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COMPLEX GLYCAN UTILIZATION PREFERENCES OF HUMAN GUT BACTERIA

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Yunus Emre Tuncil

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of

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For the degree of Doctor of Philosophy

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Date

To my lovely wife and my parents

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TABLE OF CONTENTS

	Page
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	viii
INTRODUCTION	1
CHAPTER 1. LITERATURE REVIEW: A PERSPECTIVE ON THE COMPLEXITY OF DIETARY FIBER STRUCTURES AND THEIR POTENTIAL EFFECT ON GUT MICROBIOTA	5
1.1 Introduction	5
1.2 What is Dietary Fiber?.....	6
1.3 Carbohydrate Active Enzymes (CAZymes) and Their Role in Dietary Fiber Digestion	8
1.4 Evidences That the Gut Microbiota Can Be Changed by Fiber Structures	10
1.4.1 Effect of Resistant Starch on Gut Microbial Composition	11
1.4.2 Effect of Pectin on Gut Microbial Composition	13
1.4.3 Effect of Cellulose on Gut Microbial Composition	14
1.4.4 Effect of Hemicelluloses on Gut Microbial Composition	15
1.4.5 Effect of Inulin on Gut Microbial Composition.....	17
1.5 Bacteria’s Glycan Degradation Strategies Need to be Known to Achieve Success for Intelligent Manipulation of Colonic Microbiota Composition.....	18
1.6 A Hierarchical Perspective	19
1.7 Conclusion	20

	Page
CHAPTER 2. RECIPROCAL HIERARCHICAL PREFERENCES OF HUMAN GUT SYMBIONTS TO GLYCANS ALLOW THEM TO MAINTAIN THEIR COEXISTENCE	29
2.1 Abstract.....	29
2.2 Introduction	30
2.3 Materials and Methods	34
2.3.1 Glycans Used	34
2.3.2 Growth of Bacteria Strains and Growth Curves on Pure Glycans.....	34
2.3.3 Glycan Exposure, Growth in Glycan Mixture, and Sample Collection.....	35
2.3.4 Measuring the Remaining Glycans	36
2.3.5 Transcript Analysis of PUL genes by Quantitative PCR (qPCR).....	38
2.3.6 qPCR Enumeration of Competing Species in Co-Cultured Experiments.....	38
2.3.7 Thin-Layer Chromatography (TLC) Analysis	38
2.3.8 Preparation of ARAB-containing Media Predigested by <i>B. thetaiotaomicron</i> and <i>B. ovatus</i> and Growth Experiments	39
2.3.9 Data Analysis	40
2.4 Results and Discussions.....	40
2.4.1 <i>B. ovatus</i> and <i>B. thetaiotaomicron</i> Exhibit Reciprocal Hierarchical Preferences to Glycans as Measured Directly by Carbohydrate Depletion	40
2.4.2 Gene Expression Does Not Always Truly Measure Corresponding Glycan Depletion	43
2.4.3 Different Species Exhibit Variable Sensitivities to Amount of Glycans	44
2.4.4 Hierarchical Orders Remained Generally the Same, When These Organisms Grew Together on Same Glycan Mixture	44
2.4.5 Different Hierarchical Preferences of Bacteria Species Allow Them to Maintain Their Coexistence in the Competitive Environment.....	45
2.4.6 Co-Culturing Enabled <i>B. ovatus</i> to Utilize Previously Inaccessible ARAB due to Cross-Feeding.....	46

	Page
2.5 Conclusion	49
2.6 Supporting Information	60
CHAPTER 3. STRUCTURE OF A GLYCAN AFFECTS ITS PRIORITIZATION BY HUMAN COLONIC BACTERIA.....	72
3.1 Abstract.....	72
3.2 Introduction	73
3.3 Materials and Methods	76
3.3.1 Glycans Used	76
3.3.2 Determination of Structural Features of Starch Samples.....	77
3.3.2.1 ¹ H NMR spectroscopy.....	77
3.3.2.2 Amylose and amylopectin content	78
3.3.2.3 Molecular size distribution.....	78
3.3.3 Growth of Bacteria Strains, and Growth Curves on Pure Glycans	79
3.3.4 Growth in Glycan Mixture and Sample Collection in Time-Course Assay	79
3.3.5 Measuring the Remaining Glycans	80
3.3.6 Transcript Analysis of PUL Genes by quantitative PCR (qPCR).....	81
3.4 Results	81
3.4.1 Growth Rate of <i>B. ovatus</i> on Starch is Significantly Correlated to its Molecular Size	83
3.4.2 Starch Structure Affects its Prioritization by <i>B. ovatus</i> in a Glycan Mixture..	84
3.4.3 <i>B. ovatus</i> Preferentially Utilizes Amylose Over Amylopectin	86
3.5 Discussion.....	87
3.6 Supplementary Information.....	97
CHAPTER 4. DEGRADATION OF SOME DIETARY FIBERS BY HUMAN GUT MICROBIOTA IS DELAYED WHEN PRESENTED IN A MIXTURE.....	100
4.1 Abstract.....	100
4.2 Introduction	101
4.3 Materials and Methods	104
4.3.1 Dietary Fibers Used.....	104

	Page
4.3.2 <i>In-vitro</i> Fermentation	105
4.3.3 Total Gas Production and pH Change Measurements, and Sample Collection for SCFA, Carbohydrate Depletion and DNA Analysis	106
4.3.4 SCFA Analysis.....	106
4.3.5 Measuring the Glycan Disappearance.....	107
4.3.6 DNA Extraction	108
4.3.7 16S rRNA Gene Sequencing.....	108
4.3.8 Bioinformatics.....	109
4.4 Results and Discussions.....	109
4.4.1 Microbiota Composition of Fecal Samples Before Fermentation	110
4.4.2 Delay of Degradation of Some Dietary Fibers When in a Mixture	111
4.4.3 SCFA Production During <i>In Vitro</i> Fermentation.....	114
4.4.4 Composition of Fecal Microbiota Community After <i>in Vitro</i> Fermentation of Dietary Fibers	116
4.4.4.1 Effects of <i>in vitro</i> fecal fermentation of dietary fibers on α -diversity of microbial communities	116
4.4.4.2 Effects of <i>in vitro</i> fecal fermentation of dietary fibers on β -diversity of microbial communities	117
4.4.4.3 Changes in microbiota composition after <i>in vitro</i> fermentation of given dietary fibers.....	118
4.5 Conclusion	121
4.6 Supplementary Information.....	132
OVERALL CONCLUSION.....	139
LIST OF REFERENCES	141
VITA.....	160

LIST OF TABLES

Table	Page
Table 1.1 Chemical structures of dietary fibers, degradation enzymes, CAZymes families	22
Table 2.S1 Primers used in this study.....	68
Table 2.S2 Chopped meat broth formulation.....	70
Table 2.S3 Bacteroides minimal media (MM) formulation.....	61
Table 3.1 Structural features of starches used, and growth parameters of <i>B. ovatus</i> on these starches	91

LIST OF FIGURES

Figure	Page
Figure 1.1 Possible variations in the chemical structures of dietary fibers which affects their utilization by microbiota.....	26
Figure 1.2 Chemical structure of pectin.....	27
Figure 1.3 Variations in the chemical structures of arabinoxylans from different sources and regions of the plants	28
Figure 2.1 Chemical structures of glycans used	51
Figure 2.2 Individual glycan depletion in the mixture and expression of PULs by bacteria species over time during growth.....	52
Figure 2.3 Relative abundances (% total bacteria) of <i>B. ovatus</i> (blue line) and <i>B. thetaiotaomicron</i> (green line) grown together in the mixture of six glycans, and the cell density of the media measured by absorbance (OD ₆₀₀) (red dots).....	54
Figure 2.4 Relative abundances (% total bacteria) of <i>B. ovatus</i> (blue line) and <i>B. thetaiotaomicron</i> (green line) grown together media containing the individual glycans as the only carbon source	55
Figure 2.5 Growth curves of <i>B. ovatus</i> and <i>B. thetaiotaomicron</i> grown individually in media containing different arabinan-based carbon sources[arabinose, arabinobiose, or arabinobiose:arabinan (9:1)].	56

Figure	Page
Figure 2.5 Growth curves of <i>B. ovatus</i> and <i>B. thetaiotaomicron</i> grown individually in media containing different arabinan-based carbon sources[arabinose, arabinobiose, or arabinobiose:arabinan (9:1)].	56
Figure 2.6 a) Growth of <i>B. thetaiotaomicron</i> on media containing ARAB as the only carbon source. b) Thin-layer chromatography (TLC) of ARAB breakdown products in media harvested throughout the growth phases of <i>B. thetaiotaomicron</i> . c) <i>B. ovatus</i> growth on media harvested at different time points throughout <i>B. thetaiotamicron</i> 's growth on ARAB	56
Figure 2.7 Relative abundances (% , total bacteria) of (a) wild type <i>B. ovatus</i> (blue line) and wild type <i>B. thetaiotaomicron</i> (green line) and (b) wild type <i>B. ovatus</i> (blue line) and capsular carbohydrate-free Δ CPS <i>B. thetaiotaomicron</i> (green line) grown together in a media containing arabinan as the only carbon source	59
Figure 2.S1 Monosaccharide composition of glycans used in this study as their trimethylsilyl derivatives	60
Figure 2.S2 Growth curves of (a) <i>B. ovatus</i> and (b) <i>B. thetaiotaomicron</i> on individual glycans used in this study (5 mg/ml solution).	61
Figure 2.S3 Growth curves of bacteria	62
Figure 2.S4 Amount of glucose synthesized by bacteria.....	63
Figure 2.S5 Optical densities (OD ₆₀₀) of the cultures in which <i>B. ovatus</i> and <i>B. thetaiotaomicron</i> were co-cultured on media containing (a) chondroitin sulfate, (b) polygalacturonic acid, (c) pectic galactan, (d) rhamnogalacturonan I, (e) amylopectin, and (f) arabinan.....	64

Figure	Page
Figure 2.S6 Absorbance changes of the media harvested throughout <i>B. thetaiotaomicron</i> 's growth on ARAB over time.	65
Figure 2.S7 Growth of <i>B. thetaiotaomicron</i> on arabinose and growth of <i>B. ovatus</i> on media containing arabinose predigested by <i>B. thetaiotaomicron</i>	66
Figure 3.1 a) Utilization of glycans by <i>B. ovatus</i> , when multiple glycans are present together. Illustration is made based on our previous report, b) Chemical structures of glycans used	92
Figure 3.2 Growth curves of <i>B. ovatus</i> grown on the AP-containing GM (solid line) and MH-containing GM (dashed lines)..	93
Figure 3.3 Glycan utilization and corresponding PUL expression by <i>B. ovatus</i> grown on on AP containing GM (solid lines) and MH containing GM (dashed lines)	94
Figure 3.4 Changes in the degree of branching of starches over time during which they are utilized by <i>B. ovatus</i>	96
Figure 3.S1 Monosaccharide composition of glycans used in this study as their trimethylsilyl derivatives	97
Figure 3.S2 Growth curves of <i>B. ovatus</i> grown on different starches	98
Figure 3.S3 Calculation of the growth parameters (cell density and growth rate) of <i>B. ovatus</i>	99
Figure 4.1 Initial microbiota compositions of fecal samples collected from different donors	123
Figure 4.2 Substrate depletions by fecal microbiota obtained from different donors during a time course <i>in vitro</i> fermentation.....	124

Figure	Page
Figure 4.3 Short chain fatty acid (SCFA) production by fecal microbiota obtained from three different donors during a time course <i>in vitro</i> fermentation	125
Figure 4.4 Changes in α -diversity of the fecal microbiota communities during a time course <i>in vitro</i> fermentation of the dietary fibers, as measured by Shannon's Index Calculator	126
Figure 4.5 Bray-Curtis dissimilarity of fecal microbial communities based on the relative abundances of OTUs at 97% similarity level after <i>in vitro</i> fermentation for 0, 6, and 12 h with dietary fibers	127
Figure 4.6 Phylum level changes in microbiota composition of the fecal samples after the <i>in vitro</i> fermentation for 0, 6, and 12 h with dietary fibers.....	128
Figure 4.7 Genus level changes in microbiota composition of the fecal samples after the <i>in vitro</i> fermentation for 0, 6, and 12 h with dietary fibers.....	130
Figure 4.S1 Chemical structures of glycans	132
Figure 4.S2 Monosaccharide compositions of the dietary fibers used in this study.....	133
Figure 4.S3 Total gas production by the fecal microbiota as a result of <i>in vitro</i> fermentation with dietary fibers.....	134
Figure 4.S4 Changes in pH of the media over time as a result of <i>in vitro</i> fecal microbiota fermentation with dietary fibers.....	135
Figure 4.S5 Monosaccharides found in the blank during the time course of the <i>in vitro</i> fecal fermentation	136
Figure 4.S6 Family level changes in microbiota composition of the fecal samples after the <i>in vitro</i> fermentation for 0, 6, and 12 h with dietary fibers.....	137

ABSTRACT

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Complex glycans, making up dietary fiber, have gained significance in recent years as they are the main energy source for the colonic microbiota which are physiologically important for the host health. Understanding glycan utilization strategies of the members of this community is essential to maximize beneficial health outcomes obtained from them. In order to investigate how the members of our gut microbiota utilize glycans and activate their respective polysaccharide utilization loci (PULs), we performed a series of time-course assays in which two model organisms, *Bacteroides thetaiotamicron* (*Bt*) and *B. ovatus* (*Bo*), were individually grown in a medium containing carbohydrates utilized by both bacteria: amylopectin (AP), arabinan (ARAB), chondroitin sulphate (CS), pectic galactan (PG), polygalacturonic acid (PGA), and rhamnogalacturonan I (RGI), and measured remaining substrates over time as well as PULs expression. *Bt* and *Bo* utilized some glycans before others, but with different prioritizations supporting past work that bacterial species show species-specific hierarchical preference to dietary fibers. Bacteria sensitivities to some glycans are tightly tuned to the residual concentration of polysaccharide in the medium, while others remain highly expressed even after most of the target substrates have been depleted. For

example, expression of the PGA utilization gene by *Bo* gradually reduced as PGA amount decreased in the media; however, expression of the CS utilization gene by *Bo* remained at a high level even after the substrate was reduced by about 98%. Next, the possibility of change in substrate hierarchy was explored when bacteria are in a competitive environment. Co-culturing of these organisms in the same mixture resulted in reduction in expression of some *Bt* PULs, however the hierarchical orders remained generally the same. Relative abundances of these species in the co-cultured environment remained constant throughout the exponential phase. This is, most probably, due to their different glycan priorities which allow them to maintain their coexistence. Next, a hypothesis was tested whether molecular structure of a glycan affects its place in the hierarchy. To test this, we repeated the hierarchical substrate preference test for *Bo* by substituting AP with a starch analog [maltohexaose (MH) (low molecular weight hexasaccharide derived from starch)]. AP was used after RGI by *Bo* when AP was included in the mixture, whereas MH was used before RGI, so the utilization of RGI by *Bo* was delayed in the presence of MH. These results provide information about the strategies of *Bt* and *Bo* use to utilize different glycans that are often present as a mixture in the colon, and the effects of a particular carbohydrate chemical structure on preference of use.

Finally, we questioned whether such a hierarchical preference of glycans also exists in the highly competitive environment of the colon. To answer this, we designed a series of *in vitro* fermentation studies using fecal microbiota obtained from three healthy individuals, and compared the degradation profiles of single glycans with a mixture containing an equal amount of these substrates. These results showed that, from the

polysaccharide perspective, hierarchical preferences exist in the competitive environment of the colon, and degradation of some glycans by fecal microbiota is delayed when they are present as a mixture. Therefore, this might be a logical strategy for delivering particular dietary fibers into the distal part of the colon so that the fermentative activity can be stimulated throughout the colon, and beneficial bacteria as well as more SCFA production in the distal colon can be promoted.

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INTRODUCTION

Awareness of the significance of dietary fibers on host health as it relates to the gut microbiome has increased because of numerous investigations and reports released over the last two decades. Diets rich in dietary fibers have been shown to increase the diversity of the gut microbial community (Martinez et al., 2013; Tap et al., 2015), which is associated with immunological improvements such as blood markers of inflammation, lipid metabolism and insulin responses (Martinez et al., 2013). On the other hand, inadequate consumption of dietary fibers causes the loss of some member of this community, thereby decreasing the diversity (Sonnenburg et al., 2016), which is associated with an increase in disease related biomarkers for cancer and inflammatory bowel diseases (Mosca et al., 2016).

There are also convincing findings that selective consumption of dietary fibers determines which microbial groups are favored in the gut and influence the balance of species in the colon. This is because dietary fibers differ in their chemical structures and types of linkages, and utilization ability of gut microorganisms depends on their gene content that encode carbohydrases for specific types of fiber. For instance, consumption of a long-chain hydrolyzed arabinoxylan, consisting of a linear chain backbone of xylose residues linked through $\beta 1 \rightarrow 4$ glycosidic residues and substituted with different degrees

of arabinose residues, increased the abundance of butyrogenic bacteria, namely *Roseburia intestinalis*, *Eubacterium rectale*, *Anaerostipes caccae* in the colon of humanized mice (Van den Abbeele et al., 2011a). The same trend was also observed in the colon of humans fed with an inulin supplemented diet (consisting of $\beta 2 \rightarrow 1$ linked fructose residues) (Barcenilla et al., 2000; Duncan et al., 2002; Schwartz et al., 2002). The *Bacteroides-Provotella* bacteria group was promoted by β -glucans, a repeating linear polymer of two $\beta 1 \rightarrow 4$ glucose units alternated with $\beta 1 \rightarrow 3$ glucose molecules (Hughes et al., 2008). Accordingly, there has been an increasing interest to use dietary fibers for modulation of the gut microbiota composition as a way to maximize their beneficial health effects. However, in order to achieve success in this, interaction between dietary fiber structures and the members of the gut microbiota, as well as their dietary fiber utilization strategies, need to be investigated in addition to knowing their carbohydrate requirements (Hamaker and Tuncil, 2014).

From this point of view, several studies have been conducted to investigate how an individual member of our gut microbiome (*B. thetaiotaomicron*) utilize dietary fibers when there are multiple ones available (Lynch and Sonnenburg, 2012; Martens et al., 2008; Rogers et al., 2013; Sonnenburg et al., 2006). The results from these studies revealed that bacteria species show hierarchical preferences to dietary fibers meaning that they preferentially utilize certain dietary fibers before others. A recent report demonstrated that this metabolic hierarchy is species dependent with different species showing variable prioritizations (Pudlo et al., 2015). These studies rely on transcriptional profiling of bacteria to given dietary fibers, which is not a direct measurement of fiber utilization as the abundance and activity of enzymes may be further controlled by post-

transcriptional events. There is a need to understand such preferences, particularly in terms of how closely the observed transcriptional changes mirror actual glycan utilization and their role in competitive environments. In addition to this, there is a need to investigate whether there are external factors (i.e. variations in the chemical structures of dietary fibers) that affect metabolic hierarchies of bacteria species or whether bacteria species are programmed to use dietary fibers in a set order. Filling these gaps in the field is essential for understanding strategies bacteria species use to utilize different dietary fibers. Such information would allow scientists to design dietary strategies to modulate the gut microbiota for better health in a predicted way.

Hypothesis and Specific Objectives

There are three different hypotheses developed in this thesis work. The first is that the bacteria species can change their dietary fiber preferences in competitive environments in order to provide growth advantages to themselves. In order to test this hypothesis, we first determined the hierarchical preferences of two closely related human gut symbionts, *B. thetaiotaomicron* and *B. ovatus*, in singly cultured media containing six different dietary fibers and measured the depletion of each over a time course. We then determined whether they changed their hierarchical preferences in a competitive environment by co-culturing them on a medium containing the same dietary fibers (Chapter 2).

The second hypothesis was that variation in the chemical structure of a particular glycan affects its prioritization by a bacteria species, or in other words that dietary fiber structure has an impact on its rank order in the hierarchy. To test this hypothesis, we

repeated the above mentioned hierarchical tests by substituting one of the fiber constituents in the mixture with its structurally simple counterpart (Chapter 3).

The final hypothesis was that, from the dietary fiber perspective, hierarchical preferences also exist in the broader microbiota of the colon, and that degradation of some dietary fibers by human gut microbiota is delayed when they are presented in a mixture. This might be a logical strategy to delivery dietary fibers into distal part of the colon. To test this hypothesis, we designed a series of *in vitro* fermentation studies using fecal microbiota obtained from three healthy individuals to compare the degradation profiles of single dietary fibers and a mixture containing equal amounts of these substrates (Chapter 4).

CHAPTER 1. LITERATURE REVIEW: A PERSPECTIVE ON THE COMPLEXITY OF DIETARY FIBER STRUCTURES AND THEIR POTENTIAL EFFECT ON GUT MICROBIOTA

1.1 Introduction

In recent years, dietary fibers have been shown to have the ability to change at least some portion of the gut microbiota composition (Cantarel et al., 2012; Davis et al., 2011; Gibson et al., 1995; Martinez et al., 2010; Mitsou et al., 2010; Ramirez-Farias et al., 2009; Terada et al., 1995a; Terada et al., 1995b). Yet, little is known about how this might be achieved in a predicted way. Part of the problem is that there are many factors that determine microbial populations such as the immune system (Hooper et al., 2012), gut environmental factors including pH (Duncan et al., 2009), host genetics (Turnbaugh et al., 2009), sex (Mueller et al., 2006; Yatsunenko et al., 2012), age, (Biagi et al., 2012; Mueller et al., 2006; Yatsunenko et al., 2012), and diet (De Filippo et al., 2010). As well, there is the more recent realization that functional outcomes of the microbiota may be a more important to the body and that there is a built-in microbial redundancy to meet such outcomes (Van den Abbeele et al., 2011b). Still dietary fiber represents by far the main source of energy for the bacteria of the colon, and accordingly there exists the potential for its use to maintain or improve the microbiota, particularly when dysbiosis exists, through an understanding of carbohydrate requirements,

This chapter is part of our previous publication: **Hamaker BR and Tuncil YE**. 2014. A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. *Journal of Molecular Biology* **426**:3838-3850.

interactions, and employment of strategies that would shift population compositions (Neyrinck et al., 2012; Walker et al., 2011; Zhao, 2013).

While perhaps an oversimplification of the utilization of carbohydrates by the colonic bacteria (e.g., not including the favoring of bacterial growth due to other factors such as cross-feeding), we hope this perspective paper provides a context for beginning to think better about how specific fiber structures or fiber mixtures might be used either to maintain a healthy gut microbiota, or to shift dysbiotic populations back to better balance for an improved health outcome. It is suggested that a concerted effort be made to understand a set of rules or guidelines that likely exists for microbiota manipulation, and to build a better framework for the intelligent use of fiber structures to effect positive change in microbiota composition.

1.2 What is Dietary Fiber?

Hipsley (1953) first coined dietary fiber as the non-digestible constituents making up the plant cell wall (Hipsley, 1953); only representing part of what we consider to be dietary fiber today. In 2001, the American Association of Cereal Chemists published a definition that had broad acceptance: “the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine” (2001). Thus, the term included non-starch polysaccharides, such as cellulose, pectin and gums, as well as resistant oligosaccharides such as fructooligosaccharides and galactooligosaccharides, and other carbohydrates such as resistant starch and dextrans. Moreover, the non-carbohydrate-based polymer, lignin, which is bound to cellulose in the plant cell wall;

and some animal origin carbohydrates, such as chitin, hyaluronan, and chondroitin sulfate, are also considered in this definition as dietary fibers. The 2009 Codex Alimentarius dietary fiber definition was introduced to harmonize the definition among countries (Codex, 2008), and a new analytical method was proposed to analyze all dietary fibers in one assay procedure (AOAC, 2009). The Codex definition additionally stipulates that added fiber must be shown to have a physiological benefit to health, and places the decision on national authorities whether to include indigestible carbohydrates from 3 to 9 monomeric units (oligosaccharides) as dietary fiber (Codex, 2008).

Carbohydrates, and thus dietary fibers, bear different structural features based on monosaccharide composition, anomeric configurations, linkage types, linear chain lengths, branch chain compositions, and reducing terminal attachments. Although dietary fiber polysaccharides and oligosaccharides are generally composed of a relatively small number of monosaccharides (principally glucose, galactose, mannose, fructose, arabinose, xylose, rhamnose, fucose; and some of their uronic acid forms), a range of possible linkages combinations exists between two sugar units, as well as their next level larger structural units that comprise oligomeric and polymeric structures. Because of the resulting large number of possible structures, dietary fiber is one of the most heterogeneous and diverse group of associated molecules found in nature. And, of course, variation in their chemical structures affects their utilization by gut microbiota (Figure 1.1), because the bacteria have different abilities to cleave the linkages in the structure of these complex molecules to obtain simple sugars. Chemical structures of selected dietary fibers are given in Table 1.1.

Dietary fiber utilization ability of gut microbes depends on their gene content that encodes carbohydrate active enzymes (CAZymes) for cleavage of linkage types, and associated proteins like carbohydrate-binding proteins and transporters. Bacteria in the colon utilize carbohydrate-based substrates in a highly competitive environment and those that have the necessary genes to degrade a particular substrate have ways to compete for it related to differences in enzyme activity, binding efficiency of carbohydrate-binding moieties, ability to sequester digested product, and perhaps factors like ability to colonize around fiber particles and hierarchical preference differences.

1.3 Carbohydrate Active Enzymes (CAZymes) and Their Role in Dietary Fiber Digestion

The breakdown of complex dietary fibers is controlled by a series of CAZymes. CAZymes which cleave the glycosidic bonds between sugar monomers or between carbohydrate and non-carbohydrate structures such as lignin, are classified under four different classes based on their amino acid sequence similarity (Lombard et al., 2014). Glycoside hydrolases (GHs) are a class of enzymes that cleave the glycoside linkages between two or more sugar units or between sugar and non-sugar moieties. Polysaccharide lyases (PLs) catalyze the breakage of acidic sugar units (i.e. glucuronic acid and galacturonic acid) contained in polysaccharide chains via the β -elimination mechanism. Action of these enzymes results in a formation of an unsaturated hexenuronic acid residue and a new reducing end. Carbohydrate esterases (CEs) hydrolyze the ester linkages that may be found in pectin (pectin methyl esters) and

acetylated arabinoxylans. Finally, enzymes showing auxiliary activities (AAs), a new class of CAZymes, join in lignin degradation (www.cazy.org) (Lombard et al., 2014).

