan fermentation. The top six most responsive OTUs comparison across all donors. Orange shades represent increases in abundance; blue shades represent decreases in abundance; black indicates no change with respect to the initial microbiota. OTUs not detected within an individual's inoculum are gray.

Cross-donor abundance fold change correlations revealed glucan-specific patterns. In many cases OTUs (and, sometimes, higher taxa) behaved similarly within (or even across) glucan classes.

For example, members of *Bacteroides* (OTU00017, OTU00003, OTU00004, OTU00008, OTU000014, and OTU00011) were generally strongly correlated with one another across glucan classes, with the exception of moderate inverse correlation in growth between OTU00003 and both OTU00017 and OTU00004 on polydextrose H and resistant maltodextrins J and M. However, for some OTUs (and higher taxa), the correlations in abundance fold change among members were strongly determined by substrate glucan. Similarly, in genus *Blautia* (OTU00033, OTU00007, OTU00026, OTU00023, OTU00019, and OTU00062), the level of coherence in growth response on glucans among the various OTUs depended upon the community of origin. In donor 1 communities, OTU00026 displayed significantly above-average growth (compared with other members of *Blautia*) on essentially all polydextrose and resistant maltodextrin substrates. This OTU also showed above-average increases in relative abundance in donor 2 communities, however OTU00007 showed above-average growth on resistant maltodextrins K, L, and M and OTU00062 responded strongly to polydextroses E and H and resistant maltodextrins D, G, and J (though still composing a small fraction of the community, owing to its small initial abundance). On the other glucans, the growth responses of these two OTUs were average or below. In donor 3 communities, only OTU00007 and OTU00026 show slightly faster growth than their other cousins in *Blautia*, though this effect much smaller across glucans than in donor 1 and 2 communities. Taken together, these data suggest that interactions among OTUs and complex glucans depended upon 1) the fine structure of the glucan and 2) the initial abundances of other community members.