Currently, 127 families of GHs, 22 families of PLs, 16 families of CEs, and 11 families of AAs have been identified and this list is continuously updated (www.cazy.org) (Lombard et al., 2014). One drawback for an amino acid sequence-based classification of enzymes is that families do not reflect substrate specificity of their members; thus, enzymes belonging to more than one family may show activities on a specific substrate. For example, cellulose degrading enzymes fall into the families GH1, GH3, GH5, GH7, GH8, GH9, GH44, GH48, GH51, GH74. Also, members of a given family can show activity on different substrates; for instance, the members of the GH5 family have roles in degradation of several dietary fibers, such as cellulose and arabinoxylan (Table 1.1).

CAZymes encoded in the human genome are very limited with only 17 enzymes involved in digestion of carbohydrates (8 with known functions), which are responsible for degradation of non-resistant starch, lactose, maltose and maltooligosaccharides, sucrose, and trehalose (Cantarel et al., 2012). Genomes of the resident microbes in the large intestine, on the other hand, encode a large repertoire of CAZymes which make them able to utilize dietary fibers through the fermentation process. Some bacteria can utilize relatively few carbohydrates and are termed “specialists”, and others can utilize a large number of different carbohydrate structures and are termed “generalists” (Koropatkin et al., 2012). As an example of a generalist, *Bacteroides thetaiotaomicron* encodes 269 GHs, 17 PLs, and 18 CEs (see www.cazy.org/b135.html), thus dedicating 18% of its genome for dietary fiber utilization (Martens et al., 2008). On the other hand,

Campylobacter hominis, another resident bacteria in the human colon, encodes only 6 GHs, 3 CEs, and no PLs (see www.cazy.org/b612.html); suggesting that the dietary fiber utilization ability of this bacteria is more limited, but perhaps to more commonly available substrates. The difference in the number and type of genes expressing CAZymes that microorganisms possess suggests that selective consumption of dietary fibers determines which bacterial groups are favored in the gut and influence the balance of species and strains in the colon. This forms the basis of an idea that the gut microbiota can be manipulated via dietary fibers as a way to improve health. From this perspective, the following sections will focus on how specific dietary fibers types have been shown to change in some respect gut microbial composition, and how fibers might be viewed as a collection of discrete structures that drive such changes.

1.4 Evidences That the Gut Microbiota Can Be Changed by Fiber Structures

De Filippo and co-workers (De Filippo et al., 2010) studied fecal microbiota composition of children (ages 1 – 6) from Europe, whose diet was rich in fat and low in fiber; and from rural Africa, whose diet was rich in fiber and low in fat and animal proteins. European and African children were fed with breast milk up to 1 and 2 years old, respectively. Fecal microbiota compositions during the breast milk feeding period were not significantly different from each other. However, once solid food was introduced, in the fecal samples of the African children there was a significant enrichment in Bacteroidetes and depletion in Firmicutes, and an abundance of bacteria from the genera *Provetella* and *Xylanibacter*, when compared to that of the European children. This was attributed to the fact that the Bacteroidetes members have genes

encoding cellulases and xylanases to ferment cellulose and xylans (i.e., arabinoxylans) that are found in higher amounts in the African children's diets. Conversely, *Shigella* and *Escherichia* members were more abundant in the fecal samples of the European children. The question of how fast the effects of diet on gut microbiota composition can be realized has recently been reported in both mice (Turnbaugh et al., 2009a) and humans (David et al., 2014), revealing that the responses of gut microbiota to change in diet are quick with variation in the gut microbial composition recognized in even less than 1 day.

There are numerous studies that have been conducted to examine both *in vitro* and *in vivo* effects of specific dietary fiber types on gut microbial composition, and these are discussed as follows.

1.4.1 Effect of Resistant Starch on Gut Microbial Composition

Starch is composed of two different glucose polymers, amylose and amylopectin, and, for many decades, it has been recognized that a portion can pass undigested into the large intestine creating a dietary fiber type called "resistant starch" (RS). RS is divided into four subgroups: 1) RS1, physically inaccessible starch found in whole- or partially milled grains and legumes; 2) RS2, granular starch which is tightly packed and is relatively dehydrated; 3) RS3, cooked and reassociated, or retrograded, starch (principally amylose) where accessibility to host enzymes is limited; and 4) RS4, chemically or enzymatically modified starches (Sajilata et al., 2006).

Due to the difference in their structures and accessibility, different RS types or structures within type favor different resident bacteria in the gut. For example, in a human study using RS2 and RS4, only RS4 consumption had an influence at the phyla

level with an increase in abundance of Actinobacteria and Bacteroidetes, and a decrease of Firmicutes (Martinez et al., 2010). At the species level, an increase was observed in abundance of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* with consumption of RS4, whereas RS2 consumption increased the abundance of *Ruminococcus bromii* and *Eubacterium rectale* (Martinez et al., 2010). RS3 consumption in pigs increased the relative abundance of *Faecalibacterium prausnitzii*, and reduced the number of *E. coli* and *Pseudomonas* spp (Haenen et al., 2013). The promotion of some of these species may occur due to the cross-feeding of the breakdown products. An *in vitro* co-cultured study, in which the RS (RS2 and RS3) utilization ability of four amyolytic bacteria found in human colon (*E. rectale*, *B. thetaiotaomicron*, *B. adolescentis*, and *R. bromii*) was investigated, revealed that *R. bromii* plays a crucial role to initiate the degradation of RS and releases breakdown products which are then used by other colon bacteria (Ze et al., 2012).

Even within RS types, there are subtle structural differences that have been shown to favor different bacteria. Linear chains of starch found in amylose and the external chains of amylopectin form double helices which result in a formation of different crystalline entities that can be found in both RS2 and RS3. In the A-type polymorphic form, double helices generate a more compact structure with relatively low water content, while double helices in the B-type crystallinity form a more open structure with comparably high water content. Cereal starches usually exhibit A-type x-ray diffraction patterns, while tuber starches and starches with high amylose content form the B-type (Tester et al., 2004). An *in vitro* human gut model study revealed that RS3 with B-type crystalline structure enriched *Bifidobacterium* spp., while RS3 with the A-type

polymorphic form increased numbers of *Atopobium* spp. (Lesmes et al., 2008). B-type crystallites favored higher butyrate production, which suggested that starches from tubers are more butyrogenic than those from cereals.

1.4.2 Effect of Pectin on Gut Microbial Composition

Pectin is a structurally complex heteropolysaccharide composed of a series of linked polymers [arabinan, pectic galactan, arabinogalactan, homogalacturonan, and rhamnogalacturonans (RGs) (RG type I and RG type II)], which are also known as pectic substances (Figure 1.2) (Harholt et al., 2010; Mohnen, 2008; Thakur et al., 1997). Pectins are found in substantial amounts in the primary cell wall and middle lamella of dictyledonous plants, where they may be intertwined with other cell wall components including cellulose, hemicelluloses, and lignin. Today, commercially available pectins are mostly produced from apple pomace and citrus peels. The source, as well as the extraction procedure used, results in differences in chemical structure, including degree of methylation, which subsequently affects its usage by bacteria. For instance, even though *F. prausnitzii* strains were able to grow on pectin from apple, they could not utilize pectin from citrus (Lopez-Siles et al., 2012).

Because the structure of pectin varies from source to source, a simple comparison of the effect of dietary enrichment of pectin on microbiota composition yields different results. For example, a 3 wk study showed higher abundance of *Bacteroides* spp. in the colon of rats fed a diet containing 6.5% citrus pectin (Dongowski et al., 2002); while a 4 wk study with rats fed a diet containing 7% apple pectin showed cecal microbiota enriched with species belonging to the genera of *Anaeroplasma*, *Anaerostipes*, and

Roseburia, and a decrease in the number of *Alistipes* and *Bacteroides* spp. (Licht et al., 2010). The discrepancy between these two studies highlights the need to characterize the molecular composition and even linkage structures of dietary fiber substrates for studies on their effect on the microbiota.

Besides chemical structure, pH of the colon appears to be a factor that determines the pectin utilization ability of bacteria. A recent *in vitro* study conducted to examine the competition of *F. prausnitzii* strains with major pectin utilizers (*B. thetaiotaomicron*, and *E. eligens*) found revealed that while apple pectin utilization capability of *B. thetaiotaomicron* is restricted at lower pH (pH = 6.12), *F. prausnitzii* grew well at this pH (Lopez-Siles et al., 2012).

1.4.3 Effect of Cellulose on Gut Microbial Composition

Cellulose, a β -1,4-linked glucose polymer, is the most abundant naturally occurring organic compound (OSullivan, 1997), with a degree of polymerization reaching up to 15,000 glucose units per molecule (Zhang and Lynd, 2004). It is usually not fully fermented in human gut (Slavin et al., 1981), which is attributed to its unique structure in which the molecules are linked through inter- and intra-molecular hydrogen bonds and form crystalline linear aggregates (microfibrils). Moreover, cellulose molecules are also associated with other substances such as hemicellulose pectin, and proteins, which limits their fermentability in the colon (Leschine, 1995).

Cellulose-degrading species isolated from human feces have been reported as *Clostridium* sp., *Eubacterium* sp., *Ruminococcus* sp., (Montgomery, 1988; Robert and Bernalier-Donadille, 2003; Wedekind et al., 1988), and *Bacteroides* sp. (Betian et al.,

1977). A human study showed that even though cellulose degrading microorganisms are present in the gut microbiota of all individuals, the structure of the cellulose degrading community differs largely depending on the methane status of the subjects (Chassard et al., 2010). The dominant cellulose degraders isolated from non-methane excreting subjects belonged mainly to Bacteroidetes, while they were predominantly composed of Firmicutes in methane excreting individuals (Chassard et al., 2010).

The structure of cellulose also affects its utilization by colonic microorganisms. An *in vitro* study on hydrated and crystalline cellulose degradation ability of fecal samples from five individuals showed that hydrated cellulose was degraded by all of the fecal inocula; but only one of inocula degraded crystalline cellulose (Wedekind et al., 1988). This was confirmed by Chassard and co-workers (Chassard et al., 2010), who showed that cellulose degradation by cellulolytic bacteria is dependent on type of cellulose with *Bacteroides* isolates able to utilize native cellulose, but not microcrystalline cellulose.

1.4.4 Effect of Hemicelluloses on Gut Microbial Composition

Like cellulose, hemicelluloses are found in the cell walls of higher plants where they interact with cellulose and lignin, thereby contributing to the strength of the cell wall (Scheller and Ulvskov, 2010). This major group of dietary fibers includes arabinoxylans, xyloglucans, glucomannans, galactomannans, and β -glucans. The common feature of these compounds is that most have a continuous β -1,4 linked backbone, with the exception of β -glucan which has both β -1,4 and β -1,3 linkages in its backbone. Most hemicelluloses have branched structures off the backbone that may be simple (one

monosaccharide and few linkage types) or very complex (many monosaccharides, many linkage types, and varying length of branches) (Figure 1.3). An exception are the cereal β -glucans which are not branched.

Recently, there has been an increasing interest in using arabinoxylooligosaccharides, which are hydrolyzates of arabinoxylan, and β -glucans as prebiotics due to their potential to stimulate the growth of bifidobacteria in the human colon. Prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health” (Gibson et al., 2004). Accordingly, there have been several studies conducted to examine their bifidogenic effects both *in vitro* and *in vivo*. An *in vitro* study, in which the fecal samples of 22 year old adult individuals were inoculated into a simulator of the human intestinal microbial ecosystem, revealed that arabinoxylooligosaccharide degradation in the colon was compartment specific, mainly occurring in the transverse colon (Grootaert et al., 2009). Another *in vitro* human intestinal microbial ecosystem reactor study demonstrated that compartment specificity of arabinoxylooligosaccharide degradation is dependent on their average degree of polymerization. Those with an average degree of polymerization lower than 15 were almost completely utilized in the ascending and transverse colon compartments; while of those with an average degree of polymerization higher than 15, 70% were utilized in the ascending and transverse colon compartments and 30 % in the descending colon compartment (Sanchez et al., 2009). It was further shown that arabinoxylooligosaccharides with an average degree of polymerization of 29 increased the abundance of bifidobacteria in the ascending colon, lactobacilli in both the ascending

and transverse colon, and *C. coccoides* – *E. rectale* groups in the descending colon (Sanchez et al., 2009). Arabinoxylan hydrolyzates with a degree of polymerization of 60 or higher, in humanized mice, decreased the cecal abundance of *Clostridium* clusters I/XI/XV and *Verrucomicrobia*, and increased cecal Actinobacteria (Van den Abbeele et al., 2011a). These studies demonstrate that degree of polymerization is an important factor in determining which bacteria is favored in a particular compartment of colon. Native hemicelluloses are large polymeric molecules when they enter the large intestine, however it is likely that a cross-feeding effect exists where one bacteria liberates smaller parts of polymers that then favor other bacteria.

The molecular size of β -glucan has also been shown as an important factor in favoring bacterial groups. For example, β -glucan hydrolyzates with molecular weights of 137, 150, and 172 kDa significantly increased the abundance of the *Bacteriodes-Provetella* group at 24 h of an *in vitro* study; but no significant increases were observed when given larger size hydrolyzates (230 and 243 kDa) (Hughes et al., 2008). Similarly, when the growth of seven lactobacilli strains on β -glucan hydrolysates were analyzed, five of the lactobacilli were unable to grow on hydrolyzates with a degree of polymerization greater than 3 (Snart et al., 2006).

1.4.5 Effect of Inulin on Gut Microbial Composition

The effect of inulin, the longer chain version of fructooligosaccharides, on gut microbial composition has been extensively studied using *in vitro* (Grootaert et al., 2009; Rossi et al., 2005; van de Wiele et al., 2007), animal (Juskiewicz et al., 2007; Sakaguchi et al., 1998; Van den Abbeele et al., 2011a), and human models (Gibson et al., 1995;

Harmsen et al., 2002b; Kruse et al., 1999). *In vitro* simulator models revealed that inulin modulates colonic microbial composition (Grootaert et al., 2009), and degradation of inulin mainly takes place in the ascending colon (Grootaert et al., 2009; van de Wiele et al., 2007). Chain length of inulin affects its utilization pattern. Short chain inulin (degree of polymerization of less than 10) was more rapidly degraded than long chain inulin (degree of polymerization of more than 10) (Roberfroid et al., 1998).

Inulin consumption resulted in an increase in *E. rectale*, *Roseburia intestinalis*, and *Anaerostipes caccae*, which are known butyrate producing bacteria (Barcenilla et al., 2000; Duncan et al., 2002; Schwartz et al., 2002), and a decrease in the abundance of *Akkermansia muciniphila* in the cecum of humanized rats (Van den Abbeele et al., 2011a). An *in vitro* study in which the inulin utilization ability of five butyrate-producing bacteria isolated from human fecal samples were tested, revealed that *E. rectale* was able to grow on inulin, but *R. intestinalis* was not (Duncan et al., 2002). It was suggested that *E. rectale* is the primary degrader of inulin in the colon, whereas *R. intestinalis* and *A. caccae* ferments smaller fragments of inulin (Van den Abbeele et al., 2011a), inferring that cross-feeding occurs between the members of colonic microbial community to degrade inulin.

1.5 Bacteria's Glycan Degradation Strategies Need to be Known to Achieve Success for Intelligent Manipulation of Colonic Microbiota Composition

In the highly competitive environment of the large intestine, access and sequestration of sugars from fiber molecules demands a range of strategies for fiber utilization from the perspective of the resident bacteria. This brings in the consideration

factors in addition to chemical structures that allow either individual bacteria or bacterial groups to utilize fibers and outcompete others. Complex chemical fiber structures may have resulted in a different basis for strain diversification based on selective pressure that rests instead on the complexity of the chemical structure itself. For example, the complex arabinoxylans require many enzymes to digest it [in a wheat arabinoxylan, 16 enzymes are encoded in one PUL for degradation by *B. ovatus* (Bacova_03411-50) (Martens et al., 2011), and the bacteria that digest these complex structures may have taken on a different strategy involving large complements of enzymes and associated proteins. These wide ranging differences in fiber structures can help us to think about how the gut microbiota can be manipulated, and implies that there are likely a set of rules or guidelines that one could use to change the colon microbiota composition. In this regard, a number of questions come up, including the inter-individual variation that exists in gut microbiota composition (Eckburg et al., 2005a; Qin et al., 2010; Turnbaugh et al., 2009b; Yatsunenکو et al., 2012), and must be considered to understand what needs to be done to create such rules to begin to develop the means to manipulate the microbiota in a predicted way.

1.6 A Hierarchical Perspective

Another way to view dietary fiber substrates and microbiota manipulation is to think about carbohydrate substrates in a hierarchical sense. Martens' group (Rogers et al., 2013) studied the response of *B. thetaiotaomicron*, a human gut generalist that has ability to utilize dozens of different dietary fibers, to a mixture of twelve carbohydrates for the purpose of understanding preference of utilization. They found that *B.*

thetaitaomicron uses certain carbohydrates before it does others, and proposed a “metabolic hierarchy” based on transcriptional events. For instance, the bacteria utilizes a high priority substrate like pectic galactan over a low priority substrate like α -mannan (Rogers et al., 2013).

It seems probable that a hierarchy also exists when one thinks about substrate preference in the context of the competitive pressure on bacteria in the colon. And it is to this point that one can begin to think in a broader way about manipulation of the microbiota with carbohydrate substrates. Discrete structures, in the absence of more generally utilized structures, would favor bacteria at the species or even strain level, and perhaps in this way could be used to move the microbiota in prescribed directions.

Ultimately, a dietary strategy would likely be needed to obtain desirable microbial shifts. A simple idea is that by reducing or removing broadly used and abundant carbohydrate substrates that are high in hierarchical preference, then mixtures of discrete structures would have a better chance in promoting desired microbial shifts.

1.7 Conclusion

There seems somewhat of a disconnect currently between the carbohydrate (and food) science discipline as it researches dietary fiber polysaccharides and oligosaccharides in terms of the colon microbiota and health, and the large efforts going on in clinical, microbiological, and biochemical research on the colon microbiome and its relationship to colon and whole body health. Many studies that come out of the dietary fiber area, in this respect, are designed to investigate a specific, or even novel, fiber type and to examine what it does in the colon including prebiotic effect, microbial shifts,

functional outcomes such as short-chain fatty acid amounts and profiles, and other systemic and metabolic effects. Yet, it is not easy, and perhaps is not possible, to piece such studies together to build a mechanistic framework for intelligent manipulation of the colon microbiota with dietary fibers. For one thing, these studies do not provide much information about bacterial strain level interactions with specific types of dietary fiber structures. Work towards such a mechanistic understanding of dietary fiber structures and how colon bacteria utilize them in the competitive environment of the colon requires multi-disciplinary teams that include carbohydrate scientists. Such an approach will be needed to bridge advances in the field of the microbiome and health to translational applied science.

Table 1.1 Chemical structures of dietary fibers, degradation enzymes, CAZymes families ^a

Dietary Fibers	Chemical Structures	Degradation Enzymes ^b	CAZymes Families ^c	References
Resistant Starch	Linear α -(1,4) Glu units, branched with α -(1,6) Glu	α -amylase, glucoamylase, isoamylase, pullulanase,	GH4, GH13, GH14, GH15, GH31, GH57, GH63, GH97	(Sajilata et al., 2006; Zobel and Stephan, 2006)
Pectins ^d				
<i>Homogalacturonan</i>	Linear α -(1,4) GalA units partially methylated and acetylated	Polygalacturonase, pectin methyl esterase, pectin lyase, pectin acetyl esterase	GH28, CE8, CE12, CE13, PL1, PL9, PL10	(Mohnen, 2008; Pedrolli et al., 2009)
<i>Rhamnogalacturonan (RG) I</i>	Linear α -(1,4) GalA and α -(1,2) Rha units	RG lyase, RG hydrolase, RG acetyl esterase	GH2, GH28, GH42, GH105, CE4, CE6, CE12, PL4, PL9, PL11	(Azadi et al., 1995; Mohnen, 2008; Molgaard et al., 2000; Wong, 2008)
<i>Pectic Galactan</i>	Composed of β -(1,4) Gal, β -(1,6) Gal, α -(1,4) Ara units	β -(1,4) galactanase, β -(1,6) galactanase, α -arabinosidase	GH2, GH3, GH5, GH10, GH30, GH43, GH53	(Mohnen, 2008; Suberkropp, 2005)

Table 1.1 Continued

<i>Arabinogalactan</i>	Composed of β -(1,3) Gal, β -(1,6) Gal, α -(1,5) Ara, and (1,3) Ara units	β -(1,3) galactanase, β -(1,6) galactanase, α -(1,5) arabinanase, α -L-arabinofuranosidase	GH2, GH3, GH10, GH30, GH35, GH43, GH51	(Mohnen, 2008; Suberkropp, 2005)
<i>Arabinan</i>	Composed of α -(1,5), (1,3) and α -(1,2) Ara units	α -(1,5) arabinanase, α -L-arabinofuranosidase	GH2, GH3, GH10, GH43, GH51, GH54, GH62	(Mohnen, 2008; Pedrolli et al., 2009)
<i>RG II^e</i>	Linear α -(1,4) GalA units branched with Api, Ara, AceA, Dha, Fuc, Gal, GluA, Kdo, Rha, Xyl	Not known ^e		(Mohnen, 2008; Wong, 2008)
Cellulose	Linear β -(1,4) Glu units	Endoglucanase, cellulobiohydrolases, β -glucosidases	GH1, GH3, GH5, GH7, GH8, GH9, GH44, GH48, GH51, GH74	(Gilbert, 2010)
Hemicelluloses <i>Arabinoxylan^d</i>	β -(1,4) Xyl units as the backbone with side chains of Ara units linked via α -(1,2), α -(1,3), and α -(1,5). Gal, GluA, and FerA units may also be present in the branched points	Xylanase, α -glucuronidase, α -L-arabinofuranosidase, β -xylosidase, feruloyl esterase, acetyl xylan esterase	GH3, GH5, GH7, GH8, GH10, GH11, GH39, GH43, GH51, GH52, GH54, GH62, GH67, GH115, CE1, CE2, CE4, CE6, CE7	(Dodd et al., 2011; Zerillo et al., 2013)

Table 1.1 Continued

<i>Xyloglucan</i>	β -(1,4) Glu units as the backbone with single unit side chains of α -(1,6) Xyl. Gal, and Fuc may also present in the branched point	Xylogluconase, xylosidase, β -D-gluconase, β -D-galactosidase	GH1, GH2, GH5, GH7, GH12, GH16, GH42, GH44, GH45, GH48, GH51, GH74	(Gidley and Reid, 2006; Gilbert, 2010)
<i>Glucomannan</i>	Linear or slightly branched backbone chain of β -(1,4) Man and β -(1,4) Glu	β -mannanase, β -mannosidase, β -glucosidase α -galactosidase	GH1, GH2, GH4, GH5, GH26, GH27, GH36, GH57, GH97, GH110, GH113.	(Gidley and Reid, 2006)
<i>Galactomannan</i>	Linear or slightly branched backbone chain of β -(1,4) Man and β -(1,4) Glu with side chains of α -(1,6) Gal	β -mannanase, β -mannosidase, β -glucosidase α -galactosidase	GH1, GH2, GH4, GH5, GH26, GH27, GH36, GH57, GH97, GH110, GH113.	(Gidley and Reid, 2006)
<i>β- Glucan</i>	Repeating linear polymer of two β -(1,4) Glu alternated with β -(1,3) Glu units	Licheninase, β -glucan endohydrolase, endo (1,4) β -glucanase	GH5, GH6, GH8, GH9, GH10, GH12, GH16, GH26, GH44, GH45, GH48, GH51	(Wolf et al., 1995)
Polyfructans <i>Inulin</i>	β -(2,1) Fru	Inulinase, β -(2,1) fructanlyase	GH32, GH91	(Franck, 2006)

Table 1.1 Continued

<i>Levan</i>	β -(2,6) Fru	Levanase, β -fructofuronosidase	GH32, GH68	(Uchiyama, 1993)
Gums				
<i>Carrageenan</i>	Sulfated galactans, units of β -(1,3) Gal and α -(1,4) linked 3,6 anhydro Gal	κ -carrageenase	GH16	(Collen et al., 2009)
<i>Alginate</i>	Linear β -(1,4) ManA and α -(1,4) GluA	Alginate lyase	PL5, PL6, PL7, PL15, PL17	(Wong et al., 2000)

^a Abbreviations: AceA: Aceric Acid; Ara: Arabinose; Api: Apiose; Dha: 3-deoxy-D-lyxo-2-heptulosaric Acid; FerA: Ferulic Acid; Fru: Fructose; Fuc: Fucose; Gal: Galactose; GalA: Galacturonic Acid; Glu: Glucose; GluA: Glucuronic Acid; Kdo: 3-deoxy-D-manno-2-octulosonic Acid; Man: Mannose; Man: Mannuronic Acid; Rha: Rhamnose; Xyl: Xylose; GH: Glycoside Hydrolases; PL: Polysaccharide Lyases; CE: Carbohydrate Esterases.

^b Enzymes required for complete degradation of the corresponding dietary fiber structure.

^c Possible Carbohydrate Active Enzymes (CAZymes) families that harbor the corresponding degradation enzymes for the given dietary fiber structure. For more detail, refer to CAZymes database (www.cazy.org) (Lombard et al., 2014).

^d Chemical structures of pectin and arabinoxylans are illustrated below.

^e Enzymes degrading RG II are not known (Wong, 2008); however, Martens and coworkers (Martens et al., 2011) reported that enzymes secreted by *B. thetaiotaomicron* to utilize RG II belong to enzymes families of GH2, GH28, GH33, GH43, GH78, GH95, GH105, GH106, PL1.

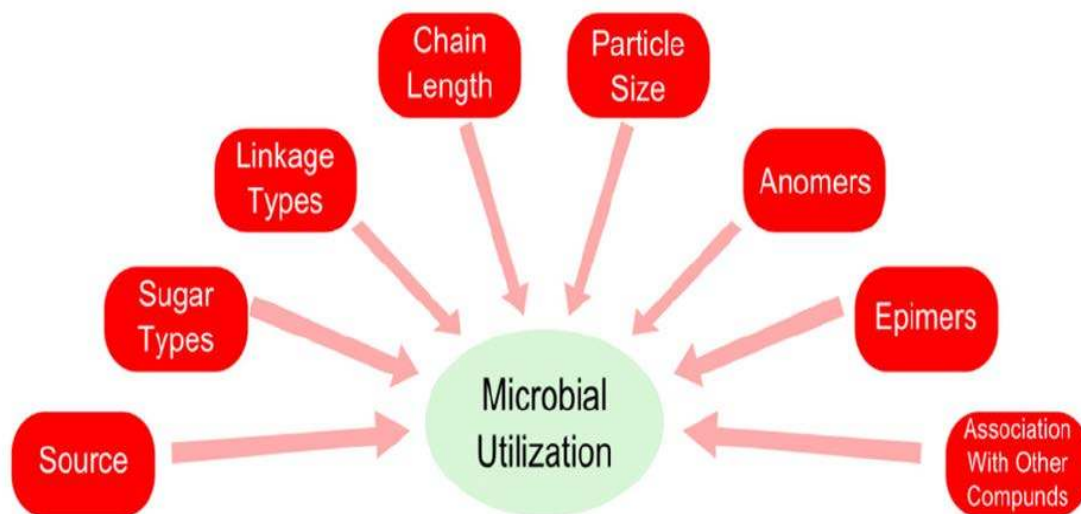


Figure 1.1 Possible variations in the chemical structures of dietary fibers which affects their utilization by microbiota.

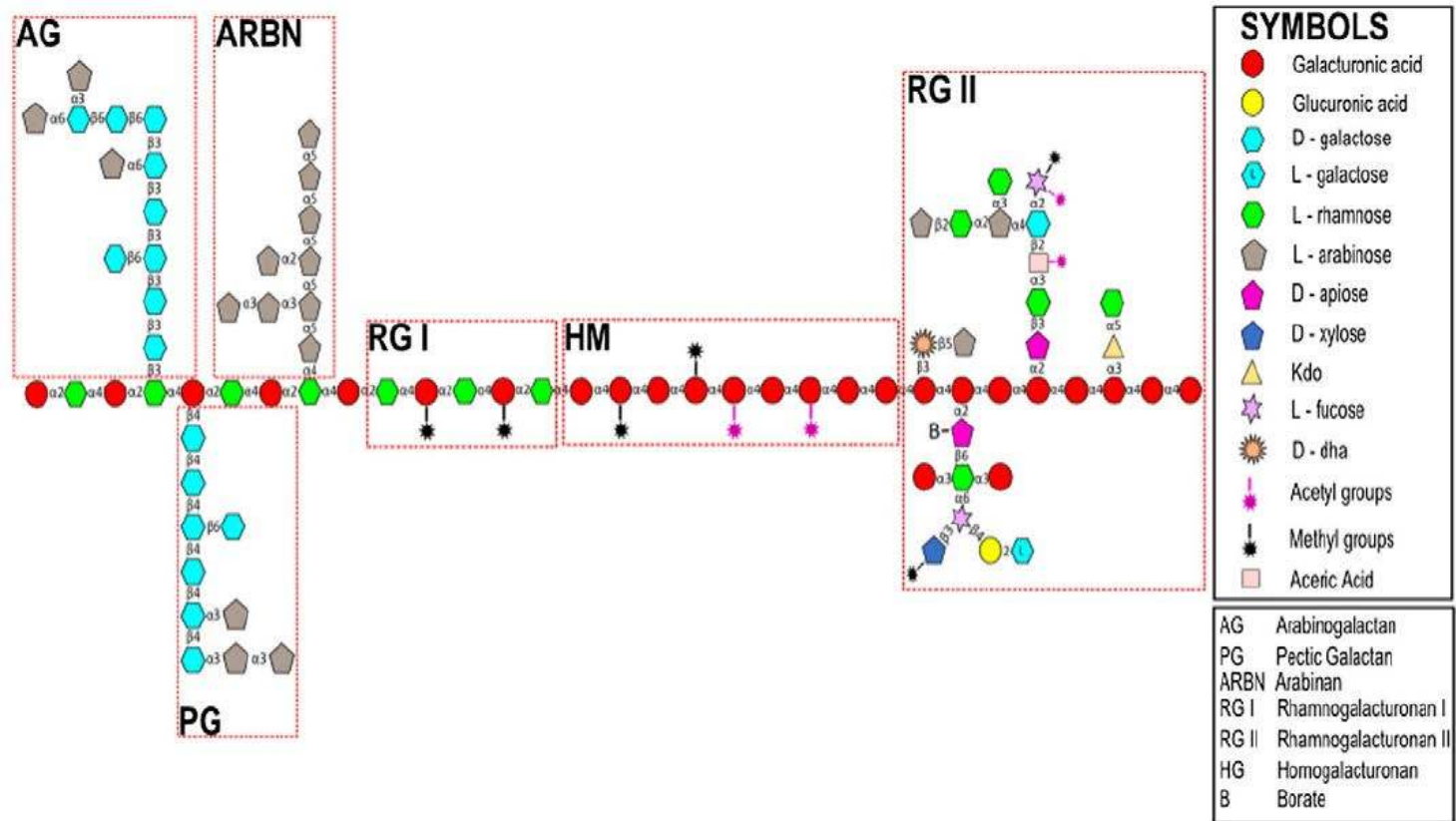
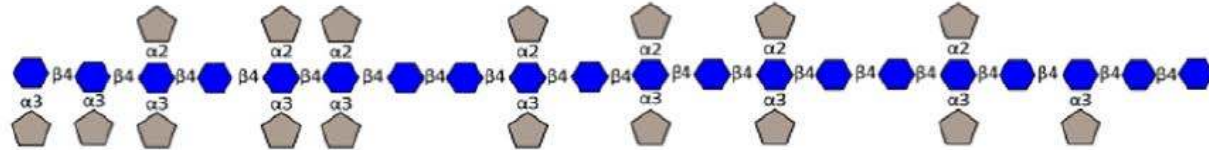


Figure 1.2 Chemical structure of pectin.

a)



b)

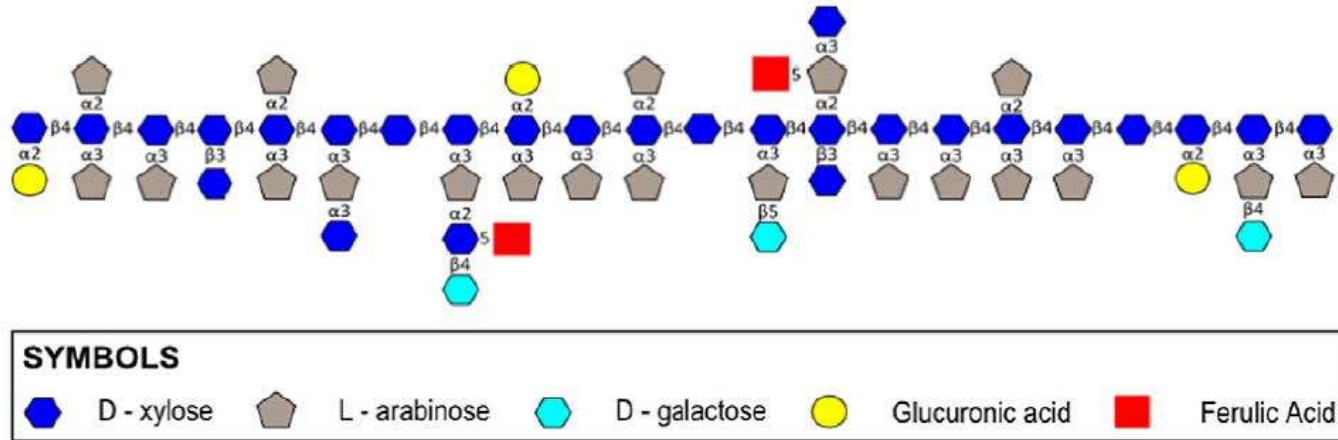


Figure 1.3 Variations in the chemical structures of arabinoxylans from different sources and regions of the plants: **a)** arabinoxylan from wheat endosperm [redrawn based on reference (Izydorczyk and Biliaderis, 1994)], **b)** arabinoxylan from maize bran [redrawn based on reference (Saulnier et al., 1995)].

CHAPTER 2. RECIPROCAL HIERARCHICAL PREFERENCES OF HUMAN GUT SYMBIONTS TO GLYCANs ALLOW THEM TO MAINTAIN THEIR COEXISTENCE

2.1 Abstract

Several human gut *Bacteroides* species have previously been shown using gene transcription studies to exhibit hierarchical preferences for glycans presented as a mixture. However, the precise biological roles of these preferences are still unknown, particularly in terms of how closely the observed transcriptional changes mirror actual glycan utilization and their consequences in competitive environments. To understand the strategies of the closely related human gut symbionts, *Bacteroides ovatus* and *B. thetaiotaomicron*, for utilizing different glycans presented as a mixture, we performed time-course assays in which both species were individually grown in a medium containing six different glycans that both are capable of degrading, and measured the remaining amounts of each substrate over time and transcription of the corresponding polysaccharide utilization loci (PULs). Both species utilized some glycans before others, but with different priorities, supporting past work that gut bacteria show species-specific hierarchical preference to glycans. Transcriptional responses to some glycans were variably sensitive to their residual concentration in the medium, with some PULs that target high-priority substrates remaining highly expressed even after their target was mostly depleted. Co-culturing of these organisms in the same mixture resulted in

reductions in expressions of some PULs by both strains; however, the hierarchical orders generally remained the same. The relative abundances of these species in co-culture on the glycan mixture remained constant during the exponential growth phase, after which *B. ovatus* became dominant. Perhaps this is due to their different glycan priorities that would allow them to maintain their coexistence. Our results provide essential information about the strategies of the microbiota to utilize different glycans, which are usually present as a mixture in the colon. Such information will be necessary to develop strategies for predicted manipulation of this community via diet for improved health.

2.2 Introduction

The human genome is limited with secretion of only eight known carbohydrate degradation enzymes, which are responsible for the digestion of lactose, sucrose, trehalose, non-resistant starch, and starch hydrolyzates (maltose, and maltooligosaccharides) (Cantarel et al., 2012). Therefore, other complex carbohydrates (also known as dietary fibers or indigestible glycans) taken into body with the meal escape from human digestion in the upper gut, and reach the colon intact where they are degraded and utilized by members of the microbiota. Through fermentation of these complex carbohydrates, gut microbes benefit the host by generating biologically important compounds, such as short-chain fatty acids. These contribute to our daily calorie requirements (McNeil, 1984). They also have been shown to impact gut epithelial health and immune system development, have anti-inflammatory and anti-carcinogenic effects (Bailon et al., 2010; Daly and Shirazi-Beechey, 2006), and protect against diet-induced obesity (Lin et al., 2012).

Indigestible dietary glycans are one of the most important parameters that determines microbial composition in the colon because the nutrient requirements of the microbiota are fulfilled mainly by these dietary components. Consumption of different types of glycans favors different bacteria or bacterial groups in the colon [reviewed in (Hamaker and Tuncil, 2014)], and these effects can be realized in less than twenty-four hours (David et al., 2014). This shift in microbial composition can be attributed to the differences in the gene contents of members of this community that enables some of them, while disfavoring others, to optimally utilize particular carbohydrates (Sonnenburg et al., 2010).

The human colonic microbiota is dominated by the members of only a few phyla, with the Firmicutes and the Bacteroidetes usually most abundant (Eckburg et al., 2005b; Ley et al., 2008; Qin et al., 2010). High abundance of the members of the Bacteroidetes phylum, especially the genus *Bacteroides*, in the colon can be attributed to their ability to catabolize a broad variety of complex carbohydrates as well as host-derived glycans (Koropatkin et al., 2012). Carbohydrate utilization ability of *Bacteroides* species is mediated by a series of gene clusters named polysaccharide utilization loci (PULs) (Bjursell et al., 2006; Martens et al., 2009). Each PUL encodes carbohydrate-binding proteins, transporters, and enzymes that target a particular polysaccharide for breakdown (Bolam and Sonnenburg, 2011; Martens et al., 2009a). *Bacteroides* species possess cognate PULs for all glycans that they are capable of degrading (Koropatkin et al., 2012; Martens et al., 2008; Martens et al., 2011), and expression of a given PUL is activated by either an oligosaccharide derived from a larger polysaccharide (Martens et al., 2011) or its monosaccharide moieties (Sonnenburg et al., 2010).

Most diets, even those represented by just one dietary fiber source, contain more than one type of polysaccharide. Therefore, optimal utilization of these different structures by gut *Bacteroides* requires activation of multiple PULs. For example, pectins from various botanical sources may be composed of arabinan, arabinogalactan, homogalacturonan, pectic galactan, and rhamnogalacturonan I and II. *Bacteroides thetaiotaomicron* expresses at least 8 of its PULs to utilize all of these pectin structures (Martens et al., 2011). This raised the question as to whether *Bacteroides* species activate all of their PULs simultaneously to utilize different glycans found as a mixture or do they have a strategy that they activate them in an order. When grown in a mixture containing different glycans, *B. thetaiotaomicron* was shown to preferentially utilize certain glycans before others (Lynch and Sonnenburg, 2012; Martens et al., 2008; Pudlo et al., 2015; Rogers et al., 2013; Sonnenburg et al., 2006). Thus, even though colonic bacteria species have the ability to utilize a number of glycans, they show hierarchical preferences to them (Rogers et al., 2013). This metabolic hierarchy is species specific with different species exhibiting variable and sometimes opposite priorities for some glycans (Pudlo et al., 2015).

On a broader scale, it is imperative that an understanding be gained of how gut bacteria utilize and compete for carbohydrate substrates in order to design diets or dietary fiber supplements that maintain or promote a healthy microbiome. Knowledge of not only what carbohydrate types individual bacteria utilize, but also *which* they choose to use in a competitive environment will be necessary to translate these fundamental findings to human health applications.

Here, in order to explore their strategies for glycan utilization when more than one type of glycans are present and demonstrate whether these strategies are changed when they are co-cultured, we have performed a series of *in vitro* assays using two closely related *Bacteroides* strains: *B. thetaiotaomicron* VPI-5482 and *B. ovatus* ATCC-8483 as model organisms, which share 96.5 % nucleotide sequence identity in their 16S rDNA genes (Martens et al., 2011) and are commonly found in the gut of adult humans (Qin et al., 2010). Direct glycan depletion measurements, along with monitoring associated PUL gene transcription, measured glycan utilization. These two methods were used because gene expression does not directly measure the output of the metabolic events as the abundances and activities of enzymes are further controlled by post-transcriptional events (Chechik et al., 2008; Gerosa and Sauer, 2011) and may be affected by other factors [for example, certain PULs of *B. thetaiotaomicron* may be involved in synthesis of capsular polysaccharides found on its surface (Martens et al., 2009b)]. Carbohydrate analysis also enabled us to understand the relationship between glycan depletion and its corresponding PUL activation.

Our results reveal that even closely related bacteria species, which possess orthologous nutrient utilization genes, exhibit different hierarchical preferences to glycans presented as a mixture. We also found that these metabolic hierarchies remain intact even when two organisms are co-cultured and the data further suggest that different glycan priorities mediate their stable coexistence. Such information provides essential knowledge about the relationship between diet and colonic microbiota, and will assist in developing strategies for intelligent manipulation of the gut microbiota for health purposes.

2.3 Materials and Methods

2.3.1 Glycans Used

Arabinan (ARAB) from sugar beet [product code (PC): P-ARAB], pectic galactan from potato (PG) (PC: P-PGAPT), polygalacturonic acid from citrus pectin (PGA) (PC: P-PGACT), and rhamnogalacturonan I from potato (RGI) (PC: P-RHAM1) were purchased from Megazyme (Megazyme International, Wicklow, Ireland). Amylopectin from maize (AP) (PC: 10120), and chondroitin sulphate from bovine trachea (CS) (PC: C9819) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The chemical structures of each glycan are illustrated in Figure 1. Sugar composition of these glycans is in Figure S1. Each of the chosen glycans had either unique monosaccharide moieties or unique linkages in their structures (Figure 2.1) that allowed for tracking disappearance during time-course assays, as noted below. Stocks for each glycan were prepared using purified water (10 mg/ml), and sterilized by autoclaving for 20 min at 121 °C.

2.3.2 Growth of Bacteria Strains and Growth Curves on Pure Glycans

B. ovatus ATCC 8483 and *B. thetaiotaomicron* VPI 5482 (ATCC 29148) strains were used for all experiments. The *B. thetaiotaomicron* capsular gene deletion strain was constructed by allelic exchange using the plasmid pExchange-*tdk* as previously described (Koropatkin et al., 2008; Rogers et al., 2013); primers for this construct were reported (Rogers et al., 2013).

B. ovatus and *B. thetaiotaomicron* were pre-grown in chopped meat broth (Table 2.S2) overnight at 37 °C in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) under an 85 % N₂, 5 % CO₂, and 10 % H₂ atmosphere for all experiments. Growth profiles of bacteria strains on pure glycans were determined using a custom carbohydrate array constructed in a 96-well format under the same conditions as described previously (Martens et al., 2011). Briefly, 100 µl of each sterilized glycan stock (10 mg/ml) were loaded into each well of a 96-well plate. One ml aliquots of bacteria cultures were centrifuged to pellet the cells, which were then washed with 1 ml of minimal media (MM) containing no carbon source [MM was prepared according to the formula in Table 2.S3, and as described by Martens et al. (2008)]. These washed cells were used to inoculate 50 ml of MM containing no carbon source. Cells (100 µl) were inoculated on each well containing 100 µl of glycan to obtain 200 µl cultures (5 mg/ml final glycan concentration). Plates were sealed in an anaerobic chamber under the atmospheric condition given above. Absorbance was measured at 600 nm (OD₆₀₀) at 10-15 min intervals for 96 h using a Powerwace HT absorbance reader coupled with a Biostack automated plate handling device (Biotek Instruments, Winooski, VT). Each species was tested in six separate carbohydrate arrays for each glycan. Data processing was handled using Gen5 software (Biotek) and Graphpad Prism® version 6 software (Graphpad Software, Inc, La Jolla, CA).

2.3.3 Glycan Exposure, Growth in Glycan Mixture, and Sample Collection

In order to prepare *B. ovatus* and *B. thetaiotaomicron* for glycan mixture exposure, cells were individually pre-grown to mid-exponential phase (OD₆₀₀ 0.5 - 0.8) in

MM containing glucose. The cells were then pelleted and washed with MM containing no carbon source prior to exposure to glycan mixtures (GM) (Rogers et al., 2013). Bacteria cells were inoculated in triplicate into an equal volume of the glycan mixture (final total glycan concentration was 5 mg/ml). For singly cultured experiments, two aliquots were removed every hour throughout their exponential phase; one of which was for measuring the remaining glycans in the media (4 ml), and the other for monitoring PUL expression over time relative to time 0 (1 ml). For the co-cultured experiment, equal amounts of *B. ovatus* and *B. thetaiotaomicron* were inoculated into same glycan mixture, and three aliquots were collected every two hours throughout the exponential phase; one for measuring the remaining glycans in the media (4 ml), one for monitoring PUL expression over time relative to time 0 (1 ml), and the last used to determine the relative abundance of each bacteria species over time (1 ml).

2.3.4 Measuring the Remaining Glycans

Collected samples for glycan analysis were dialyzed (cutoff = 1 kDa, Spectrum Laboratories, Rancho Dominguez, CA) against purified water for at least 36 h to remove vitamins and minerals from the minimal media as well as compounds possibly produced by bacteria species during growth on the glycan mixture, followed by lyophilization. Neutral and acidic monosaccharides found in the samples were determined on a weight basis using gas chromatography as their trimethylsilyl (TMS) derivatives (Doco et al., 2001). Glycosyl-linkage compositions of the samples were determined using gas chromatography coupled with mass spectrometry (7890A and 5975C inert MSD with a Triple-Axis detector, Agilent Technologies, Inc., Santa Clara, CA) (GC/MS) as their

partially methylated alditol acetate derivatives (Pettolino et al., 2012). Neutral monosaccharide amounts were confirmed as their alditol acetate derivatives using GC/MS as described previously, which also allowed us to make sure whether the methylation steps performed during glycosyl-linkage composition analysis were successful (Pettolino et al., 2012).

Over the time-course experiment, the remaining AP, CS, RGI, and PG percentages relative to their initial amounts were determined by measuring glucose, glucuronic acid, rhamnose, and galactose amounts in the samples, respectively, because these monosaccharides are signatures of the corresponding glycan found in the mixture (Figure 2.1). Similarly, remaining ARAB amount was measured by quantifying the remaining 5-arabinose linkage over time. Remaining PGA amount was calculated by subtracting total rhamnose from total galacturonic acid amount (Figure 2.1).

Members of *Bacteroides* possess the ability to synthesize capsular polysaccharides (Baumann et al., 1992; Kasper et al., 1983; Martens et al., 2009b; Xu et al., 2003). To make sure whether bacteria species tested in this study did not synthesize any glycan that could interfere with our glycan analysis, in parallel with our time-course assays, other time-course studies were done in which these bacteria species were grown in media containing mannose as the only carbon source and samples were collected for glycan analysis in each time point during their exponential phase (Figure. 2S.3d, f). These analyses revealed that both *B. ovatus* and *B. thetaiotaomicron* synthesized only glucose. Amounts of this sugar were negligible (Figure 2.S4).

2.3.5 Transcript Analysis of PUL genes by Quantitative PCR (qPCR)

PUL expression analyses were performed as previously described (Rogers et al., 2013). Primers used in this study are in Table 2.S1.

2.3.6 qPCR Enumeration of Competing Species in Co-Cultured Experiments

In the co-cultured experiment, cells were harvested by centrifugation for quantification. DNA was isolated using DNeasy® Blood and Tissue Kit according to the manufacturer's instructions (QIAGEN). DNA (10 ng) was assayed in duplicate in a Mastercycler® ep realplex (Eppendorf) using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc. Wilmington, MA) and species-specific primers for 40 cycles of 95 °C for 3 s, 55 °C for 20 s, and 72 °C for 8 s. Species-specific primers used for *B. ovatus* and *B. thetaiotaomicron* were *BACOVA03426* and *BT3854*, respectively (Table 2.S1). Purified genomic DNA standards (range; 80, 20, 2, 0.4, 0.08, and 0.01 ng) of each species were included in duplicate in each qPCR run. A standard curve generated from these standards were used to calculate the relative abundance of each species in each sample.

2.3.7 Thin-Layer Chromatography (TLC) Analysis

Thin-layer chromatography was performed using 20 cm x 20 cm glass silica gel plates (Millipore, PC:105715, EMD Millipore, Billerica, MA). Filter-sterilized samples (3 µl) were spotted on the plate. The plates were developed in a TLC tank using solution containing chloroform, acetic acid, and water at ratio of 6, 7, and 1, respectively. After development, the plates were air-dried. They were visualized by washing with a staining

solution [sulfuric acid/methanol (5:95, v/v) containing 0.03 mg/ml N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich, PC: 222488)], followed by heating at 110 – 120 °C for 5 min. Arabinooligosaccharide standards for TLC [arabinan (PC: P-ARAB), arabinobiose (PC: O-ABI), arabinohexaose (PC: O-AHE), and arabinoheptaose (PC: O-AHP)] were purchased from Megazyme (Megazyme International, Wicklow, Ireland). Arabinose (PC: A3131) was purchased from Sigma-Aldrich.

2.3.8 Preparation of ARAB-containing Media Predigested by *B. thetaiotaomicron* and *B. ovatus* and Growth Experiments

B. thetaiotaomicron was grown on ARAB-containing media (initial ARAB concentration, 5 mg/ml). Throughout its growth, media were harvested at different time points (Figure 2.6a), and then centrifuged at 10,000 rpm for 10 min, and supernatants were filter-sterilized (0.22 µ pore size).

The growth of *B. ovatus* on the filter-sterilized media were monitored using a custom carbohydrate array constructed in a 96-well format using an automated plate reader (Section 2.3.2). Filter-sterilized media (100 µl) were placed into each well of a 96-well plate. *B. ovatus* culture for inoculation was pre-grown to mid-exponential phase (OD₆₀₀ 0.5) on MM containing glucose, and 10 ml aliquots were centrifuged to pellet cells, which were then washed with MM containing no carbon source. These washed cells were used to inoculate 10 ml of MM containing no carbon source. Cell suspensions (100 µl) were inoculated in wells containing 100 µl of filter-sterilized media to obtain 200 µl cultures. Plates were sealed and loaded into an automated plate reader (Section

2.3.2). As a negative control, 200 μ l aliquots containing equal volume of filter-sterilized media and MM (containing no bacteria cells) were also placed into the plates (Figure 2.S6). As a positive control, growth of *B. ovatus* was monitored on arabinose-containing media predigested by *B. thetaiotaomicron* (Figure 2.S7b). The arabinose-containing media predigested by *B. thetaiotaomicron* at the different growth stages were obtained by harvesting cultures throughout *B. thetaiotaomicron*'s growth on arabinose as the only carbon source (Figure 2.S7a), which were filter-sterilized before *B. ovatus* inoculation as noted above.

2.3.9 Data Analysis

Statistical analysis was done using SAS version 9.4 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was performed at $\alpha=0.05$ significance level to determine differences among the samples. Least significant difference (LSD) test at $\alpha=0.05$ was used to test whether mean differences were statistically significant.