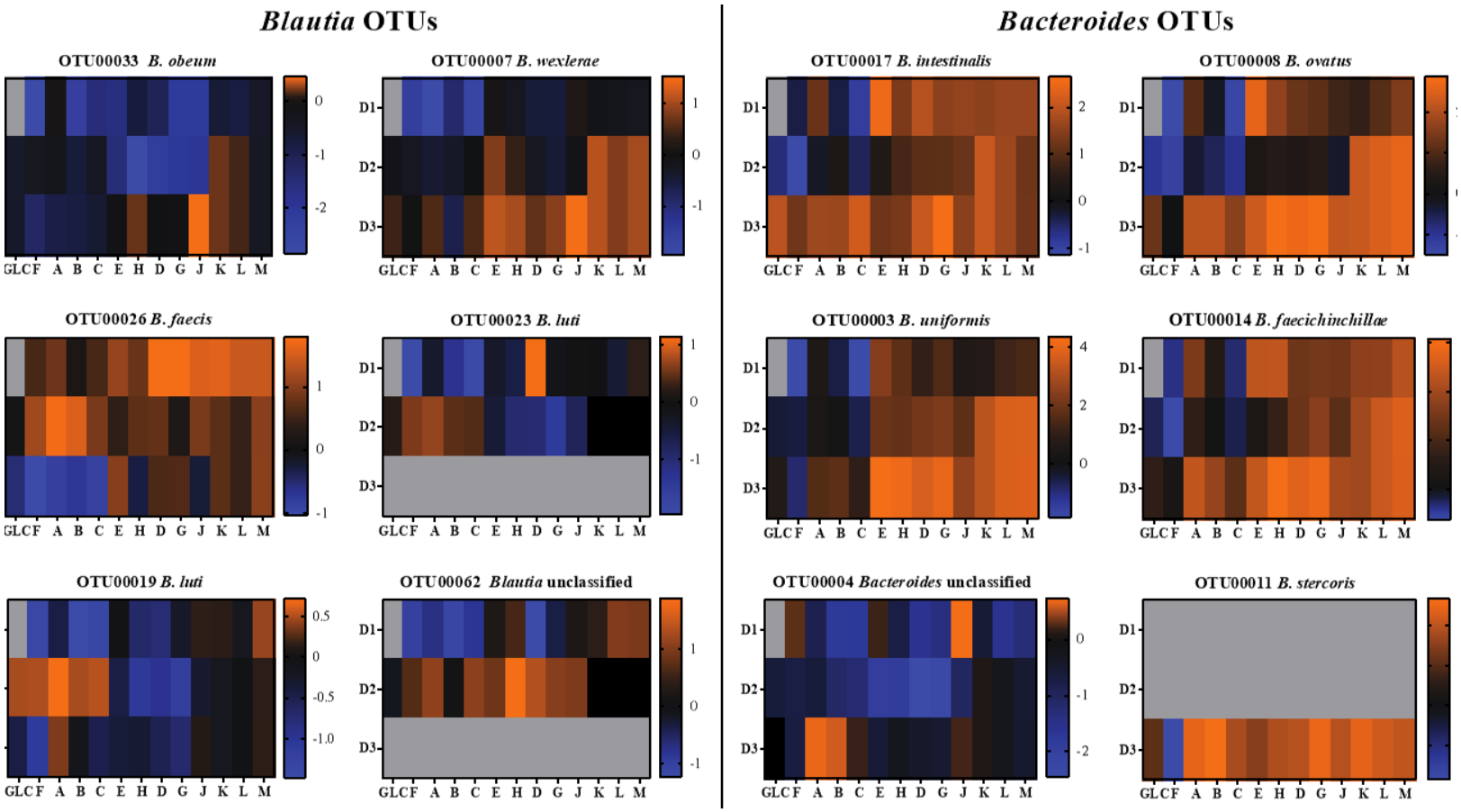

Figure 3.11 Log2-transformed fold change of OTU abundances after glucan fermentation. Comparison of OTUs belonging to the genera Blautia and Bacteroides spp.

3.4 Discussion

In nature, variation in polysaccharide structure is overwhelmingly associated with differences in the ratios of glycosyl residues that compose the polymer (starch and glycogen being notable exceptions) (Koropatkin et al., 2012). For example, arabinoxylans extracted from bran of three classes of wheat exhibited coordinate variation in sugar composition and linkage structure (Tuncil et al., 2020). These variables may exert independent influences upon microbial community responses to complex substrates. Therefore, in this study we aimed to separate these to variables by using glucans that varied in fine structure, but were composed entirely of glucose, as substrates for gut microbiota in *in vitro* fermentations. Although some natural homoglycans (for example, amylose and amylopectin) contain just one or two linkage types and have a relatively simple chemical structure although they are large polysaccharides, the glucans tested here displayed significant variability in linkage complexity despite being composed of a single sugar. In principle, different linkage structures among glucans may distinctly impact microbiome structure and function due to enzyme specificities, which vary among organisms, and therefore target different microbes (Cantu-Jungles & Hamaker, 2020). Differences in glycosidic linkage types even among glucose disaccharides has been demonstrated to significantly alter the SCFA outputs of fecal microbiota (Harris et al., 2017), suggesting that even these simple glucans are metabolized differently or by different microbes. Furthermore, in consumption of disaccharides, differences in transport are unlikely to be very influential; in contrast, as glucan sizes increase, the potential for specialization around transport of specific types of oligosaccharides, in addition to hydrolysis of specific linkages, to govern division of labor and maintenance of diversity in microbiomes increases (Lindemann, 2020b). Broadly, our data supported the hypothesis that variation in fine polysaccharide structure, independent of variation in the sugars that compose the polymer, results in different composition and function of fermenting gut microbiota.