2.4 Results and Discussions

2.4.1 *B. ovatus* and *B. thetaiotaomicron* Exhibit Reciprocal Hierarchical Preferences to Glycans as Measured Directly by Carbohydrate Depletion

Direct measurements of carbohydrate utilization by gut bacteria is achieved by analyzing the disappearance of the individual nutrients in a mixture, leading to an understanding of strategies that bacteria use to access energy to survive in the competitive environment of the gut. However, direct measurements have been infrequently applied due to their difficulty, especially in the context of polysaccharides,

which may share overlapping monosaccharide content and therefore require additional linkage analysis. Hierarchical preferences of the model gut symbiont, *B. thetaiotaomicron*, to glycan mixtures were previously determined based on transcriptional data (Lynch and Sonnenburg, 2012; Martens et al., 2008; Pudlo et al., 2015; Rogers et al., 2013; Sonnenburg et al., 2006). Here, we decided to further examine utilization and competition for glycans using glycan depletion analysis in conjunction with transcriptional profiling and to also include a related organism, *B. ovatus*, for comparison. First, *in vitro* time course studies were done on *B. ovatus* and *B. thetaiotaomicron* which were individually grown on a mixture of glycans that they both are capable of degrading (Martens et al., 2011) (Figure 2.S2). This mixture contained chondroitin sulphate (CS), polygalacturonic acid (PGA), pectic galactan (PG), rhamnogalacturonan I (RGI), arabinan, and amylopectin (AP), each at equal concentrations with total carbohydrate (5 mg/ml) being the limiting nutrient (Figure 2.1). These glycans were chosen because they either have unique monosaccharides or unique glycosidic linkages in their structures (Figure 2.1) that allowed for tracking their disappearance during the time course experiments. Media and bacterial cells were collected at every hour during the exponential growth phase of *B. ovatus* and *B. thetaiotaomicron* (Figure 2.S3a and 2.S3b, respectively) to measure the remaining glycans (red lines in Figure 2.2a for *B. ovatus* and Figure 2.2b for *B. thetaiotaomicron*) and expression of PULs targeting each glycan (blue lines in Figure 2.2a for *B. ovatus* and green lines in Figure 2.2b for *B. thetaiotaomicron*).

Our results reveal that each species utilizes the presented glycans in a hierarchical order (Figure 2.2a and 2.2b). Throughout the experiment, *B. ovatus* revealed four

different PUL expression and glycan depletion trends (Figure 2.2a). The first one was rapid recognition and utilization exemplified by CS and PGA. The second trend was observed in PG where recognition was very quick, but the degradation started at a later time. The third was characterized by RGI where gene expression and utilization occurred at a later time after the most preferred glycan, CS, was gone. Lastly, for AP, a gradual decrease in its amount was seen as a gradual increase occurred in the respective PUL.

B. thetaiotaomicron showed three glycan recognition and utilization trends that were similar in some aspects with *B. ovatus*, though different in others. The first trend, represented by CS and PG, shows recognition and utilization occurring very rapidly after exposure to the mixture. The second trend was characterized by PGA and AP, where PULs displayed a transient rise upon exposure to the glycan mixture followed by further increases at later times; which was consistent with glycan depletion where small amounts of PGA and AP were utilized within the first two hours followed first by no significant depletions ($P < 0.05$) until mid-exponential phase, and then decreases in amount of remaining substrates. RGI and ARAB exemplified the third trend where the expression of PULs were rapid, followed by a plateau and then by further increase; degradation of these glycans occurred mostly later. *B. thetaiotaomicron* seemed to prepare its utilization systems upon exposure to the glycan mixture, but did not necessarily degrade the target glycan when utilizing the prioritized CS and PG.

In agreement with Pudlo et al. (2015), these data reveal that hierarchical preferences are variable between species, whereby different species show different and sometimes reciprocal priorities for some glycans. Interestingly, the direct glycan depletion data showed hierarchical rankings that were not exactly the same as found in

transcriptional analysis. Specifically, *B. thetaiotaomicron* began to utilize PG right after exposure to the mixture and degradation of PGA occurred at an intermediate time (Figure 2.2b), while the opposite trend was observed for *B. ovatus* (Figure 2.2a). Gene expression did not disclose these differences in glycan utilization as both bacteria activated the corresponding PULs for PG and PGA immediately upon exposure to the mixture (Figure 2.2a blue lines for *B. ovatus*, and 2.2b green lines for *B. thetaiotaomicron*). Collectively, these observations suggest that additional mechanisms exist beyond transcriptional sensing to modify glycan utilization trends – which could include variations in translation/secretion rates for these mostly extracellular system, along with enzyme or transporter efficiencies.

2.4.2 Gene Expression Does Not Always Truly Measure Corresponding Glycan Depletion

Increase in PUL expression does not always mean that bacteria species utilize the respective glycan. For instance, even though *B. ovatus* highly expressed the corresponding PUL for PG right after exposure to the glycan mixture (> 100-fold induction), significant reduction ($P < 0.05$) in its amount occurred only 8 h of incubation (Figure 2.2a). A similar result was also seen in *B. thetaiotaomicron* for utilization of ARAB where it highly expressed the corresponding PUL in the first hour of incubation (254-fold induction), and where significant reductions in ARAB occurred later (Figure 2b). This can be attributed to the fact that gene expression is not a direct measure of metabolic events as the abundance and activity of enzymes may be further controlled by post-transcriptional events (Chechik et al., 2008; Gerosa and Sauer, 2011). Martens et al.

(2009b) reported that certain PULs of *B. thetaiotaomicron* are also coordinately regulated with synthesis of capsular polysaccharides found on its surface (Martens et al., 2009b)]. These results show that in the study of glycan utilization strategies of bacteria species, carbohydrate analysis should be included in the experimental design.

2.4.3 Different Species Exhibit Variable Sensitivities to Amount of Glycans

Bacteria sensitivities to some glycans are tightly tuned to the residual concentration of polysaccharide in the medium, while others remain highly expressed even after most of the target substrate has been depleted. For instance, over time the expression of the *B. ovatus* PUL targeting PGA continuously dropped as its remaining amount decreased (Figure 2.2a), indicating that *B. ovatus* has the ability to adjust its PUL targeting PGA based on the amount of available substrate found in the environment. Conversely, the *B. ovatus* PUL corresponding to CS degradation remained at a high level until 98 % of it was consumed (Figure 2.2a), indicating that this PUL is highly sensitive to even a quite small amount of CS.

2.4.4 Hierarchical Orders Remained Generally the Same, When These Organisms Grew Together on Same Glycan Mixture

The experiments above reveal that human gut symbionts show species-specific hierarchical preferences to glycans presented as a mixture, but do not answer whether these metabolic hierarchies change when they are in a competitive environment. *B. ovatus* and *B. thetaiotaomicron* were co-cultured on the same glycan mixture and their gene expression profiles as well as glycan depletions (red lines in Figure 2.2c) were

monitored every two hours for 16 h. Transcriptional profiles of the bacteria species for corresponding glycans in the competitive environment (blue lines for *B. ovatus* and green lines for *B. thetaiotaomicron* in Figure 2.2c) were compared to that in the singly cultured experiments (Figure 2.2a for *B. ovatus* and Figure 2.2b for *B. thetaiotaomicron*).

Overall expressions levels (fold changes relative to time zero) of PULs in both *B. ovatus* and *B. thetaiotaomicron* decreased when they were co-cultured compared to those observed when they were individually cultured (Figure 2.2), however general expression patterns were similar to those of the singly cultured bacteria (Figure 2.2a and 2.2b). This indicates that human gut symbionts are ‘programmed’ to utilize glycans in a hierarchical order when they are presented together, and these metabolic hierarchies do not change even if they are in competitive environment.

2.4.5 Different Hierarchical Preferences of Bacteria Species Allow Them to Maintain Their Coexistence in the Competitive Environment

We also monitored the stability of the co-cultured community over the course of 16 h by determining the relative abundance of each bacteria species by quantitative PCR using species-specific primers. There was no significant change ($P < 0.05$) observed in the relative abundance of the two species within the first 10 h (Figure 2.3), during which CS, PGA, PG, and AP were mainly depleted (Figure 2.2c, red lines).

We wished to investigate what factors contribute to the stability of the community during these 10 h, rationalizing that it could either be: 1) mutual utilization of the same sugars without one species dominating, or 2) in the time frame when one species was utilizing a particular glycan, another was using a different glycan for the purpose of

reducing competition. The organisms were co-cultured in a medium containing each of the individual glycans as the only carbon source and the relative abundance of each species was determined to understand which of these scenarios was responsible for the stability of the community. In all cases, there was a trend towards favoring of *B. ovatus* over *B. thetaiotaomicron* on the individual glycans, although some substrates (pectic galactan and amylopectin) promoted more equal competition of *B. thetaiotaomicron* (Figure 2.4a-d). These findings support the second hypothesis that *B. theta* was able to persist in the complex mixture because the two competing bacterial species were able to utilize different glycans at certain points in a non-competitive manner. This could be attributed to the fact that these species exhibit reciprocal priorities for some glycans (Figure 2.2a and 2.2b). Thus, variable hierarchical preference of human gut symbionts to glycans could be an important strategy to maintain their coexistence in the competitive environment of the colon, and suggests that consumption of mixtures of glycans, rather than only one type, is optimal for maintaining microbial diversity.

2.4.6 Co-Culturing Enabled *B. ovatus* to Utilize Previously Inaccessible ARAB due to Cross-Feeding

Next, we investigated how *B. ovatus* outcompeted *B. thetaiotaomicron* after 10 h (Figure 2.3) when utilization of ARAB mainly occurred (Figure 2.2c, red lines), and considering that *B. ovatus* was unable to degrade ARAB when it was its sole carbon source (Figures 2.1a and 2.S2a). It seemed likely there was a syntrophic interaction between *B. ovatus* and *B. thetaiotaomicron* that enabled *B. ovatus* to utilize previously inaccessible ARAB. To test the ARAB cross-feeding hypothesis, we first monitored *B.*

ovatus and *B. thetaiotaomicron* growth on arabinose (building block of ARAB) and arabinobiose (disaccharide in which two arabinose units are joined via a α -1,5 glycosidic linkage, and is an intermediate compound possibly released as a result of ARAB degradation by bacteria). *B. ovatus* utilized arabinose and arabinobiose as efficiently as *B. thetaiotaomicron*, while it did not utilize ARAB (Figure 2.5). Martens et al. (2011) showed that oligosaccharide signal molecules with degree of polymerization of ≥ 5 activate *B. thetaiotaomicron*'s ARAB utilization PUL (Martens et al., 2011), and accordingly growth patterns were observed on arabinose and arabinobiose with a small amount of ARAB incorporated with arabinobiose. Organisms were grown individually on a media containing arabinobiose and ARAB at a ratio of 9:1. With a low amount of ARAB present, *B. thetaiotaomicron* grew substantially better on arabinobiose while *B. ovatus* did not. We then decided to investigate whether *B. thetaiotaomicron* releases ARAB degradation products such as arabinooligosaccharides into the media while growing on ARAB, which are potentially utilized by *B. ovatus*. For this, we grew *B. thetaiotaomicron* alone on a media containing ARAB as the only carbon source and collected samples at different growth stages [3 samplings at its early-exponential phase (EEP), 1 sampling at its mid-exponential phase (MEP), 2 samplings at its late-exponential phase (LEP), and 1 sampling at its stationary phase (SP)] (Figure 2.6a). The supernatants of the media were then monitored for the presence of arabinooligosaccharides using TLC (Figure 2.6b). The results showed that *B. thetaiotaomicron* releases ARAB breakdown products into the media, which have variety of chain lengths, but mainly arabinooligosaccharides having lower than 7 arabinose units (Figure 2.6b, columns 6 through 12). In order to examine whether *B. ovatus* can utilize *B. thetaiotaomicron*

released ARAB breakdown products, we monitored *B. ovatus* growth on filter-sterilized ARAB breakdown products obtained throughout *B. thetaiotaomicron*'s growth on ARAB as described above (Figure 2.6a). *B. ovatus* did not grow on the media obtained at the EEP1 and EEP2 growth stages (Figure 2.6c), perhaps attributed to a paucity of released ARAB breakdown products which would be sufficient to trigger the utilization machinery of *B. ovatus*. Notably, *B. ovatus* grew on the media obtained after the EEP2, with different degrees of growth rate culminating high growth rate in the LEP2 media. These clearly indicates that *B. ovatus* efficiently grows on ARAB breakdown products released into media by *B. thetaiotaomicron*. No growth of *B. ovatus* was observed in the media obtained in the SP (Figure 2.6c), indicating that *B. thetaiotaomicron* had used all of the ARAB breakdown products. Collectively, these data show that cross-feeding occurred when these organisms were co-cultured, through release of ARAB degradation products of DP < 7 and considerably > 2 that supported growth of *B. ovatus*.

We had also considered that *B. ovatus* might utilize the capsular polysaccharides of *B. thetaiotaomicron*, when there was no other carbohydrate that it can access in the environment (i.e., ARAB). To test this, *B. ovatus* was co-cultured with a capsular polysaccharide-free mutant (Δ CPS) *B. thetaiotaomicron* (deletion of all eight of the capsular polysaccharide synthesis gene clusters) in a medium containing ARAB as the only carbon source, and collected samples every 4 h until the optimal density of the culture reached steady state level (Figure 2.S5f) in order to quantify the stability of the community (in parallel, as a control, wild type *B. thetaiotaomicron* was co-cultured with *B. ovatus* in a medium containing the same glycan). In both cases, *B. ovatus* was able to

outcompete *B. thetaiotaomicron* (Figure 2.4a and 2.4b) indicating that capsular polysaccharides did not have an observable effect on community stability.

2.5 Conclusion

Determining the glycan utilization strategies of the members of our gut microbial community has become a major research focus due to the essential role of these dietary compounds on community structure and their ability to be non-invasively altered via our diet. Most of these studies rely on transcriptional profiling of bacteria to given glycans. However, complete understanding of these strategies requires monitoring the changes in glycan amount because, as experimentally demonstrated in this study, gene expression does not always truly measure glycan utilization by bacteria species. Using carbohydrate depletion analysis in conjunction with transcriptional profiling enabled us to investigate thoroughly the strategies of *B. ovatus* and *B. thetaiotaomicron* to metabolize complex glycans presented as a mixture. Our results show for the first time that hierarchical preferences of bacteria to dietary glycans are protected when they are in a competitive environment further suggesting that bacteria are ‘programmed’ to utilize different glycans found in a mixture in an order; and furthermore variations in hierarchical preferences for glycans may allow bacterial coexistence in a competitive environment. For bacteria utilizing the same set of dietary glycans, reciprocal processing allows them to access foods simultaneously and survive in the competitive environment of the colon.

Additionally, the bacteria species exhibited variable sensitivities to different glycans whereby the gene recognition of certain glycans was concentration dependent, while others were recognized even in low residual amounts. This might be an important

concept when attempting to favor a specific bacteria or bacterial groups in the colon by dietary supplementation. One would need to make sure that a given amount of a particular glycan would be enough for the targeted bacteria to recognize.

Chemical structures of a particular glycan type show structural variation from source to source. In the future, it will be essential to investigate whether the metabolic preferences given to glycans shown by bacteria species are structure dependent or not. The current and future studies will provide essential information about the metabolic activities and strategies of bacteria species to utilize different polysaccharides, which often present as mixtures in a complex environment like colon. Such knowledge could play a pivotal role in designing dietary interventions to promote desired microbial shifts for better health.

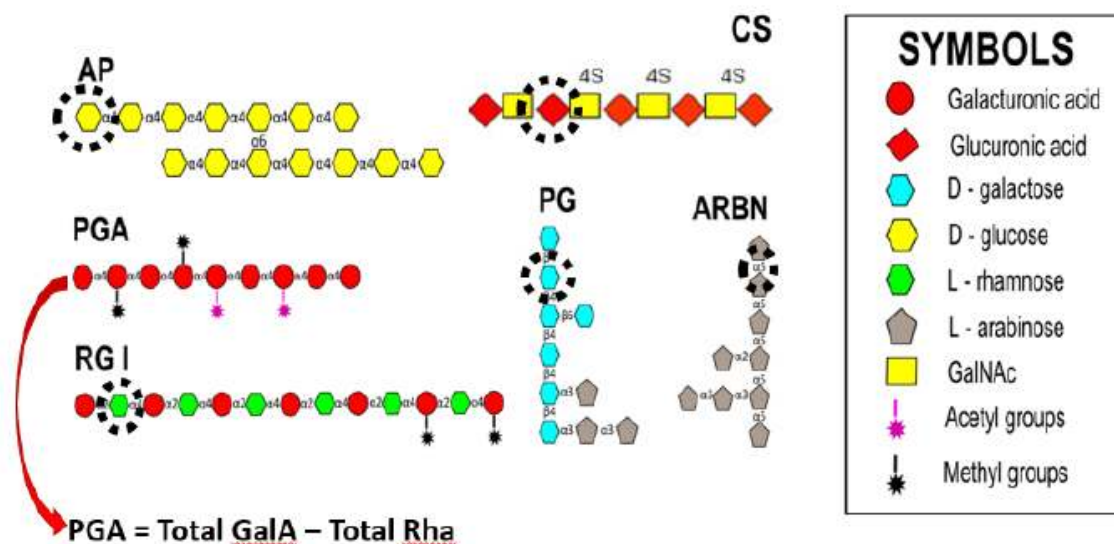


Figure 2.1 Chemical structures of glycans used. Chemical structures of glycans used. Dashed circles indicate the monosaccharides or linkages that were used to measure the remaining amount of corresponding glycan in the media throughout experiments. Remaining AP, CS, RGI, and PG relative to their initial amounts were determined by measuring the glucose, glucuronic acid, rhamnose, and galactose amounts in the samples, respectively. The remaining ARAB amount was measured by quantifying the remaining 5-arabinose linkage over time. The remaining PGA amount was calculated by subtracting total rhamnose from total galacturonic acid. Abbreviations: AP, amylopectin; ARAB, arabinan; CS, chondroitin sulphate; PG, pectic galactan; PGA, polygalacturonic acid; RGI, rhamnogalacturonan I.

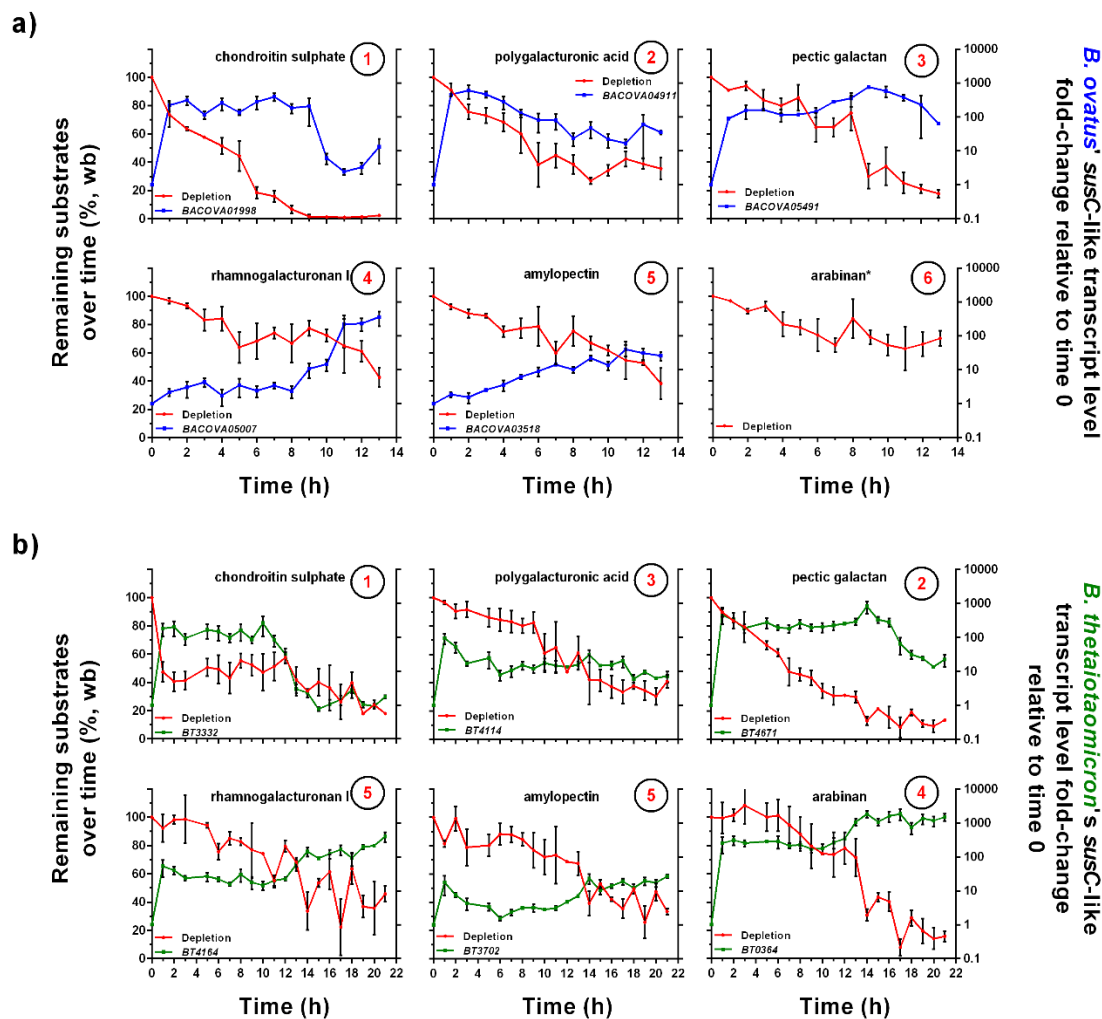


Figure 2.2 Individual glycan depletion in the mixture and expression of PULs by bacteria species over time during growth. **a)** Substrate depletion by *B. ovatus* (red lines), and temporal expression of PULs (blue lines). **b)** Substrate depletion by *B. thetaiotaomicron* (red lines), and temporal expressions of PULs (green lines). Circled numbers refer to hierarchical ranking order based on the following criteria: 1) depletion slope of each glycan to the midpoint of time-course experiment, and 2) final remaining level of each glycan. In **(a)** and **(b)** rankings of rhamnogalacturonan I and amylopectin could not be differentiated.

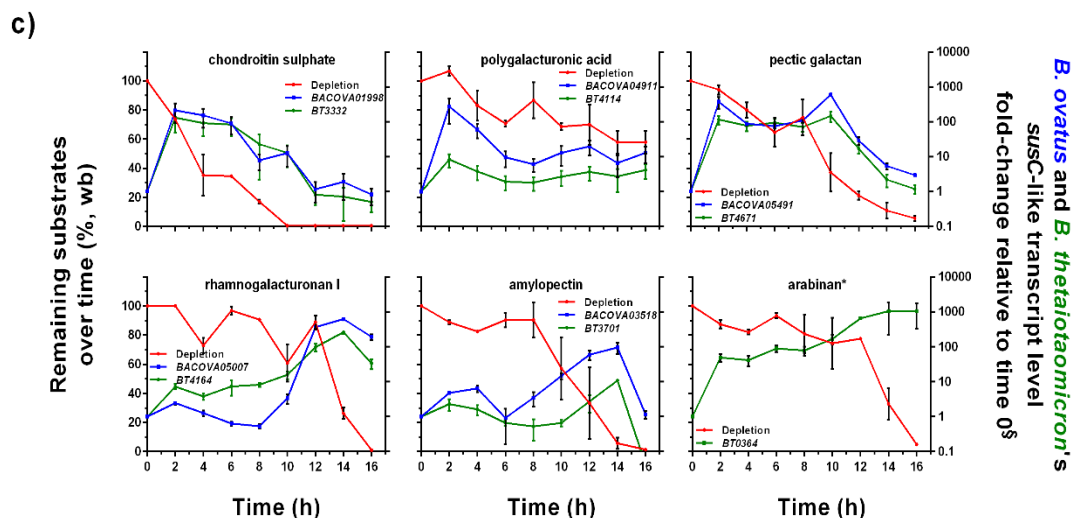


Figure 2.2 Continued. **c)** Substrate depletion by *B. ovatus* and *B. thetaiotaomicron* grown together (red lines), and temporal expressions of *B. ovatus* PULs and that of *B. thetaiotaomicron* PULs (blue and green lines, respectively) (co-cultured study). All analyses were performed using samples collected throughout exponential phase of corresponding bacteria (growth curves are given in Figure 2.S3a-c). Transcript levels were determined using qPCR against *susC*-like genes representing PULs which are triggered by each of the six glycans. Lines showing transcript level changes are described with locus tag numbers of corresponding genes. All transcript level changes were relative to time 0, prior to exposure to the mixture of six glycans. Error bars represent the standard error of the mean of three separate replicates. *The arabinan utilization PUL of *B. ovatus* not known. § In the co-cultured environment, *BT3701* gene expression (*susD*-like transcript level) was measured to determine the *B. thetaiotaomicron*'s responses to AP, rather than using the *BT3702* gene (*susC*-like transcript level), because there was cross hit during qPCR analysis (data not shown). No attempt was made to re-measure the *susD*-like transcript level of *B. thetaiotaomicron* in response to AP, since the expression of *susC* and *susD* during its growth on AP were not significantly different (Koropatkin et al., 2008).

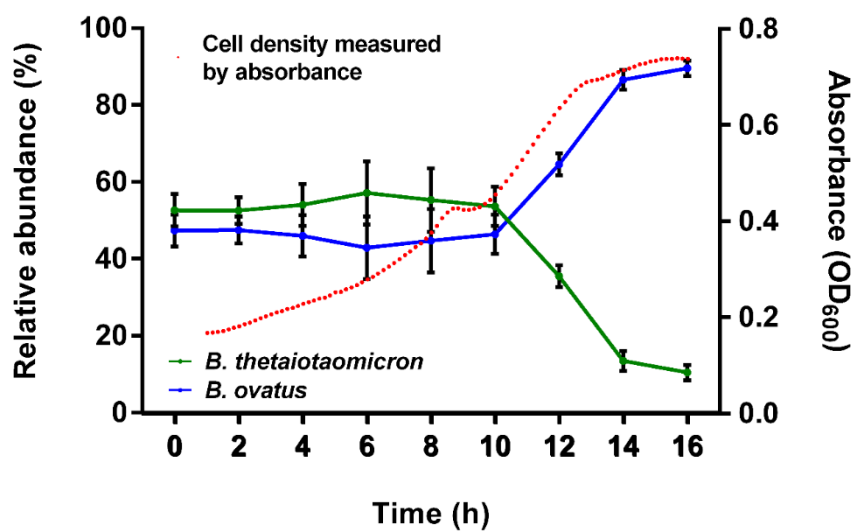


Figure 2.3 Relative abundances (% total bacteria) of *B. ovatus* (blue line) and *B. thetaiotaomicron* (green line) grown together in the mixture of six glycans, and the cell density of the media measured by absorbance (OD₆₀₀) (red dots). Abundances were determined using qPCR with species-specific genes (*BACOVA03426* for *B. ovatus*; *BT3854* for *B. thetaiotaomicron*). Error bars represent the standard error of the means of three separate replicates.

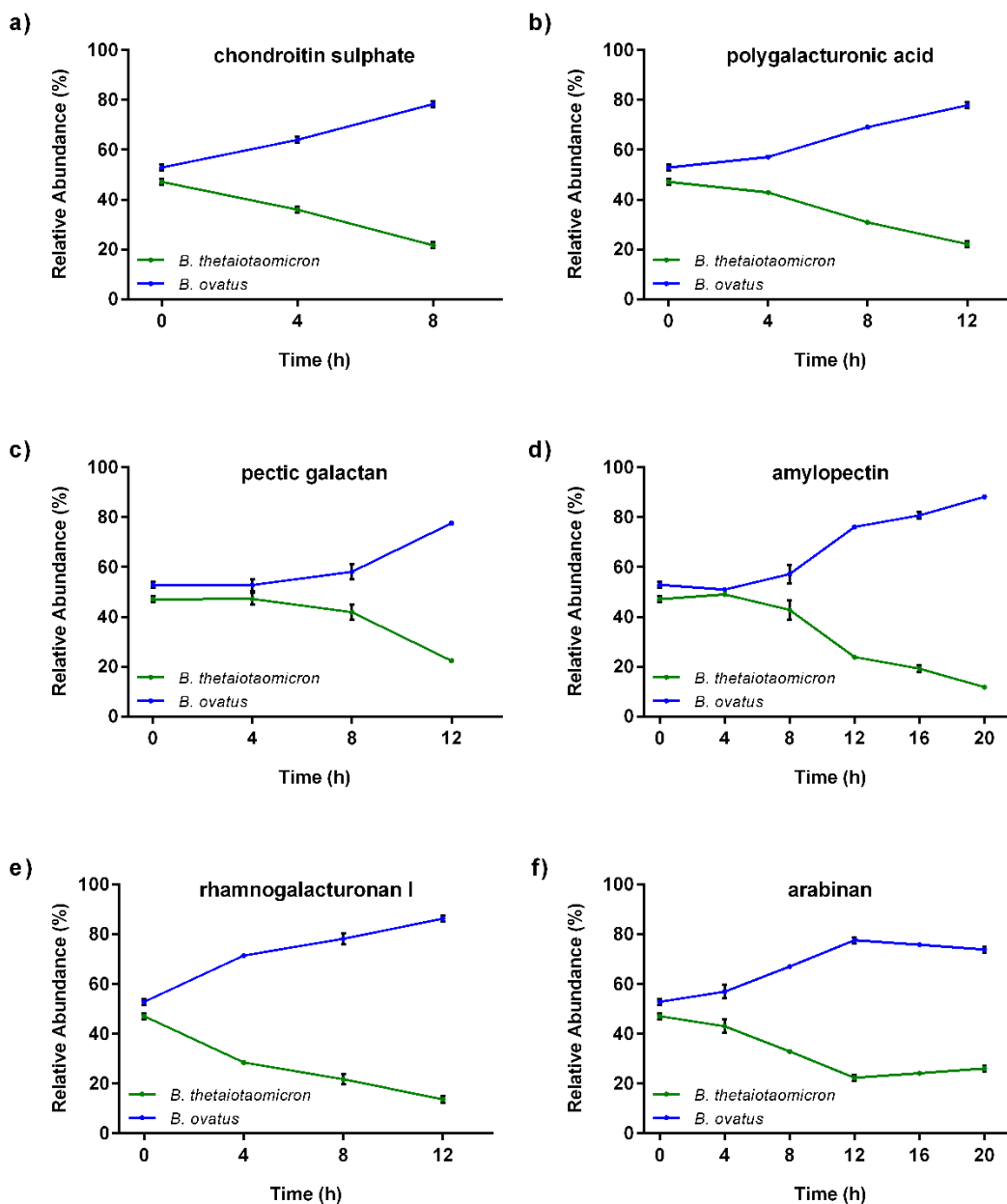
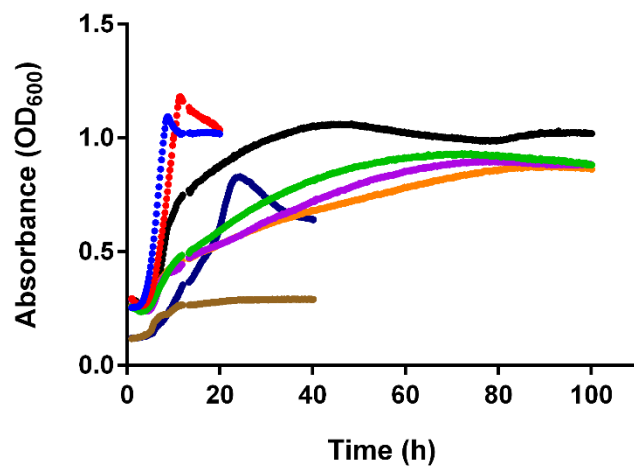


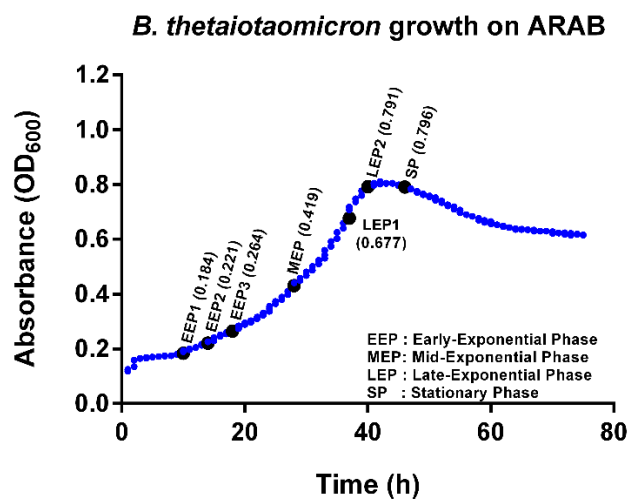
Figure 2.4 Relative abundances (% total bacteria) of *B. ovatus* (blue line) and *B. thetaiotaomicron* (green line) grown together media containing the individual glycans as the only carbon source. Abundances were determined using qPCR with species-specific genes (*BACOVA03426* for *B. ovatus*; *BT3854* for *B. thetaiotaomicron*). Error bars represent the standard error of the means of three separate replicates.



- *B. ovatus* on arabinose
- *B. thetaiotaomicron* on arabinose
- *B. ovatus* on arabinobiose
- *B. thetaiotaomicron* on arabinobiose
- *B. ovatus* on arabinobiose:arabinan (9:1)
- *B. thetaiotaomicron* on arabinobiose:arabinan (9:1)
- *B. ovatus* on arabinan
- *B. thetaiotaomicron* on arabinan

Figure 2.5 Growth curves of *B. ovatus* and *B. thetaiotaomicron* grown individually in media containing different arabinan-based carbon sources [arabinose, arabinobiose, or arabinobiose:arabinan (9:1)].

a)



b)

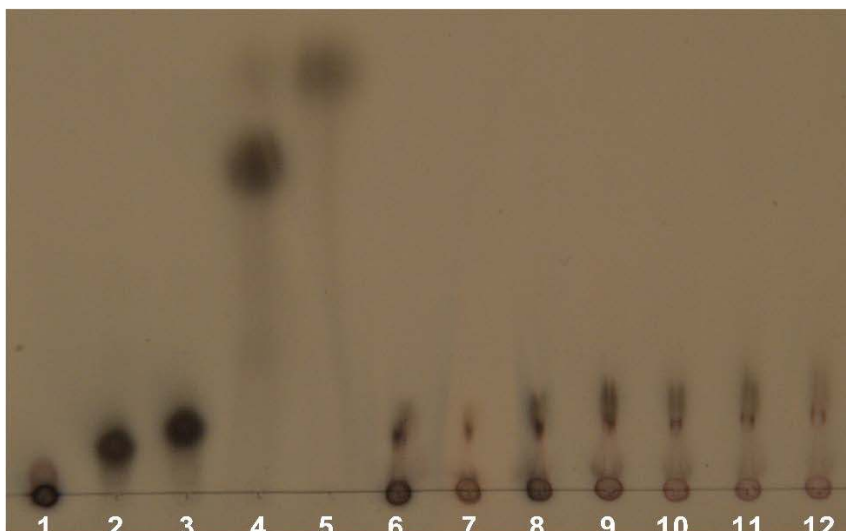


Figure 2.6 **a)** Growth of *B. thetaiotaomicron* on media containing ARAB as the only carbon source. Thicker black dots indicate the points where cultures were harvested for subsequent analysis. Numbers in the parentheses at each harvesting points refer to absorbance values. **b)** Thin-layer chromatography (TLC) of ARAB breakdown products in media harvested throughout the growth phases of *B. thetaiotaomicron*. Column 1 through 5 represents the external standards: 1, arabinan; 2, arabinoheptaose; 3, arabinohexaose; 4, arabinobiose; 5, arabinose. Columns 6 through 12 are samples: 6, EEP1; 7, EEP2; 8, EEP3; 9, MEP; 10, LEP1; 11, LEP2; 12, SP.

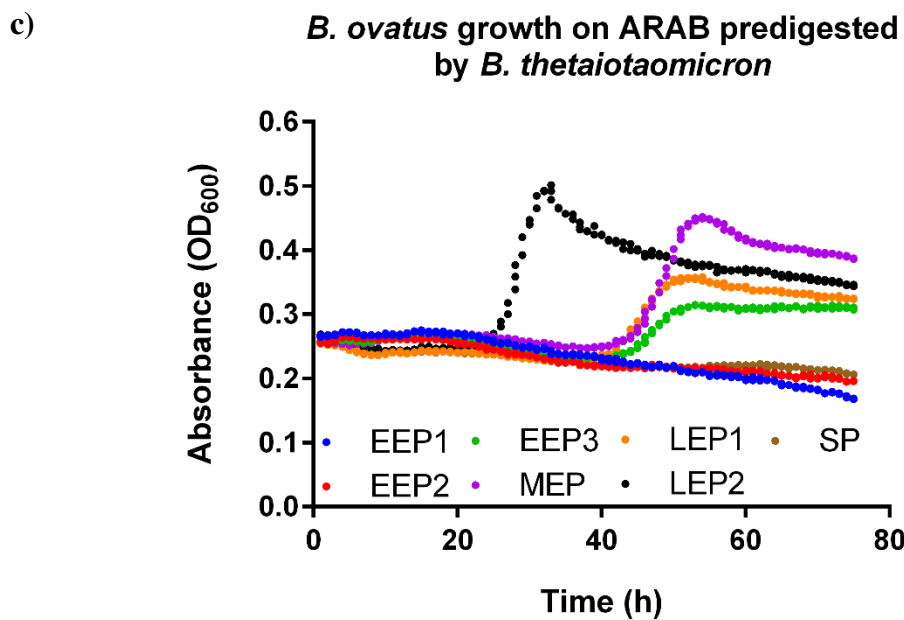


Figure 2.6 Continued. c) *B. ovatus* growth on media harvested at different time points throughout *B. thetaiotaomicron*'s growth on ARAB. For instance, EEP1 refers to *B. ovatus* growth on media harvested at the early-exponential phase of *B. thetaiotaomicron*'s growth on ARAB.

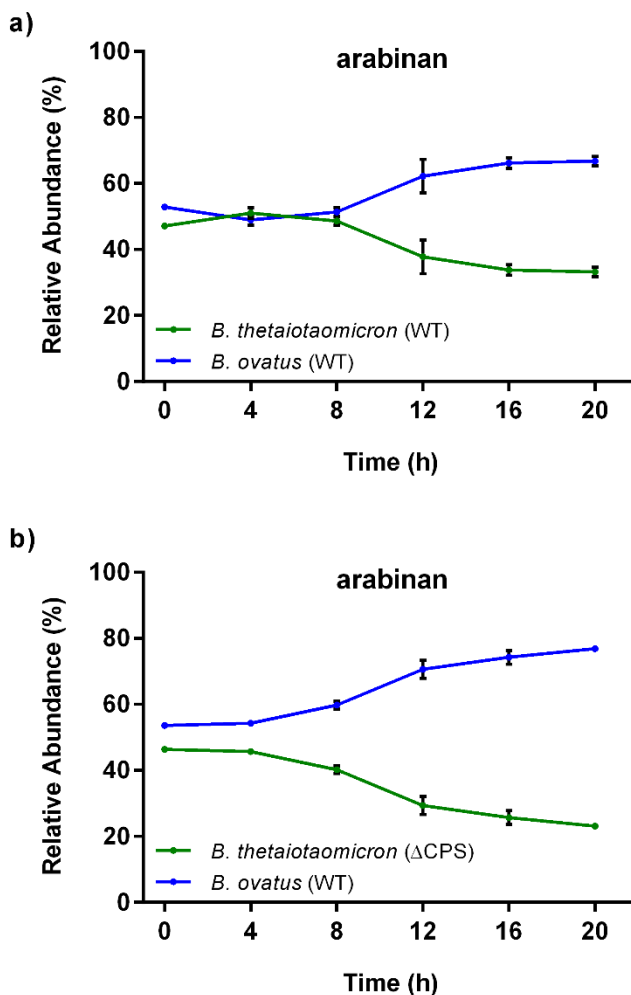


Figure 2.7 Relative abundances (% total bacteria) of **a)** wild type *B. ovatus* (blue line) and wild type *B. thetaiotaomicron* (green line) and **b)** wild type *B. ovatus* (blue line) and capsular carbohydrate-free Δ CPS *B. thetaiotaomicron* (green line) grown together in a media containing arabinan as the only carbon source. Abundances were determined using qPCR with species-specific genes (*BACOVA03426* for *B. ovatus*; *BT3854* for *B. thetaiotaomicron*). Error bars represent the standard error of the means of three separate replicates.

2.6 Supporting Information

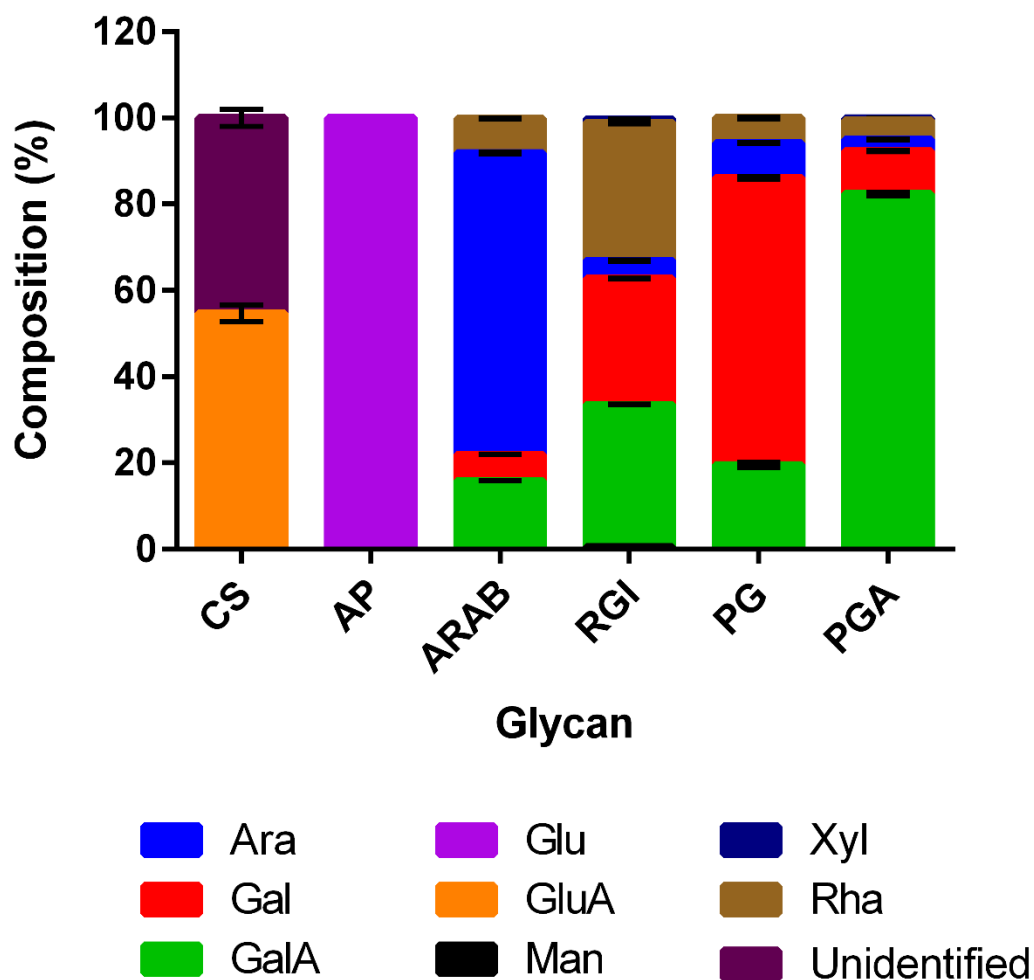


Figure 2.S1 Monosaccharide composition of glycans used in this study as their trimethylsilyl derivatives. Error bars represent the standard error of the means of two separate replicates. Abbreviations: **Ara**, arabinose; **Gal**, galactose; **GalA**, galacturonic acid; **Glu**, glucose; **GluA**, glucuronic acid; **Man**, mannose; **Rha**, rhamnose; **Xyl**, xylose.

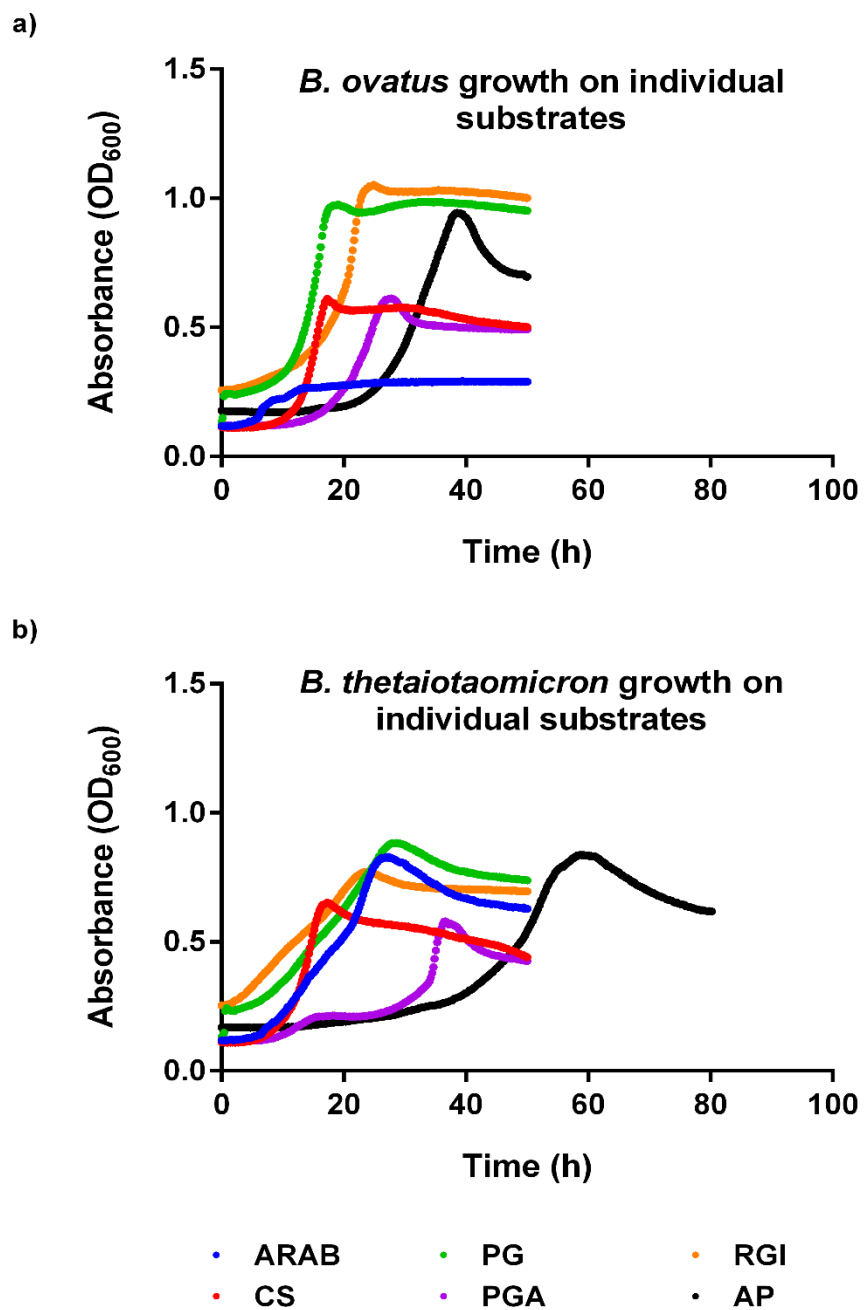


Figure 2.S2 Growth curves of a) *B. ovatus* and b) *B. thetaiotaomicron* on individual glycans used in this study (5 mg/ml solution).

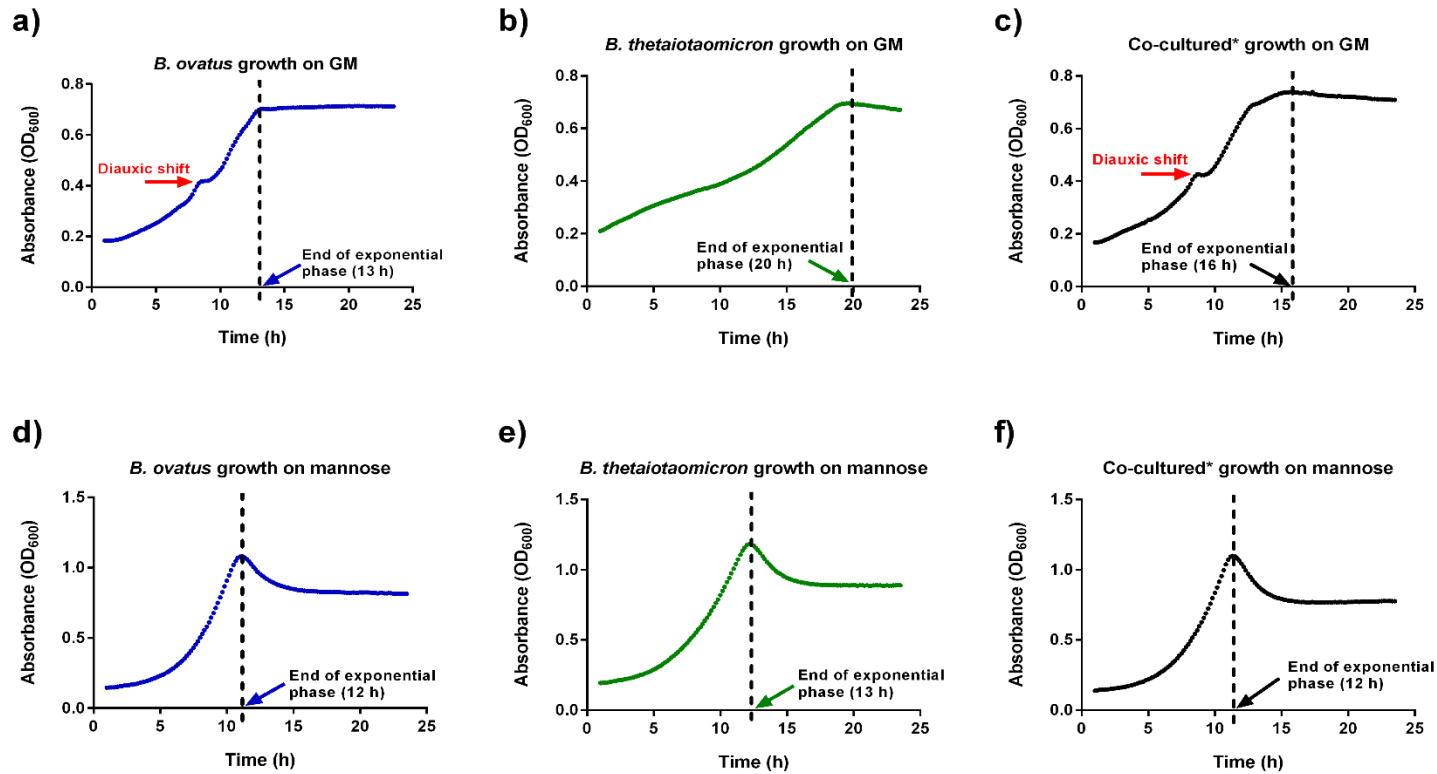


Figure 2.S3 Growth curves of bacteria. **a)** *B. ovatus* growth on mixture containing the six glycans (GM). **b)** *B. thetaiotaomicron* growth on GM. **c)** Growth curves of *B. ovatus* and *B. thetaiotaomicron* co-cultured on GM. **d)** *B. ovatus* growth on mannose. **e)** *B. thetaiotaomicron* growth on mannose. **f)** Growth curves of *B. ovatus* and *B. thetaiotaomicron* on mannose. End of exponential phase for each media is shown with a dashed line.