These results demonstrate that microbiome responses to structurally distinct resistant glucans depend upon both fine glucan structure and community context, and community metabolic phenotypes emerge from the interaction of the two. With respect to metabolic outcomes, predominant SCFA outputs were largely determined by resistant glucan class (mixed-linkage α-glucans, resistant maltodextrins, and polydextroses), although some donor-dependent differences were observed among individual glucans within a class. Importantly, whether propiogenesis or butyrogenesis dominated in fermentation of a resistant glucan class was idiosyncratic to donors

and inversely correlated, suggesting 1) microbiomes have potential to produce either SCFA, but that 2) microbiomes are poised to produce one of the two from any given glucan class. It is important to account for differences in digestion of these glucans in the upper gastrointestinal tract, which may differentially alter the glucan structures finally encountered by gut microbiota (Baer et al., 2014); however, these priming effects may underlie variable individual metabolic responses to dietary resistant glucans.

With respect to community composition, however, individual glucans within a class markedly diverged from one another in donor-dependent ways. Although we observed some consistency in responses across glucan classes at higher taxonomic levels, these relationships often shifted when individual glucans were considered independently. For example, members of genus *Bacteroides*, when considered at the level of glucan class, were overrepresented in cultures on resistant maltodextrins, with some species instead preferring polydextroses. When individual glucans were considered, however, this shifted to alternate preferences for resistant maltodextrins G and M, indicating that preferences of these OTUs for substrates within the resistant maltodextrin class was not equivalently high. Similarly, family *Lachnospiraceae* was a linear discriminant of polydextroses with respect to glucan classes, but of resistant maltodextrin D when all glucans were considered individually. Members of the genera *Bacteroides* and *Parabacteroides* as well as *Anaerostipes* (within *Lachnospiraceae*) have been observed to increase in abundance in feeding trials using some types of resistant starches (Barouei et al., 2017; Deehan et al., 2020; Hu et al., 2016; Martínez et al., 2010; Nishimura et al., 2018; Trachsel et al., 2019; Upadhyaya et al., 2016). In addition, these taxa appear to respond to resistant maltodextrins and polydextrose in some feeding trials (although often other fibers are co-administered, which may obscure the effect of the glucans) (Baer et al., 2014; Hoeflinger et al., 2015; Lefranc-Millot et al., 2012; Maragkoudaki et al., 2020; Tran et al., 2019; Whisner et al., 2016), so their general increases in abundance in cultures consuming resistant maltodextrins and polydextroses in our *in vitro* fermentations is not particularly surprising. However, to our knowledge, previous studies have not rigorously compared resistant glucans for differential effects on gut microbiome community structure; intriguingly, however, variant resistant glucans have been observed to ferment different fecal SCFA concentrations in rat feeding trials (Weaver et al., 2010), which suggests microbiome specificities as a hypothetical mechanism.

Underlying these higher-taxon differences, divergences among initial microbiota and glucan structures became even more pronounced when considered at the OTU level. To extend the previous example, relative abundance fold changes among *Bacteroides* OTUs varied as a function of both glucan and initial microbiota composition; although members of *Bacteroides* were responsive across individuals, the specific OTU-glucan associations varied. Interestingly, however, in many cases the same OTUs responded strongly to glucans across multiple donors (in some cases, all three) irrespective of initial abundances, suggesting fundamental associations exist for some OTU-glucan pairs. Together, these data suggest that species may have preferences (possibly based on hydrolytic enzyme or transporter gene content) for specific glucan structures; however, it further suggests that an organism's fitness on these substrates depends on competitive (or, possibly, cooperative) interactions with other species in the community. It should be noted that organisms can compete (and cooperate) in ways that are independent of carbohydrate consumption (for example, in exchange of terminal fermentation products (Belenguer et al., 2006; Bernalier-Donadille, 2010)), and this study cannot separate these mechanisms of competition. However, to my knowledge, the degree of correspondence between specific OTUs (within responding higher taxa) for varying glucan structures and the influence of community context has heretofore not been demonstrated.