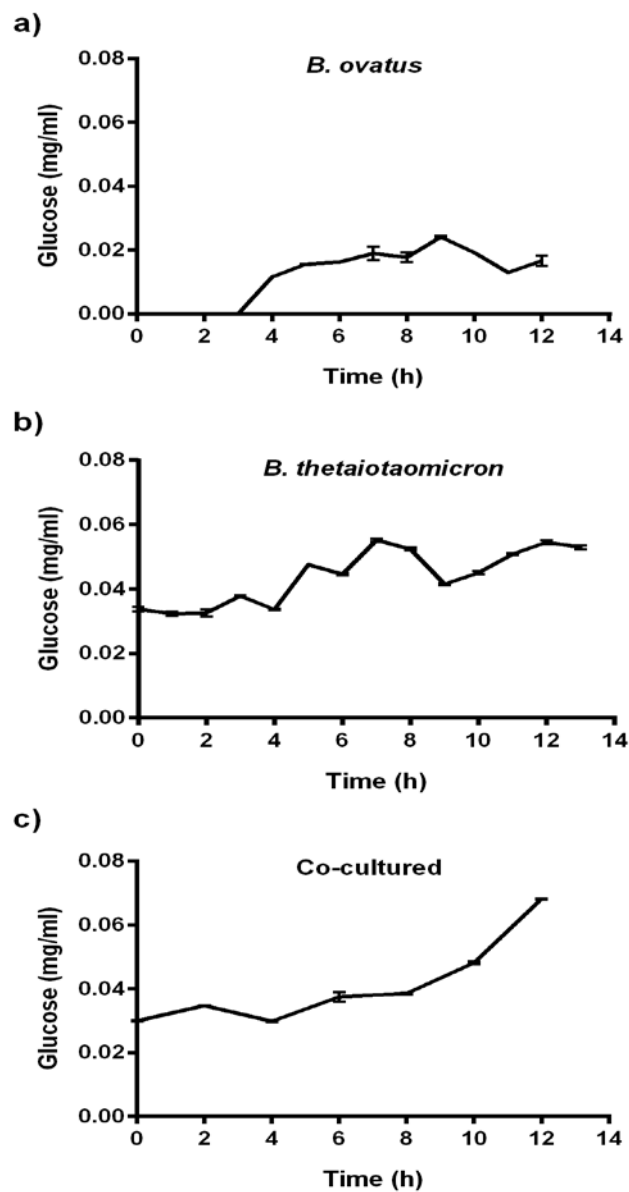


Figure 2.S4 Amount of glucose synthesized by bacteria. **a)** Amount of glucose synthesized by *B. ovatus* grown in a medium containing mannose as only carbon source. **b)** Amount of glucose synthesized by *B. thetaiotaomicron* grown in a medium containing mannose as only carbon source. **c)** Amount of glucose synthesized by *B. ovatus* and *B. thetaiotaomicron* grown together in a medium containing mannose as only carbon source. Error bars represent the standard error of the means of two separate replicates.

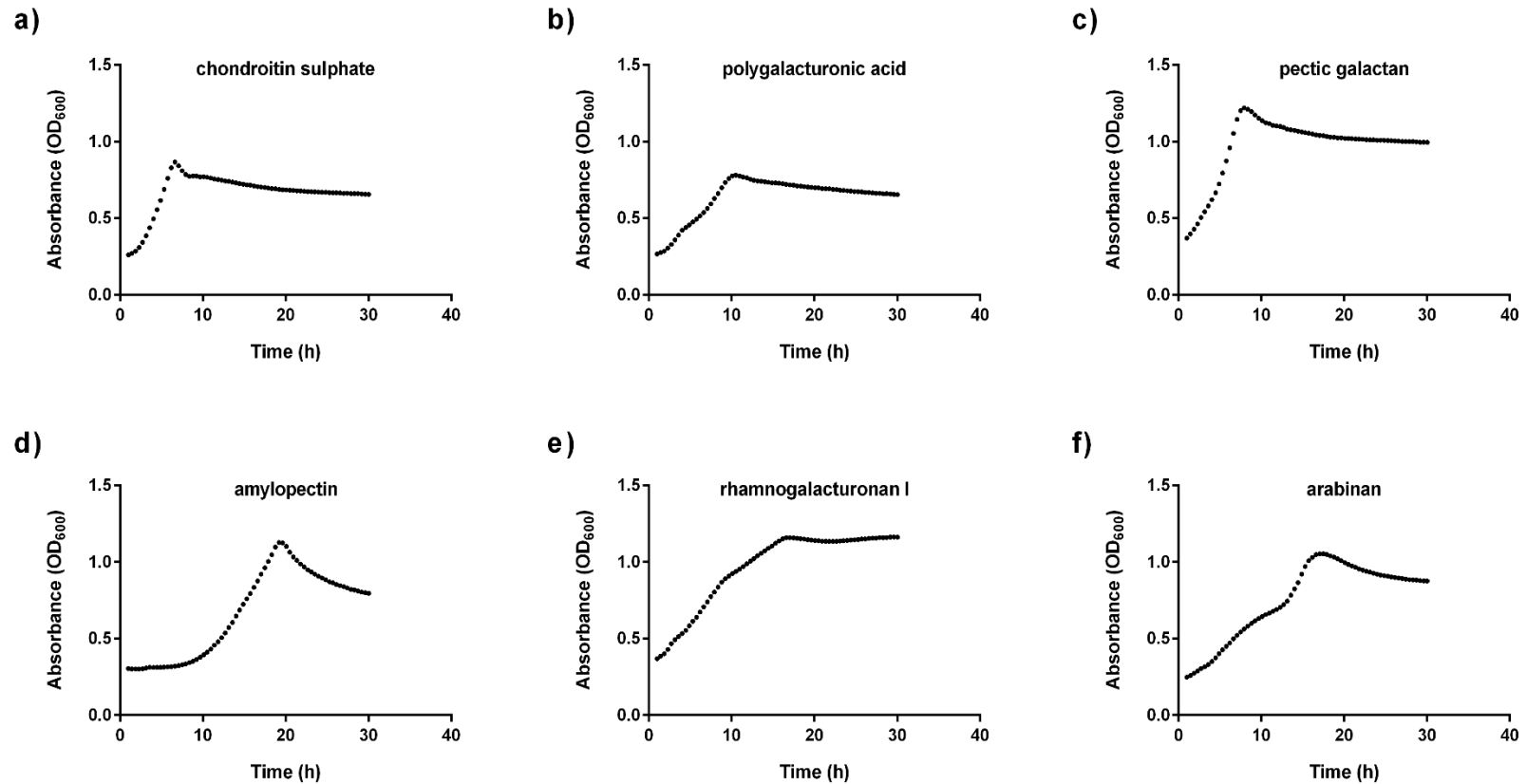


Figure 2.S5 Optical densities (OD₆₀₀) of the cultures in which *B. ovatus* and *B. thetaiotaomicron* were co-cultured on media containing **a)** chondroitin sulfate, **b)** polygalacturonic acid, **c)** pectic galactan, **d)** rhamnogalacturonan I, **e)** amylopectin, and **f)** arabinan.

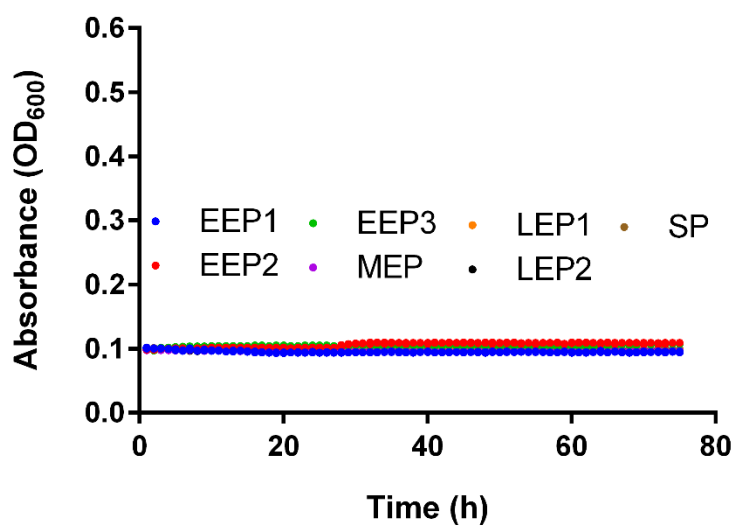


Figure 2.S6 Absorbance changes of the media harvested throughout *B. thetaiotaomicron*'s growth on ARAB over time (represents the negative control for Figure 2.6). Thick black dots in Figure 2.6a indicate the points where the media were harvested. For example, EEP1 refers to absorbance change of the media harvested at the early-exponential phase of *B. thetaiotaomicron*'s growth on ARAB.

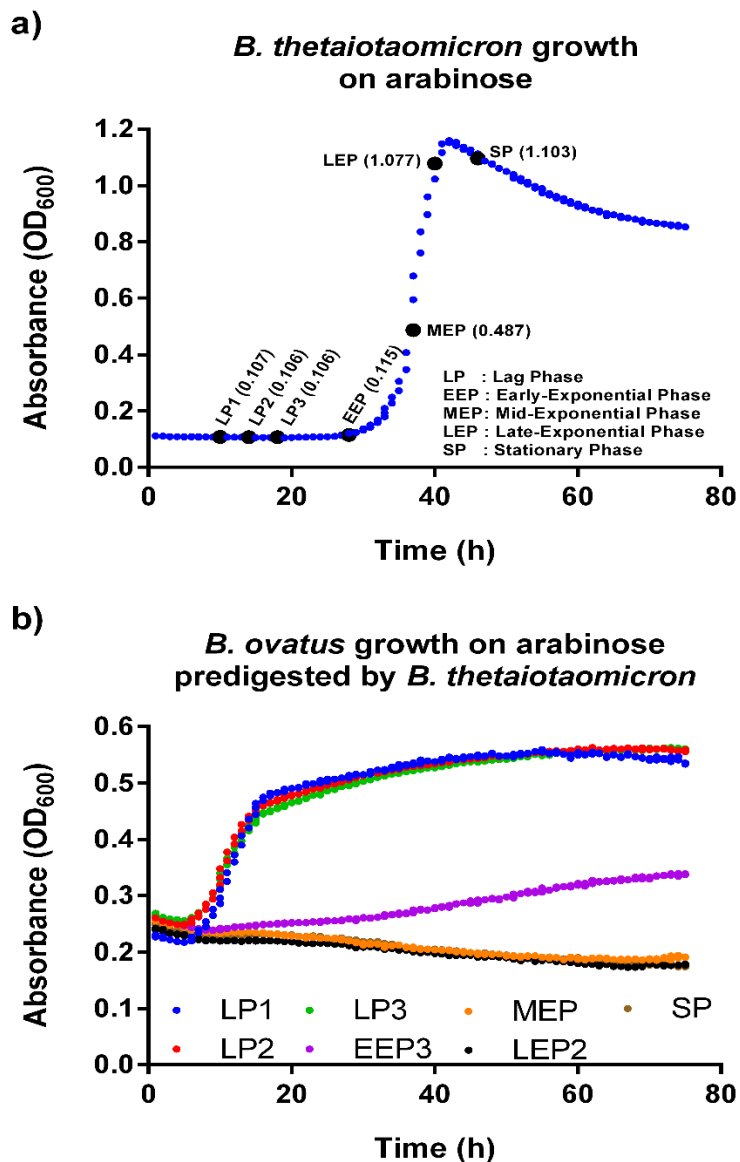


Figure 2.S7 Growth of *B. thetaiotaomicron* on arabinose and growth of *B. ovatus* on media containing arabinose predigested by *B. thetaiotaomicron*. [a) and b) represent the positive controls for Figure 2.6] a) Growth of *B. thetaiotaomicron* on media containing arabinose as the only carbon source. Thick black dots indicate the points where the media was harvested for monitoring the growth of *B. ovatus*. The numbers in the parentheses at each harvesting points refer to absorbance values.

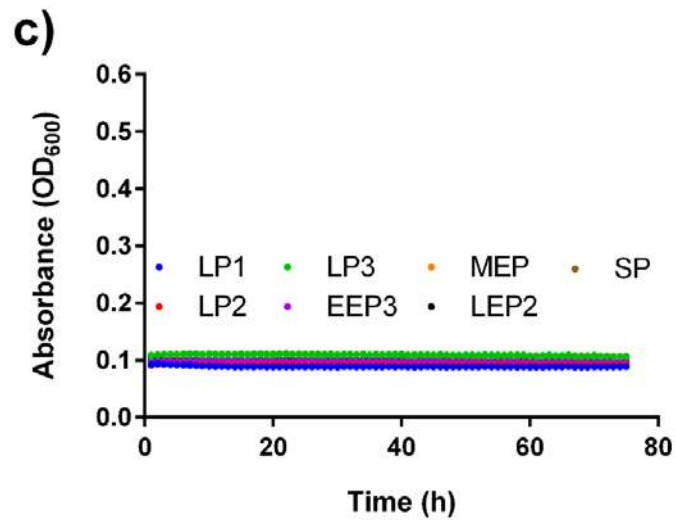


Figure 2.S7 Continued. **b)** *B. ovatus* growth on media harvested at different time points throughout *B. thetaiotaomicron*'s growth on arabinose. For example, LP1 refers to *B. ovatus* growth on media harvested at the lag phase of *B. thetaiotaomicron*'s growth on arabinose. **c)** Absorbance changes of the media harvested throughout *B. thetaiotaomicron*'s growth on arabinose over time (this represents the negative control for Figure 2.S7a).

Table 2.S1 Primers used in this study

Primer Name	Sequence (written 5' to 3')	Use
<i>B. ovatus</i>		
BACOVA01998 F	CACTCGGGATGGCAGGAATG	CS PUL expression
BACOVA01998 R	CAATGAACCGGAAGGGAAATAGC	CS PUL expression
BACOVA04911 F	CAAAGCTTTAGCCAACCAAT	PGA PUL expression
BACOVA04911 R	ACCGTCTGCACGTACTGTAA	PGA PUL expression
BACOVA05491 F	ATCCGTGTGCGTGGTGGTG	PG PUL expression
BACOVA05491 R	TTCGGGTTGATGAGGCTAAGGA	PG PUL expression
BACOVA05007 F	TCCAGCCTGTCCGACCCTACTAT	RGI PUL expression
BACOVA05007 R	CCCCAAACGTTCGGACTTACC	RGI PUL expression
BACOVA03518 F	CGTTATACCGCCTCCGTCAATC	AP PUL expression
BACOVA03518 R	ATAGCCCCGTCGTCAGCATAAC	AP PUL expression
16S F	TTTGTGGCGGGGTAACGGCC	qPCR normalization
16S R	CTTGGCTGGTTCAGGCTCTCG	qPCR normalization
BACOVA03426 F	CCTGCCAATGCGGAAGAATG	Enumeration - specific to <i>B.</i> <i>ovatus</i>
BACOVA03426 R	AGCCGCTGCAAATAACTGACAA	Enumeration - specific to <i>B.</i> <i>ovatus</i>
<i>B. thetaiotaomicron</i>		
BT3332F	TGTTCCCGGAGCCAGTGTTT	CS PUL expression
BT3332R	TTCGTCCAGCGTTTTAGTATCTTCTTT	CS PUL expression
BT4114F	CGCAACGGAAGCACTAACAGG	PGA PUL expression

Table 2.S1 Continued

BT4114R	GGGAAGCCGTCTACAATAAATAAA	PGA PUL expression
BT4671F	ATGGTTTCATCGCCCGAAGAG	PG PUL expression
BT4671R	GCGTATGGTTGAGACAGATGTAGG	PG PUL expression
BT4164F	GAAATGTAATGAATGATGCAAAAGGTAG A	RGI PUL expression
BT4164R	CGAAACGTC CGTGGAAGAAAGTA	RGI PUL expression
BT3702F	GCTATTGGCGGGGCATTGG	AP PUL expression
BT3702R	CAGCGGATTTTGGGGAGAGTTCG	AP PUL expression
BT0364F	TGAATGGCGGTAAGGTAAAAGAACA	ARAB PUL expression
BT0364R	CGGGCCGGAAGCGAGTAG	ARAB PUL expression
16S F	ATGGTATAATCAGACCGCATGG	qPCR normalization
16S R	GAGCCGTTACCTCACCAACTGC	qPCR normalization

Table 2.S2 Chopped meat broth formulation*

Components	Amount
Beef Extract	2.5 g
Pancreatic digest of casein	7.5 g
Yeast extract	1.25 g
K ₂ HPO ₄	1.25 g
L-Cysteine	250 mg
Fructose	250 mg
Galactose	250 mg
Glucose	250 mg
Mannose	250 mg
N-Acetyl Glucosamine	250 mg
Xylose	250 mg
Vitamin K ₃	250 µg
Vitamin B ₁₂	2.5 µg
Hematin/L-Histidine stock	7.6 µM/800 µM
Balch's vitamins	2.5 ml
Trace mineral solution	2.5 ml
Purine and pyrimidine solution	2.5 ml
Amino acid solution	2.5 ml

* All components were dissolved in ddH₂O and brought to 250 ml, pH 7.2. Broth was sterilized by filtration and stored at 37 °C in an anaerobic chamber under the atmospheric conditions described in the text prior to use.

Table 2.S3 Bacteroides minimal media (MM) formulation*

Constituents	Amounts
10X Bacteroides salts**	10 ml
L-Cysteine	200 mg
100 mM MgCl ₂	100 µl
0.8 % CaCl ₂	100 µl
Hematin	100 µl
Fe ₂ SO ₄	100 µl
Vitamin K ₃	100 µl
Vitamin B ₁₂	50 µl

* All components were mixed and brought to 50 ml with ddH₂O, pH 7.2. MM was sterilized by filtration and stored at 37 °C in an anaerobic chamber under the atmospheric conditions described in the text prior to use.

** 10X Bacteroides salts were formulated by dissolving potassium phosphate monobasic (136 g), sodium chloride (8.75 g), and ammonium sulfate (11.25 g) in 1 L of ddH₂O. pH was adjusted to 7.2 using concentrated HCl or NaOH. 10X Bacteroides salts were sterilized by filtration and stored at room temperature

CHAPTER 3. STRUCTURE OF A GLYCAN AFFECTS ITS PRIORITIZATION BY HUMAN COLONIC BACTERIA

3.1 Abstract

Bacteroides ovatus, a common human gut symbiont, has the ability to utilize a broad range of dietary fibers, or glycans, and utilizes them in a hierarchical order. However, the substrate factors affecting such a metabolic hierarchy remain unclear. In order to determine whether glycan structure has an impact on its rank order in the hierarchy, a mixture containing amylopectin (AP), arabinan, chondroitin sulphate, pectic galactan, polygalacturonic acid, and rhamnogalacturonan I (RGI) was used and, in another test, AP was substituted with a small-sized starch analog, maltohexaose (MH) (low molecular weight hexasaccharide contained within AP). Carbohydrate disappearance was measured as well as *B. ovatus* PUL expression. RGI was used before AP by *B. ovatus*, whereas utilization of RGI was delayed in the presence of MH. PUL expression corresponding to each glycan was consistent with these findings, indicating that the structural feature of a glycan is important in determining when it will be utilized by the bacteria. Hierarchical preference was not only observed among different glycans, but was found within the glycan molecular forms as shown in starch with *B. ovatus* utilization of amylose before amylopectin. Starch experiments also revealed that *B. ovatus* growth rate and lag time are dependent on specific structural aspects of starch, and

in particular, were significantly correlated with molecular size ($r = -0.63$, $P < 0.05$ and $r = 0.74$, $P < 0.01$, respectively). Thus, structural features of individual glycans influence both hierarchical preference and the progression of digestion of dietary fibers. This new knowledge regarding glycan substrate utilization patterns will be an important component of larger strategies that will be developed to make changes in microbiota community structures for reasons of health.

3.2 Introduction

By number, the majority of dietary carbohydrates, excluding glycemic starch and starch products, sucrose, and lactose, are not degraded and absorbed in the proximal intestine due to the lack of the genes in the human genome that secrete their corresponding enzymes for digestion. These dietary components (herein referred to as glycans) reach to the colon where they become the main energy sources for thousands of different microbes residing within (collectively known as colonic microbiota). Through fermentation, these symbionts produce short chain fatty acids that are absorbed into the body thereby contributing our daily energy requirements (McNeil, 1984), and which also stimulate the immune system (Flint et al., 2012a; Hooper et al., 2012). Disruption of this host-microbiome symbiosis - which may occur due to various reasons such as consumption of diets low in glycans (Sonnenburg et al., 2016) - has been reported to lead to formation of detrimental health outcomes such as colon cancer (Zhu et al., 2013), cardiovascular diseases (Wang et al., 2011), obesity (Ridaura et al., 2013; Zhao, 2013), and inflammatory bowel diseases (Macfarlane et al., 2009).

Glycans influence the human colonic microbiota composition (David et al., 2014; De Filippo et al., 2010; Kovatcheva-Datchary et al., 2015; Martinez et al., 2015) and selective consumption favors certain bacteria or bacterial groups in the gut (Gibson et al., 1995; Koropatkin et al., 2012). This can be attributed to the fact that glycan utilization ability of bacteria species depends on their gene content that encode carbohydrases for specific types of fiber (Sonnenburg et al., 2010). Accordingly, the idea of manipulating the gut microbiota composition as a way to improve health via diet has gained importance. Achievement of a predicted way to change the gut microbiota for purposes of health will require an advance understanding of the rules governing diet and microbiota interactions [Hamaker and Tuncil, 2014 (Chapter 1); Martens et al., 2014].

A dominant and stable component of the gut microbiota is the Bacteroidetes phylum (Eckburg et al., 2005b; Faith et al., 2013; Qin et al., 2010), of which there is particular importance attached to the genus *Bacteroides* which possess a high number of genes enabling them to utilize a broad numbers of glycans (Martens et al., 2011; Sonnenburg et al., 2010). For this reason, members such as *B. thetotaomicron* and *B. ovatus* are termed generalists (Koropatkin et al., 2012). Glycan utilization ability of these bacteria are mediated by transcription of polysaccharide utilization loci (PUL) that encode corresponding enzymes for particular glycans (Martens et al., 2008; Martens et al., 2011). A question of whether a generalist is capable of activating all (or multiple) of its PULs for degradation of different glycans presented as a mixture has become the central point of several studies (Lynch and Sonnenburg, 2012; Pudlo et al., 2015; Rogers et al., 2013; Sonnenburg et al., 2006). The generalist, *B. thetaiotaomicron*, does not activate all of its PULs; instead, it preferentially utilizes glycans in a hierarchical manner,

when they are presented as a mixture. We have also investigated hierarchical utilization of glycans in *B. ovatus* and its related expression required PULs to understand directly from carbohydrate depletion how it utilizes glycans in a mixture [arabinan (ARAB), amylopectin (AP), chondroitin sulphate (CS), pectic galactan (PG), polygalacturonic acid (PGA) and rhamnogalacturonan I (RGI) (Figures. 3.1a and 3.1b)] (Chapter 2). The mechanistic understanding why such a preference exists has been partially revealed in *B. thetaiotaomicron*, showing that glycosyl residues released from highly preferred glycans as a result of corresponding PUL activity are transferred into cytoplasm where they repress the expression of PULs targeting low priority glycans through blocking of a small intergenic region in the transcript-autonomous region (Pudlo et al., 2015). When glycosyl residues of preferred glycans are no longer available, expression of PULs targeting low priority glycans was resumed (Pudlo et al., 2015).

An unexplored detail regarding indigestible, fermentable glycans and how they are hierarchically utilized is the variation in molecular structure within glycan classes. This aspect could affect prioritization because carbohydrates can bear different structural features such as monosaccharide composition, anomeric configuration of individual saccharides, ring size, length and complexity of members and linkages of linear chains, number and kind of branch points, and physical structures (Chapter 1) (Hamaker and Tuncil, 2014). A chemical structure of a particular glycan even exhibits variation from source to source due to genotype and environment, which subsequently affects its utilization by microbiota. For instance, arabinoxylans obtained from corn bran are more complex in structure insofar as having a more complicated array of monosaccharides and linkage combinations than sorghum bran arabinoxylan related to the latter being

fermented faster *in vitro* by colonic microbiota (Rumpagaporn et al., 2015). Ease or fastness of fermentation is likely a factor that places a glycan high on the hierarchical preference list of a gut symbiont and within a glycan type it seems reasonable that structural variants would change their preference ranking.

To test this hypothesis, glycan preferences in *B. ovatus* ATCC-8483 were tested with individual different glycan molecular structures. Then, two time-course *in vitro* experiments were done in which *B. ovatus* was grown in a mixture containing above mentioned glycans, and with substitution of one of the constituent of the mixture with its counterpart having different and simpler chemical structure. *B. ovatus* response was shown to be tightly tuned to glycan molecular structure. We then showed that molecular structure of a glycan is an important factor that determines when it will be recognized and utilized by bacteria, thereby affecting its place in the hierarchy. These results provide new insight into how variation in molecular structure of a glycan affects bacterial responses to it at the strain level.

3.3 Materials and Methods

3.3.1 Glycans Used

ARAB, PG, PGA, and RGI were purchased from Megazyme (Megazyme International, Wicklow, Ireland). AP and CS were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Sources, chemical structures, sugar compositions and product codes of each glycan were reported previously (Chapter 2). Maltohexaose [product code (PC): M1024] and maltoheptaose (PC: M1025) were purchased from TCI America (TCI America Chemicals, Portland, OR). Potato, pea, and corn starches were

gifted by Roquette America, Inc. (Gurnee, IL). Rice and wheat starches were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Waxy potato and tapioca starches were obtained from a commercial source. Waxy corn starch was gifted by Tate & Lyle Inc., (Lafayette, IN). Waxy rice starch was obtained from Dr. Pinthip Rumpagaporn from the Department of Food Science and Technology, Kasetsart University (Bangkok, Thailand). Waxy wheat starch was extracted from waxy wheat varieties provided by Dr. Craig F. Morris of the Western Wheat Quality Laboratory (Ref # 07-034-0006) (Pullman, WA) as described previously (Reddy and Seib, 2000).

3.3.2 Determination of Structural Features of Starch Samples

3.3.2.1 ^1H NMR spectroscopy

^1H NMR spectroscopy was used to determine the degree of branching (DB) of waxy starch samples as previously described (Tizzotti et al., 2011) with some modifications. Starch samples (5 mg) were dissolved in 750 μl d_6 -DMSO (Sigma, PC:156914) containing 0.5 % lithium bromide (Sigma, PC: 213225). The mixture was transfer to a 5 mm NMR tube using a Pasteur glass pipet. Immediately before acquisition of the NMR spectra, 50 μl of d_1 -TFA (Sigma, PC: 152005) were added to the waxy starch samples. ^1H NMR spectra of samples were measured at 340 K on a Bruker Avance NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a TXI probe, operating at a frequency 800.13 MHz with an approximately 8 μs , 90° pulse, repetition time of 9.15 s (composed of an acquisition time of 3.15 s and a relaxation delay of 6 s)

and number of scans of 32. DB of samples were calculated using the following formula as previously described (Zhang et al., 2014)

$$DB(\%) = \frac{I_{\alpha-(1,6)}}{I_{\alpha-(1,6)} + I_{\alpha-(1,4)}}$$

[$I_{\alpha-(1,6)}$ is the integral of α -(1,6) linkages, and $I_{\alpha-(1,4)}$ is the integral of α -(1,4) linkages].

Prior to DB determination, samples collected in time course assays for DB determination were dialyzed (cutoff = 1 kDa, Spectrum Laboratories, Rancho Dominguez, CA) against purified water for at least 36 h to remove vitamins and minerals from the minimal media, followed by lyophilization.

3.3.2.2 Amylose and amylopectin content

Amylose and amylopectin contents of waxy starch samples were calorimetrically determined in triplicate using an amylose/amylopectin assay kit (Megazyme, PC: K-AMYL) according to manufacturer's instructions (Megazyme International, Wicklow, Ireland).

3.3.2.3 Molecular size distribution

Sample preparations were carried out as previously described (Zhang et al., 2006). Molecular size distribution of waxy starch samples was determined using a high-performance size exclusion chromatograph equipped with multi-laser scattering and refractive index detectors (HPSEC-MALS-RI)[a DAWN DSP-F laser photometer fitted with argon laser at $\lambda = 488$ nm with a K-5-129 flow cell (Wyatt Technology, Santa Barbara, CA) and an Optilab 903 interferometric refractometer (Wyatt Technology, Santa

Barbara, CA)], a pump (model LC-10AT vp, Shimadzu Corp., Columbia, MD), and syringe sample loading injector (model 7125, Rheodyne Inc., Catati, CA) as previously reported (Zhang et al., 2006). A dn/dc value of 0.146 was used in molecular size calculations and data processing was done using ASTRA software (Version 4.9, Wyatt Technology, Santa Barbara, CA).

3.3.3 Growth of Bacteria Strains, and Growth Curves on Pure Glycans

B. ovatus ATCC 8483 strain was used in all experiments. *B. ovatus* was pre-grown in chopped meat broth at 37 °C in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) under a 85 % N₂, 5 % CO₂, and 10 % H₂ atmosphere for all experiments. Growth curves were performed in 200 µl cultures in a 96-well plate using a Powerwace HT absorbance reader coupled with a Biostack automated plate handling device (Biotek Instruments, Winooski, VT) under the conditions described previously (Rogers et al., 2013). *B. ovatus* was tested in eight separate carbohydrate arrays for each glycan. Data processing was handled using Gen5 software (Biotek) and Graphpad Prism® version 6 software (Graphpad Software, Inc, La Jolla, CA).

3.3.4 Growth in Glycan Mixture and Sample Collection in Time-Course Assay

B. ovatus was grown to mid-exponential phase (OD₆₀₀ 0.5 – 0.8) in minimal media (MM) containing glucose, followed by washing in MM-no carbon prior to exposure to glycan mixtures. The bacteria cells were then delivered into glycan mixtures as previously described (final total glycan concentration was 5 mg/ml) (Rogers et al., 2013). Two aliquots were sampled every hour during *B. ovatus*' growth on glycan

mixture; one of which was for measuring the remaining glycans in the media (2 ml), and the other for monitoring PUL expression over time relative to time 0 (1 ml). Growth and expression studies were conducted in the laboratory of collaborator Dr. Eric Martens, University of Michigan Medical School, Ann Arbor.

3.3.5 Measuring the Remaining Glycans

Collected samples (0.5 ml) were lyophilized for glycan analysis. Neutral monosaccharides found in these samples were determined as their alditol acetate derivatives (Pettolino et al., 2012) using gas chromatography coupled with mass spectrometry (7890A and 5975C inert MSD with a Triple-Axis detector, Agilent Technologies, Inc., Santa Clara, CA) (GC/MS). Prior to preparing trimethylsilyl (TMS) derivatives, the remaining 1.5 ml of collected samples for glycan analysis were dialyzed (cutoff = 1 kDa, Spectrum Laboratories, Rancho Dominguez, CA) against purified water for at least 36 h. This was because preliminary analysis showed that presence of vitamins and minerals that come from minimal media, as well as compounds possibly produced by bacteria species during growth on the glycan mixture, interfere with TMS derivatization and gas chromatography analysis (data not shown). Dialyzed samples were then lyophilized. TMS derivatives of these samples were prepared as described previously (Doco et al., 2001) and analyzed on a gas chromatograph with a DB-5 capillary column (Agilent Technologies, Santa Clara, CA) to determine the acidic monosaccharide and rhamnose contents of the samples on a weight basis. Even though the method of TMS derivatives of sugars is suitable for simultaneous determination of neutral and acidic sugars, this was not used because maltohexaose used in one of the time-course assays has

a molecular weight of 942 Da (Table 3.1) and it would be removed during dialysis. Glycosyl-linkage profiles of the dialyzed samples were carried out as their partially methylated alditol acetate derivatives using GC/MS (Pettolino et al., 2012).

Over the time course, the disappearance AP (or MH) and PG percentages relative to their initial amounts were calculated by quantifying the glucose and galactose amounts in the samples as their alditol acetates, respectively. Similarly, the remaining ARAB amount was quantified by measuring the remaining 5-arabinose linkage over time. Residual PGA amount was determined by subtracting total rhamnose from total galacturonic acid amount calculated as their TMS derivatives. Remaining CS amounts were determined by tracking the remaining glucuronic acid amounts as their TMS derivatives. These residues are signatures of each corresponding glycan and the measured glycosyl residues were used to calculate the amount remaining of the corresponding glycans.

3.3.6 Transcript Analysis of PUL Genes by quantitative PCR (qPCR)

PUL expression profiles were carried out as previously described (Rogers et al., 2013). Primers used in this study were given in Table 2.S1.

3.4 Results

In glycan depletion experiments, *B. ovatus* preferentially utilized glycans in a hierarchical manner, when grown in a mixture containing amylopectin, arabinan, chondroitin sulphate, pectic galactan, polygalacturonic acid and rhamnogalacturonan I (Figure 3.1a) (Chapter 1). This concurs with transcriptional analysis showing hierarchical

preference of glycans in *B. thetotaomicron* (Martens et al., 2008; Pudlo et al., 2015; Rogers et al., 2013; Sonnenburg et al., 2006). In order to test whether such a preference is a glycan structure dependent or not, the previous hierarchy study was done with substitution of only one of the glycans in the mixture with a change to a simpler molecular structure, but within the same class of compounds. Starch amylopectin was chosen because there are many different types of starches, even commercially available, which bear different structural features (Buleon et al., 1998; Hoover, 2001; Tester et al., 2004). The comparator was maltohexose, a short α -1.4-glucose-linked linear chain which can itself be found in small quantity within the amylopectin molecule and is representative of longer linear chains found in abundance. Responses were then monitored to wide variety of starches to understand how structural variants affect their utilization.

Also, in the design of the second study on starch variants, cooked, gelatinized starches were used, even though it is known that such starches are generally digested in the small intestine and do not reach the colon. This was because of the analytical technique used to measure bacterial growth requires that the substrate be soluble. Regardless of this seeming drawback, the study provided important information on how bacterial responses alter when the structure of starch changes. Additionally, it should be noted that raw starches do either partially or fully enter the colon as resistant starch and contain a similar range of molecular structures though in a granular form. Such structure-bacterial function information is translatable to discovering and understanding the degradation and utilization activities of glycan substrates in the colon (Anderson and Salyers, 1989a, b; Cameron et al., 2014; Koropatkin et al., 2008; Reeves et al., 1996).

3.4.1 Growth Rate of *B. ovatus* on Starch is Significantly Correlated to its Molecular Size

In order to understand how variations in the starch structure affect growth of *B. ovatus*, 13 different starch samples bearing different structural features (5 waxy varieties, 6 normal varieties, and 2 starch analogs) were used (Table 3.1). Waxy varieties tested were from corn (2 different), wheat, potato, and rice. Normal starches were from corn, wheat, potato, rice, tapioca, and pea. Starch analogs were maltoheptaose (7 glucose units connected via α -1,4 linkages) and maltohexaose (MH) (6 glucose units connected via α -1,4 linkages). The rationale for our selection of MH as the smallest molecule is based on an early report done on another *Bacteroides* member, *B. thetaiotaomicron*, revealing that the starch utilization system (SUS) (series of protein complexes required for glycan utilization by the *Bacteroides* members, as explained in more detail below) can be dispensable during growth on starch polymers shorter than 6 glucose units (Koropatkin et al., 2008). Responses of *B. ovatus* to incubation on the different starches provided growth parameters, growth rate and maximum culture density (Table 3.1).

Variations in the structure of starches significantly affected growth parameters of *B. ovatus* ($P < 0.01$) (Table 3.1), generally revealing highest growth rate on the starch analogs followed by normal varieties of starch. Growth of *B. ovatus* on maltoheptaose and MH were four and two times faster than on the target native amylopectin, respectively ($P < 0.01$); though the maximum cell densities on the starch analogs were lower than on amylopectin. Correlation analysis between structural features of the starch samples and *B. ovatus* growth parameters showed a negative correlation between starch

molecular size and *B. ovatus* growth rate ($r=-0.63$, $P=0.022$) and positive correlation with lag time ($r=0.74$, $P=0.004$). No significant correlations were observed between maximum density and any of the structural features ($P < 0.05$), and similarly growth rate was not correlated with degree of branching or amylose/amylopectin content. Thus, *B. ovatus* responds and grows better on starches of smaller molecular size. Maltohexose was chosen as the substituting molecule for amylopectin in the further hierarchical studies described below.

3.4.2 Starch Structure Affects its Prioritization by *B. ovatus* in a Glycan Mixture

To investigate the effect of glycan structure on its prioritization in a glycan mixture (GM), *B. ovatus* was grown on a mixture containing amylopectin (AP), arabinan (ARAB), chondroitin sulphate (CS), pectic galactan (PG), polygalacturonic acid (PGA), and rhamnogalacturonan I (RGI); and in another experiment AP was substituted with MH. Growth was monitored anaerobically (Figure 3.2). Media and bacterial cells were collected every hour during the exponential growth phase on the glycan mixtures and measurements were made on remaining glycans (glycan depletion) and expression of PULs targeting each glycan.

Consistent with our hypothesis, *B. ovatus* revealed different growth patterns on AP and MH-containing GMs with the differences observed in the second half of the growth period (Figure 3.2; dashed line for growth on MH containing GM and solid line for growth on AP containing GM). Diauxic growth was observed, which is defined as a metabolic shift between the most- and least-preferred glycans, in which the early growth phase was prolonged by substituting AP with MH. This suggests that one of the least

preferred glycans other than AP, perhaps RGI or ARAB, is less utilized in the glycan mixture and, in turn, is responsible for the formation of diauxic growth pattern.

Growth of *B. ovatus* on GM containing MH compared to GM containing AP revealed almost identical degradation patterns for highly preferred glycans, CS, PGA, and PG, showing that utilization orders of the most-preferred glycans were not altered (Figure 3a). The simpler MH degradation occurred earlier than AP, while RGI degradation in MH-containing GM was later. Substituting AP with MH delayed the utilization of RGI by *B. ovatus*, clearly showing a shift in hierarchical order due to structural complexity. These data were consistent with a delay in PUL expression of *B. ovatus*' PULs expression of RGI when MH was present (Figure 3.3b), while PULs targeting CS, PGA, and PG showed similar expression and repression patterns in both glycan mixtures. The PUL for starch (*BACOVA03518*) was expressed 3 h earlier when MH was substituted for AP. Likewise, the expression of the RGI targeting gene (*BACOVA05007*) was delayed when MH was present from a gradual increase starting at 3 h after incubation and reached its maximum level at 6 h after incubation, to a sudden increase in its expression between 6 and 7 h. At this later time, the expression of *BACOVA03518* was shut down after incubation in the GM containing MH. These data clearly show that glycan structure is an important factor in determining its prioritization by bacteria species.

When *B. ovatus* was grown in the GM containing AP, a pause in the growth curve was observed between 4.5 and 5.5 h of incubation (Figure 3.2, solid line) at which time the expression of *BACOVA05007* started to increase (Figure 3.3b, solid line). Actual depletion of RGI started after growth was resumed (Figure 3.3a, solid line). On the other hand, when *B. ovatus* was grown in the GM containing MH, prolonged early growth were

observed and the pause in growth was observed later between 5.5 and 7.5 h (Figure 3.2, dashed line). At this time, expression of *BACOVA05007* began to increase (Figure 3.3b, dashed line) and the depletion in RGI amount started at this point when the growth was resumed (Figure 3.3a, dashed line). RGI was, therefore, responsible for the formation of the diauxic shift.

3.4.3 *B. ovatus* Preferentially Utilizes Amylose Over Amylopectin

Within the starch class there are two principal molecules, amylose and amylopectin, and *B. ovatus* was found to first prefer the simpler amylose structure (linear) and then the more complex amylopectin structure (highly branched). This was seen in diauxic growth patterns on normal starch (containing both amylose and amylopectin, and not waxy starch (only amylopectin) (Figure 3.S2). *B. ovatus* was grown on two of the six normal starch varieties tested earlier with normal corn and wheat starches as only carbon sources. Media were collected at different time points (Figures 3.4a and 3.4c) and changes in the degree of branching of starches were monitored using ^1H NMR. As amylose was prioritized, an increase was observed in the degree of branching. In both starches, there were large increases in degree of branching in early growth time points (first two columns in Figure 3.4b and 3.4d). These increases continued until the pause points in the exponential growth curves were observed [OD_{600} of 0.467 for normal corn starch (Figure 3.4b) and OD_{600} of 0.555 for normal wheat starch (Figure 3.4d)], showing that *B. ovatus* catabolized amylose at early stages growth phases, rather than amylopectin. After the exponential growth phase resumed, there were significant decreases observed in the degree of branching of the starches [between OD_{600}

of 0.467 and 0.585 for normal corn starch (Figure 3.4b), and between OD₆₀₀ of 0.555 and 0.572 for normal wheat starch (Figure 3.4d)], suggesting that amylopectin, in both cases, was starting to be utilized by *B. ovatus* after the diauxic shift. It seems that in the time interval when the pauses were observed, *B. ovatus* was preparing itself for degradation of α -1,6 linkages in amylopectin. The decrease in the degree of branching of the residual starch was not constant, and increase in its values were reinitiated after particular time points [after OD₆₀₀ of 0.585 for normal corn starch (Figure 3.4b) and after OD₆₀₀ of 0.572 for normal wheat starch (Figure 3.4d)]. It could be that *B. ovatus* firstly attacked branch points (α -1,6 linkages) of amylopectin and released linear chains, which were then rapidly utilized by *B. ovatus*.

3.5 Discussion

Bacteroides species residing in our colon have the ability to utilize many complex glycans. The acquisition of these nutrients by these symbionts are facilitated by a series of protein complexes composed of digestive enzymes, binding proteins, transporters, and regulators (Martens et al., 2009a). These are universal among *Bacteroidetes* (Koropatkin et al., 2012; Martens et al., 2009a), and are called Sus-like systems after the starch utilization system (Sus) discovered in *B. thetaiotaomicron* (Anderson and Salyers, 1989a, b; Cho and Salyers, 2001; D'Elia and Salyers, 1996; Martens et al., 2009a; Tancula et al., 1992). Sus-like systems are encoded by a cluster of genes at a polysaccharide utilization loci (PUL). The *Bacteroides* members possess a broad range of PULs, each of which is specific to its analogous glycan (Koropatkin et al., 2012). When multiple glycans are present, *Bacteroides* (*B. theta* and *B. ovatus*) do not activate all of the corresponding

PULs simultaneously; instead it prioritizes the utilization of some before others (Figure 3.1) (Chapter 2) (Martens et al., 2008; Pudlo et al., 2015; Rogers et al., 2013; Sonnenburg et al., 2006). Even though the logic why such a metabolic hierarchy exists is not known, one possible explanation for this phenomenon might be that the most-preferred glycans are more easily accessible and degradable compared to the least-preferred glycans, because bacteria are programmed to obtain maximum benefits from the metabolic pathways by spending minimum energy costs (Chubukov et al., 2012). It is not also known whether there are external factors affecting this metabolic hierarchy or bacteria are just simply programmed to utilize the glycans in such an order.

Our current findings demonstrate that structural features of a glycan are an important determinant for its rank order in the hierarchy. When *B. ovatus* was grown on the glycan mixture, it was previously shown to prioritize the constituent glycans as shown in Figure 1 (Chapter 2). This was confirmed in the present study (Figure 3.3). By substituting AP with its simpler and smaller linear analog, MH, its utilization ranking also changed (Figure 3.3a) and corresponded to an earlier expression of its PUL. Expression of the RGI PUL was accordingly shifted backward (Figure 3.3b). Presumably the greater accessibility and degradability to MH than to RG1 by *B. ovatus* caused the change in prioritization. We conclude that when multiple glycans are present, prioritization order of a particular glycan by a bacteria species may be manipulated by substituting it with a counterpart having different structural features.

In a more detailed manner, specific structural aspects of starch were found to affect *B. ovatus* growth. Growth rate and lag phase on a range of starches were significantly correlated with molecular size ($r = -0.63$, $P = 0.022$ and $r = 0.74$, $P = 0.004$,

respectively), with *B. ovatus* generally growing faster on lower molecular size starches (Table 3.1). Perhaps *B. ovatus* is able to catabolize lower molecular size starches faster than higher molecular size starches due to the nature of its SUS. This might be due to different affinities of starch binding proteins found on its cell surface to various structural features of starch. A recent survey in which the growing ability of wild type *B. thetaiotaomicron* and a mutant *B. thetaiotaomicron* deficient in Sus-E, -F, and -G binding proteins (Δ SusEFGsurf*) on starches with different molecular size were compared and revealed that while the Δ SusEFGsurf* strain had severe growth defects on starches with high molecular size, it was more adept at growth on that with lower molecular size (Cameron et al., 2014). This suggests that utilization of high molecular size starches requires more protein activities, which may slow growth. Since the starch utilization PUL is homologous between *B. ovatus* and *B. thetaiotaomicron* (Martens et al., 2011) and both have common enzymatic mechanisms for starch degradation (Degnan et al., 1997), it seems reasonable to exist also in *B. ovatus*.

In addition to preferring to utilize some glycans before others, *B. ovatus* also conferred prioritization to amylose before amylopectin (Figure 3.4). Better enzyme activities of *B. ovatus* starch degrading enzymes on low molecular size starches, as discussed above, might be the reason [amylose is a much smaller molecule having a molecular size range of 160 – 490,000 Da versus amylopectin with a molecular size range of 50×10^6 to 500×10^6 Da (Zobel, 1988)]. A second possibility is that *B. ovatus* starch binding proteins may bind amylose stronger than amylopectin, thus causing it to prioritize amylose. The Sus-D binding protein of *B. thetaiotamicron* specifically recognizes α -helical structure of amylose molecules suggesting that affinity of Sus-D to

amylose could be stronger than amylopectin (Koropatkin et al., 2008). As stated above, a previous study revealed that glycan prioritization by bacteria species occurs because the monosaccharide units of the most-preferred glycans repress the transcription of the genes responsible for catabolism of the least-preferred glycans by blocking the small intergenic region in a transcript-autonomous region (Pudlo et al., 2015). The finding here, however, suggests that besides monosaccharide composition, there are other structural aspects of glycans including their linkage profile that affect prioritization.

Our findings indicate that chemical structure of a glycan is an important factor that governs the glycan-microbiota relationships and specifically prioritization of use. This work highlights that structural features of glycans need to be taken into account to complement knowledge gained from genomic and metabolic information for more accurate prediction of fiber action on bacteria in the colon. This adds further knowledge to the formulation of a predictive strategy of choosing dietary fibers to promote bacteria that have been based on identifying the gene content of individual strains that encode carbohydrate-active enzymes (CAZymes) for the degradation of given glycan(s) (El Kaoutari et al., 2013; Larsbrink et al., 2014; Sonnenburg et al., 2010). Further work on dietary fiber utilization and competition of microbiota will bring us closer to developing strategies for intelligent manipulation of the gut microbiota through dietary intervention for improved health.

Table 3.1 Structural features of starches used, and growth parameters of *B. ovatus* on these starches ¹

Substrates	Structural Features of Starch Samples				Growth of <i>B. ovatus</i> ²	
	Degree of Branching	Molecular Size	Amylose (%)	Amylopectin (%)	Growth Rate (OD ₆₀₀ /h) ³	Density (OD ₆₀₀ max) ⁴
Waxy Rice	4.37	4.49 x 10 ⁸	0.46 d	99.54 a	0.0295 ef	0.9113 e
Waxy Wheat	4.17	1.64 X 10 ⁸	0.80 d	99.20 a	0.0212 gh	0.7923 gf
Waxy Potato	2.88	3.26 x 10 ⁸	1.72 d	98.28 a	0.0340 ed	0.9043 e
Waxy Corn	4.13	4.54 x 10 ⁸	1.32 d	98.68 a	0.0152 i	0.6050 h
Amylopectin from corn	4.49	1.07 x 10 ⁸	0.80 d	99.20 a	0.0353 d	1.2802 a
Normal Rice	2.99	1.46 x 10 ⁸	11.78 c	88.22 b	0.0241 gh	0.8958 e
Normal Tapioca	2.66	4.42 x 10 ⁸	12.23 c	87.77 b	0.0381 d	0.9204 e
Normal Corn	2.66	4.50 x 10 ⁸	22.19 ab	77.81 cd	0.0257 gf	0.8236 f
Normal Potato	2.59	1.68 x 10 ⁸	13.75 c	86.25 b	0.0543 c	1.0443 b
Normal Wheat	2.80	3.95 x 10 ⁸	19.47 b	80.53 c	0.0206 h	0.7697 g
Normal Pea	2.44	1.32 x 10 ⁸	23.43 ab	76.57 cd	0.0501 c	1.0048 c
Maltohexaose	nd	9.90 x 10 ² [§]	nd	nd	0.0666 b	0.8953 e
Maltoheptaose	nd	1.15 x 10 ³ [§]	nd	nd	0.1437 a	0.9668 d
LSD⁵	nd	nd	2.9526	2.9526	0.0046	0.0344

¹ Samples with the same letter on the same column are not significantly different (P < 0.01). nd: not determined.

² Actual growth curves of *B. ovatus* on each substrate are given in Figure 3.S2.

³ Growth rates were calculated with following formula: [(OD_{max} - OD_{min}) / (T_{max} - T_{min})]; where, OD_{max} is the point at which OD₆₀₀ reached its maximum; OD_{min} is the minimum OD₆₀₀ measured; T_{max} and T_{min} are the corresponding time values for each absorbance (Figure 3.S3).

⁴ Density were calculated with the following formula: (OD_{max} - OD_{min}) (Figure 3.S3).

⁵ LSD: Least Significant Difference. [§] Molecular sizes of maltohexaose and maltoheptaose are provided by the manufacturer.