If these observations of strain specificity and context dependence in competition among fecal microbiota for variant resistant glucan structures *in vitro* are recapitulated in gut microbiomes *in vivo*, it suggests two related corollaries: 1) that outcomes of resistant glucan feeding trials are initially highly individual, based upon initial microbiome structure, but 2) over time, extended resistant glucan feeding should cause gut microbiomes to converge as the most efficient organisms increase in abundance across individuals. Further, it would suggest that this level of individual variability is likely to obscure outcomes of specific interactions between gut microbiota and variant fiber structures. As previous studies have asserted, it suggests that responses to well-defined fiber structures may be predictable given known microbiome community structures (Korpela et al., 2014), and potentially even for novel fibers that have not yet been tested (by comparing their structures to microbiome responses to other structures). Future experiments should endeavor to determine whether the specificities among different OTUs and glucan structures are recapitulated *in vivo*, and whether these differences result in divergent physiological outcomes.

CHAPTER 4. CONCLUSIONS

This study was intended to determine the extent to which commercial resistant glucan products vary in fine chemical structure and degree to which these glucans impact the composition of gut microbiota and consequent metabolic output *in vitro*. The fermentation of these glucans resulted in changes in microbiota from the consumption of each specific glucan and across donors.

In the broader scientific literature, starch-derived glucans are often presumed to be metabolized in similar ways, owing to their similarities in overall composition and structure (Swanson et al., 2020). However, in the first part of my study I demonstrated that different manufacturing processes produce glucans that indeed vary in fine structural arrangements (e.g., glycosidic bonds types, degree of polymerization), therefore hypothetically altering the way they are fermented by microbiota. Production processes for resistant glucans vary in the use of heat, acid/alkali, and catalysts (for polydextroses) which may each influence the relative abundance of non-branched, single- and multiple-branched linkages and which influence the overall complexity of the glucan and its resistance to fermentation by gut microbiota. Using mass spectrometry, I was able to identify each glucan's degree of polymerization (based upon the glucose monomer), which revealed a range across the glucans, but with similarities within glucan categories. The chemical characterization of this study helped me identify and classify the glucans, resulting in three categories: polydextroses, resistant maltodextrins, and other mixed linkage α-glucans. The polydextroses and resistant maltodextrins had a higher abundance of single- and multiple-branched linkages compared to the mixed linkage α-glucans. Together, these fine structural data revealed underlying differences among glucan structures that may be relevant to fermentation by microbiota, which may vary in their abilities to hydrolyze certain bond types or import certain oligomers (Lindemann, 2020b). This is relevant to our understanding of diet influences on health and the design of food for improved health, as it raises the concern that the assumption of interchangeability of resistant glucans – and, more broadly, added fibers – may not be true. This further suggests increased focus on the manufacturing processes used to obtain these fibers and their relationship to fine structure and opportunity to manipulate and optimize these processes for targeted influences on certain gut species and overall gut health.

The second part of this study consisted of evaluating the degree to which the observed glucan fine structure impacts both the composition and metabolic outputs of gut microbiota

fermenting them. The results from this study demonstrated that community metabolic output (SCFAs) is strongly influenced by initial gut microbial composition, despite the same glucan loading across donors' cultures. Specifically, some fibers were most propiogenic in fermentation by some donors' microbiota and most butyrogenic in others; this revealed that SCFA output is not a property of fiber type, but rather a product of fiber-microbiome interactions. This revelation of structural specificity sheds light on "non-responder" phenotypes in human fiber feeding studies, as a potential mechanism (Abrams et al., 2007). Despite the idiosyncrasy of SCFA production, some inferences can be drawn across glucan categories; for example, acetate which was typically the most abundant SCFA produced from all glucans, was indeed produced in highest amount in fermentation of the mixed linkage α-glucans, this category did not produce a significantly above-average production of butyrate or propionate. In contrast, the resistant maltodextrins and polydextroses elicited a higher production of both butyrate and propionate, but to varying extents across donors. Together, this result illustrates that metabolism of resistant glucans is emergent and depends strongly upon prior microbiome configuration, suggesting that many different organisms within a community have significant access to this carbon source. As such, this may argue that responses of individuals to dietary resistant glucans over short periods may be responsive to glucan class (e.g. relatively simple mixed-linkage α-glucans vs. more complex resistant maltodextrins and polydextroses) but not, in fact, be very strongly responsive to glucan fine structure. As such, if many initial communities are well-poised to consume resistant glucans, the output of this fermentation will reflect whatever initial microbiome structure exists. Further *in vivo* feeding studies are necessary to demonstrate the extent to which glucan fine structure influences microbiome responses over these periods.

However, the seeming idiosyncrasy observed in overall community response to glucans revealed underlying similarity in OTU responses across individuals to specific structures, especially with respect to less-abundant OTUs within communities. For example, members of abundant family *Bacteroidaceae* were increased in abundance with resistant maltodextrins and polydextroses across all three donors' fermentations. However, even within glucan categories, members of specific families were sometimes preferential in their growth on one glucan over another, demonstrating that subtle structural variations influence the way organisms respond and revealing microbial differentiators of both resistant maltodextrins and polydextroses both at the family and OTU level. As such, specific growth responses to individual glucans displayed both

evidence of specificity and context-dependence. For example, one *Parabacteroides* OTU consistently responded strongly to polydextrose E across donors, whereas an *Anaerostipes* OTU responded strongly to polydextrose H and resistant maltodextrins J-M in donor 1 communities, only to polydextrose H in donor 2, and exhibited no better than average growth on any glucan in donor 3 microbiota. Altogether these results indicate that species (via their computational proxies, OTUs) are not equivalent in their access to – and competition for – different resistant glucan structures. This result suggests the possibility that, over long consumption periods, different resistant glucans may preferentially select for different taxa in ways that may have influences on health. I propose that longer-term *in vitro* fermentations be performed to determine whether long-term utilization of these different structures selects for different communities. *In vitro* experiments, however, can suggest this hypothesis but cannot realistically test it; I propose that further longer-term *in vivo* feeding studies should be explored to determine whether the specificities observed among different OTUs for glucan structures hold in a host-associated context and, more importantly, whether these differences result in divergent physiological outcomes.

Overall, my study revealed that, although differences in fine branching structure of the different glucans appear minor, they may have significant impacts on microbial utilization by different species, which may, in turn, influence the diversity, abundances, and activity of microbial populations. This result may change our thinking about fiber-microbiome interactions, especially with respect to synthesized and added fibers, in some key ways. First, as dominant SCFA formation from fibers is typically considered industrially as a property of the fiber in question, my data suggest that there may, in fact, be divergent (but possibly categorizable) responses within human populations based upon initial microbiome posture. Thus, population-scale responses to specific added fibers may be very population-specific across diets, regions, and cultures. However, regardless of their initial abundances, certain microbial taxa were responsive to specific glucan structures across donors, implying that these added fiber structures may, over time, select more strongly for certain gut microbiome taxa and overall compositions. This further suggests that added fiber responses of microbiota may be adaptable, given long periods of dietary acclimation.

Altogether, this research helps us understand and predict to what extent microbial structure and composition are influenced by subtle chemical configurations as well as how gut microbiome communities may respond and adapt differentially to subtly different resistant glucan structures. These studies highlight a future need in increased structural specificity in fiber-microbiome

interaction studies, as well as data science approaches to identify the chemical features of fibers that influence microbiome responses. This study may undergird strategies to design resistant glucans for targeted gut microbiome responsive by optimizing manufacturing processes. Further, they suggest that resistant glucans may be paired with their highly-responsive taxa in synbiotic strategies to improve gut microbiome responsiveness and metabolic products, which may, in time, be used in dietary strategies to ameliorate gut-related and chronic illnesses and to improve overall gut health.

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