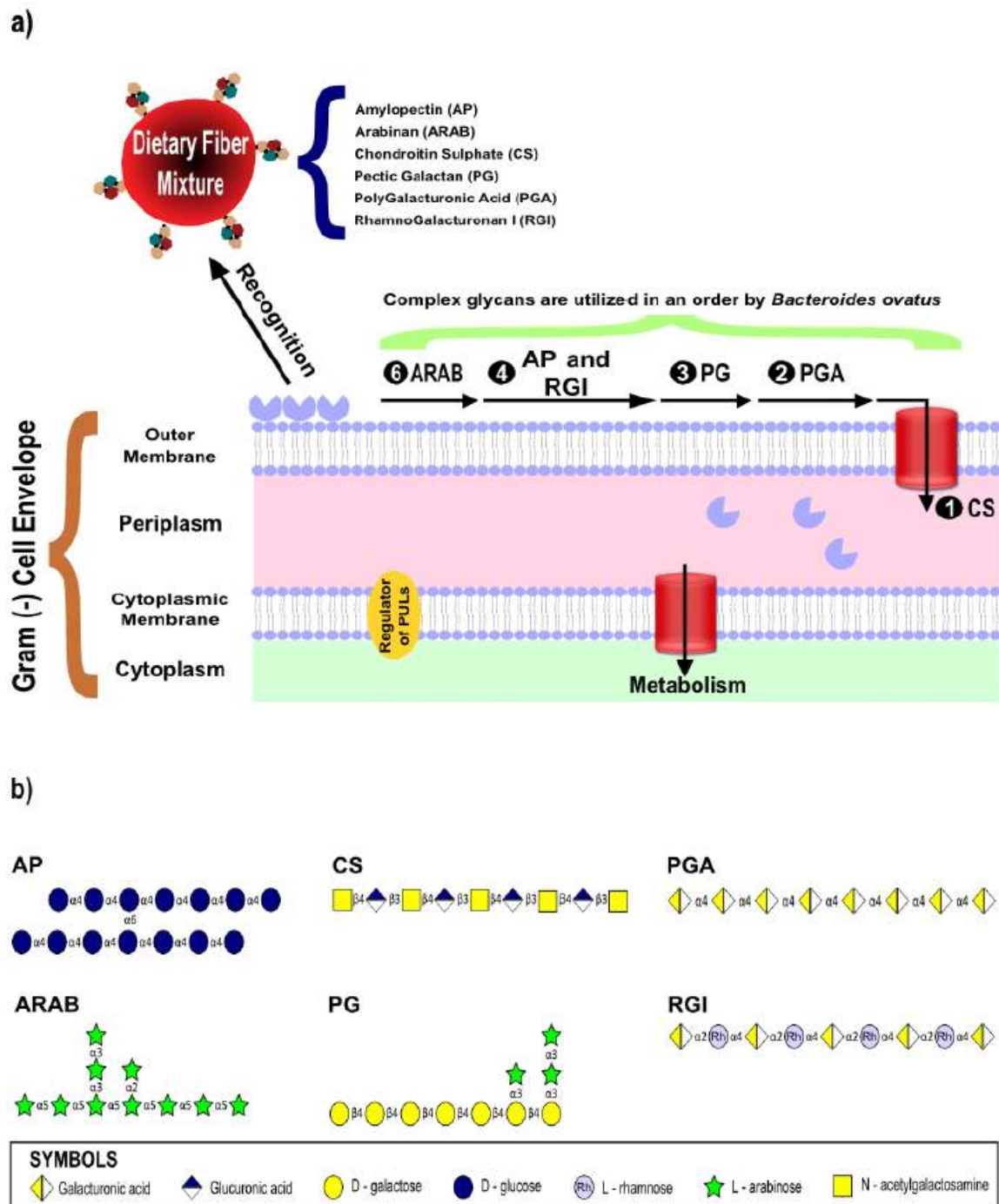


Figure 3.1 a) Utilization of glycans by *B. ovatus*, when multiple glycans are present together. Illustration is made based on our previous report (Chapter 2), b) Chemical structures of glycans used (monosaccharide composition of each glycan are given in Figure 3.S1).

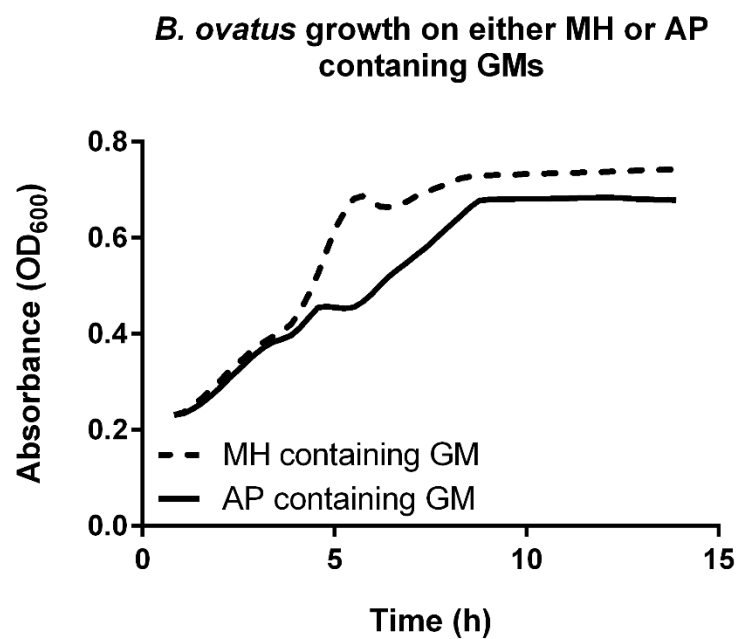


Figure 3.2 Growth curves of *B. ovatus* grown on the AP-containing GM (solid line) and MH-containing GM (dashed lines). Abbreviations: **AP**, amylopectin; **GM**, glycan mixture; **MH**, maltohexaose.

a)

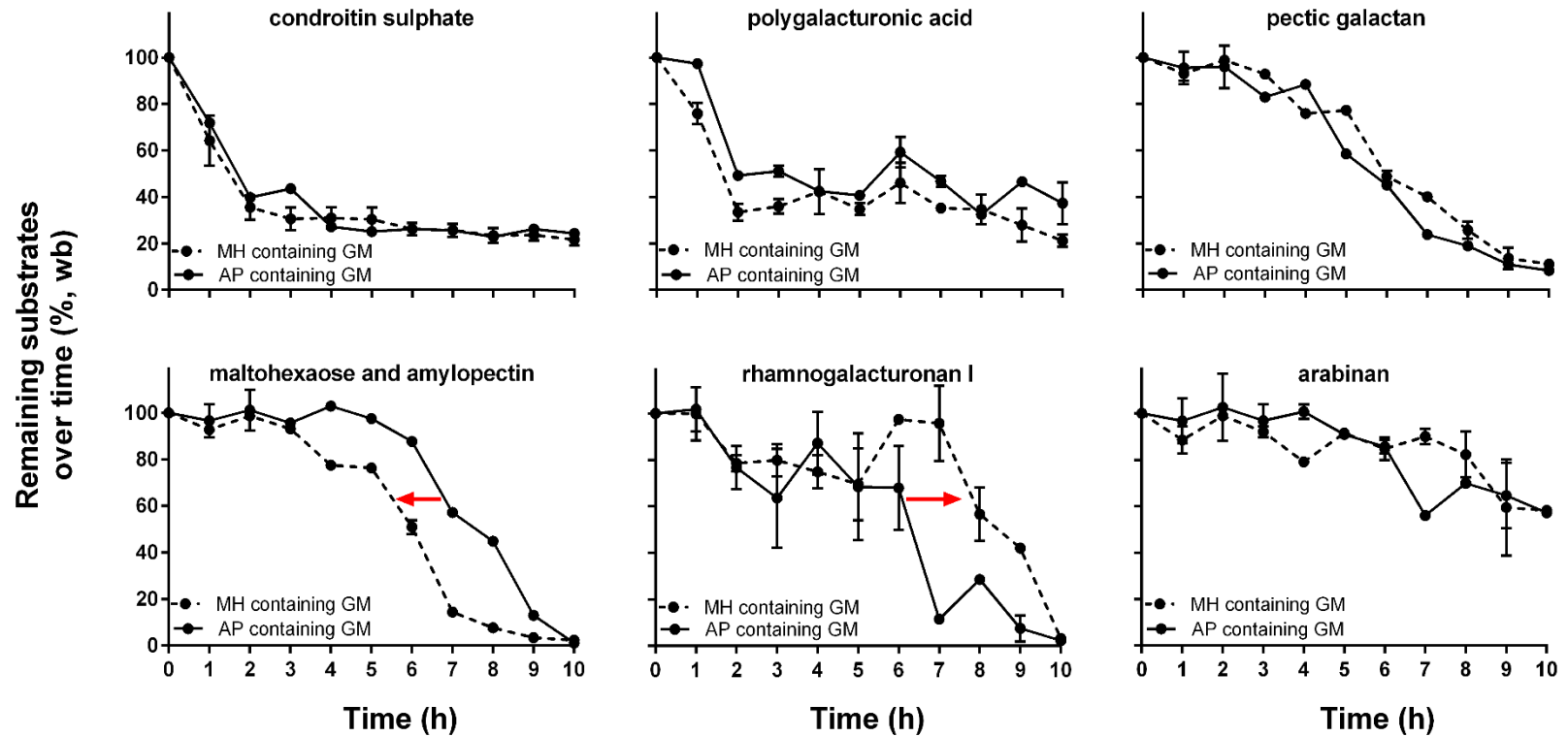


Figure 3.3 Glycan utilization and corresponding PUL expression by *B. ovatus* grown on on AP containing GM (solid lines) and MH containing GM (dashed lines). **a)** Substrate depletion, **b)** temporal expressions of PULs. All analyses were performed using samples collected throughout exponential phase of each bacterium (Figure 2).

b)

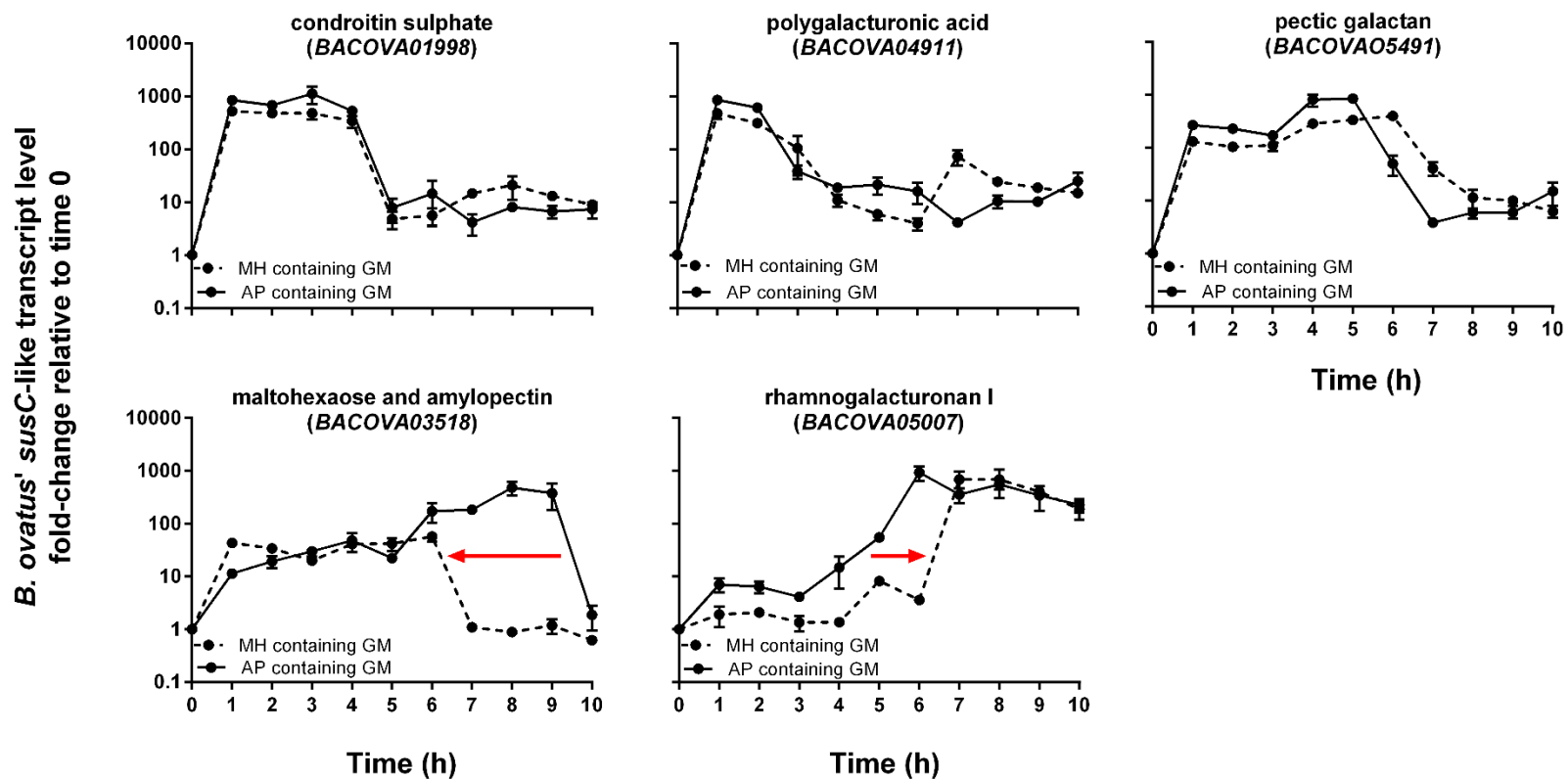


Figure 3.3 Continued. Lines showing transcript level changes are described with locus tag numbers of corresponding genes. All transcript changes are shown relative to time 0. Error bars represent the standard error of the mean of three separate replicates. *The arabinan utilization PUL of *B. ovatus* is not known. Abbreviations: **AP**, amylopectin; **GM**, glycan mixture; **MH**, maltohexaose; **PULs**, polysaccharide utilization loci.

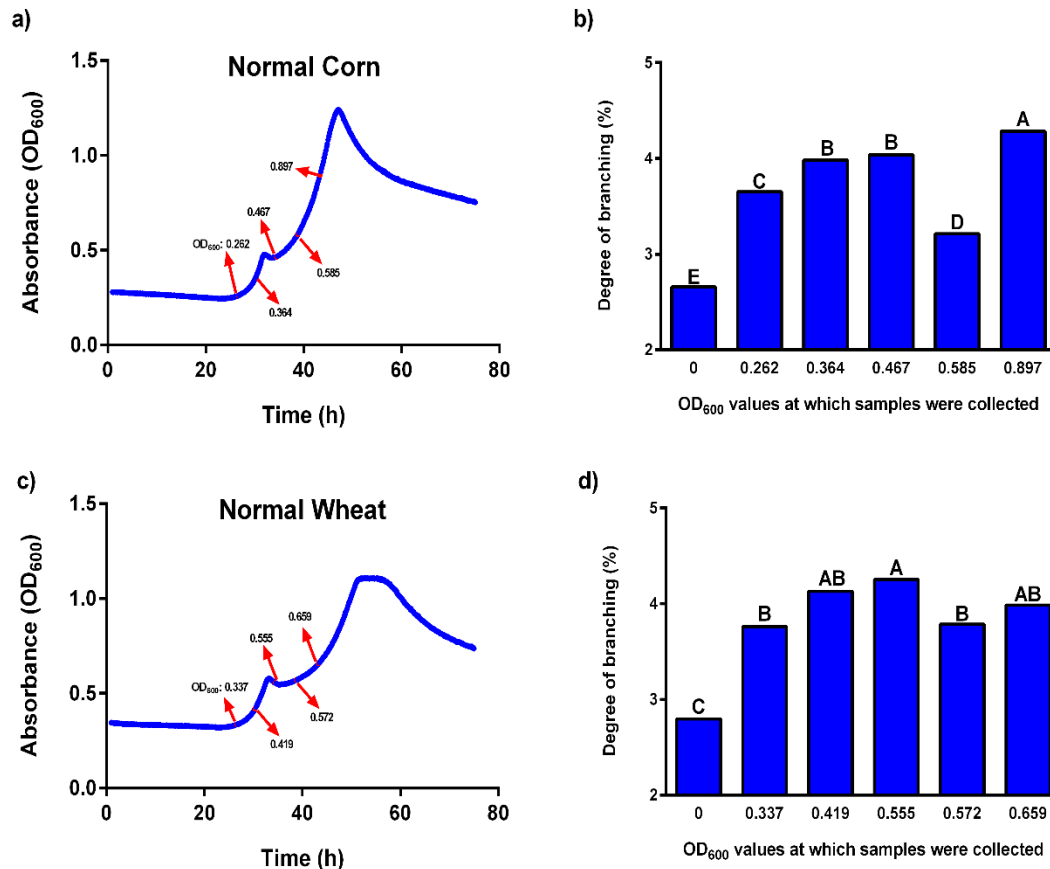


Figure 3.4 Changes in the degree of branching of starches over time during which they are utilized by *B. ovatus*. Growth curves of *B. ovatus* on **a)** normal corn starch and **b)** changes in the degree of branching of corn starch over time. Growth curves of *B. ovatus* on **c)** normal wheat starch and **d)** changes in the degree of branching of wheat starch over time. Red arrows represent the points at which media were collected for determination the degree of branching using ^1H NMR. Pairwise comparisons of the means were performed using least significance difference (LSD) test at the significance level (α) of 0.05 ($n = 2$). Bars with the same letter are not significantly significant.

3.6 Supplementary Information

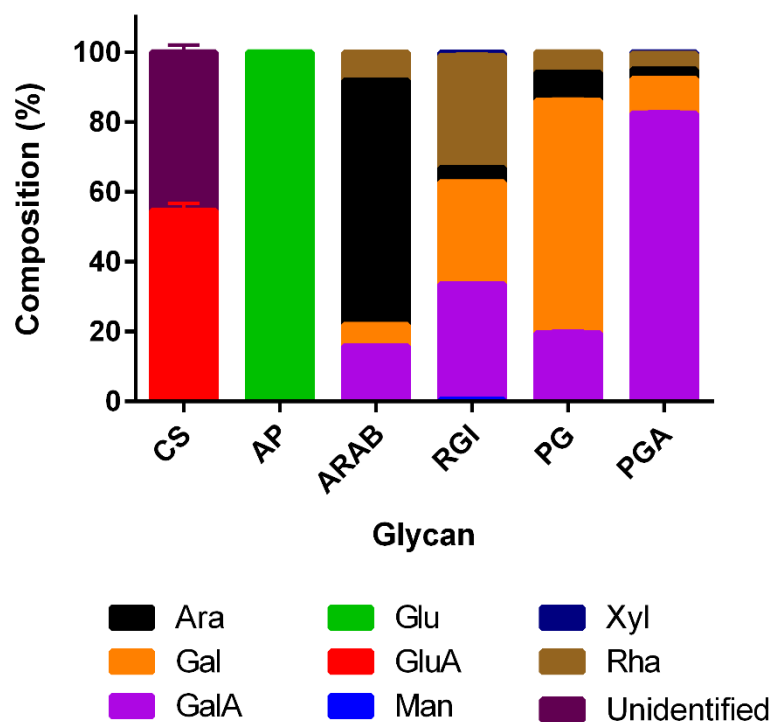


Figure 3.S1 Monosaccharide composition of glycans used in this study as their trimethylsilyl derivatives. Error bars represent the standard error of the means of two separate replicates. Abbreviations: **Ara**, arabinose; **Gal**, galactose; **GalA**, galacturonic acid; **Glu**, glucose; **GluA**, glucuronic acid; **Man**, mannose; **Rha**, rhamnose; **Xyl**, xylose.

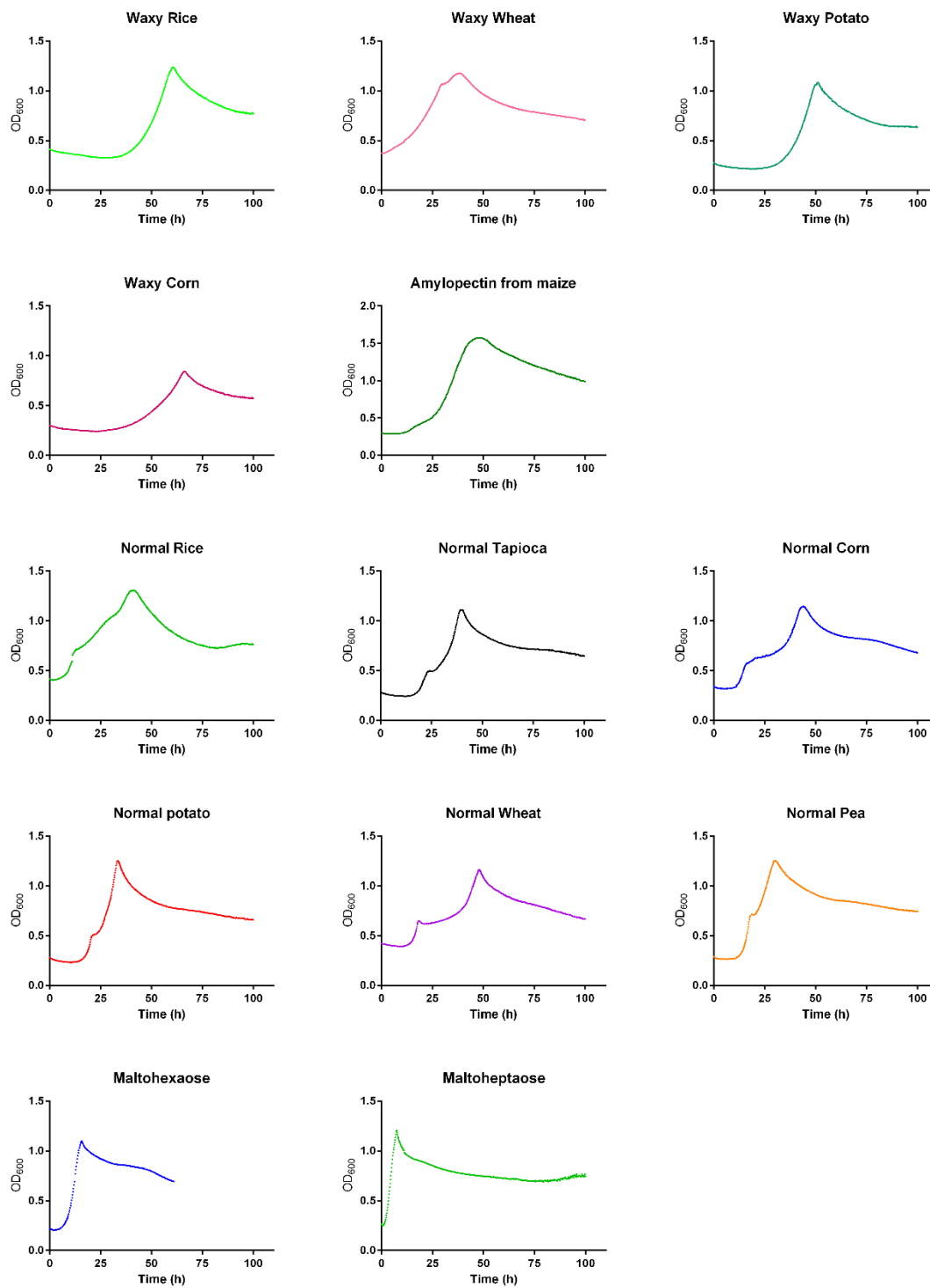
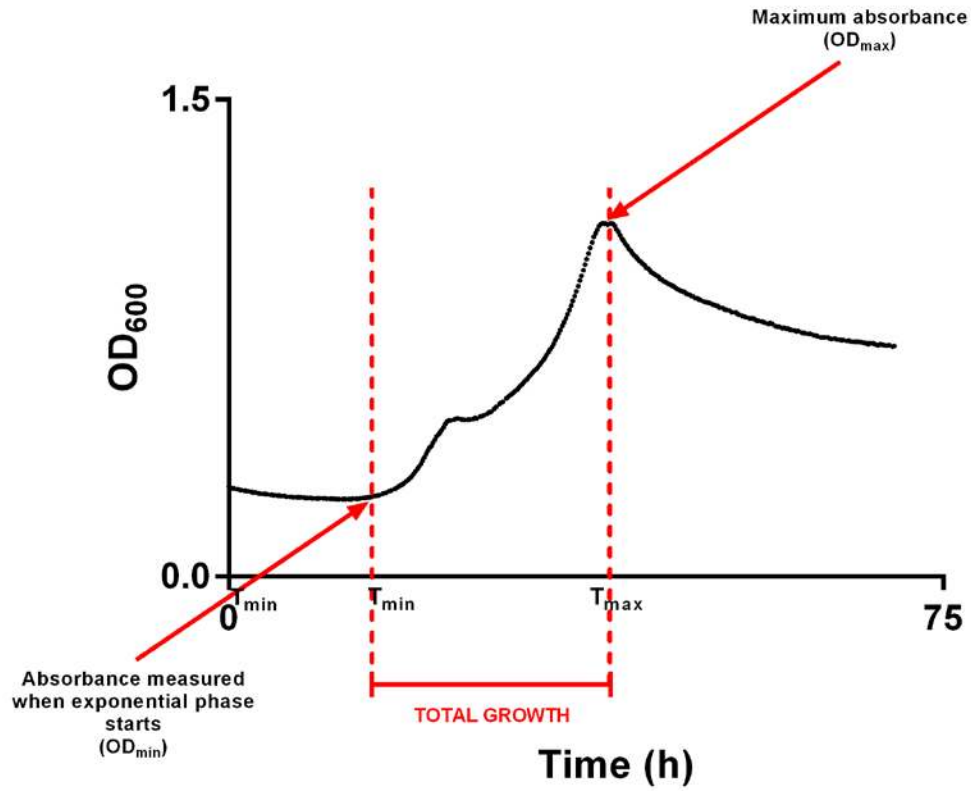


Figure 3.S2 Growth curves of *B. ovatus* grown on different starches



$$\text{Total Growth Rate} = \frac{(OD_{max} - OD_{min})}{(T_{max} - T_{min})}$$

$$OD_{max} \text{ (maximum cell density)} = OD_{max} - OD_{min}$$

$$\text{Lag time} = T_{min} - T_0$$

Figure 3.S3 Calculation of the growth parameters (cell density and growth rate) of *B. ovatus*.

CHAPTER 4. DEGRADATION OF SOME DIETARY FIBERS BY HUMAN GUT MICROBIOTA IS DELAYED WHEN PRESENTED IN A MIXTURE

4.1 Abstract

Designing strategies for delivering dietary fibers into distal colon is considered important to prevent the formation or treat colonic diseases such as cancer and ulcerative colitis. Here, we hypothesized that degradation of certain dietary fibers by the colonic microbiota will be delayed when they are presented in a mixture due to hierarchical utilization of other fibers first. Such action would provide prolonged fermentation and short chain fatty acid (SCFA) production throughout the colon. To test the hypothesis, an *in vitro* fermentation study was done individually using fecal microbiota obtained from three healthy donors using single dietary fibers [arabinoxylan, chondroitin sulphate (CS), galactomnan (GM), polygalacturonic acid (PGA), xyloglucan (XG)] and a mixture containing an equal amount of each of the substrates. Degradation of CS, PGA, and XG were delayed when present in the mixture. At the end of the fermentation period, in all donor groups, >30% of CS and PGA remained unfermented in the mix, while >95% of these dietary fibers were utilized when tested singly. Two donors microbiota had significantly higher butyrate levels when incubated with most of the plant-based fibers. 16S rRNA gene sequencing analysis showed that the different SCFA responses of donors

were due to their different fecal microbiota compositions. This supports our past work showing that the amount of SCFA produced from a particular dietary fiber is determined by the fecal microbiota compositions of individual. Mixing different types of dietary fibers might be a logical strategy for delivering particular dietary fibers into distal part of the colon for improved health. Inter-individual differences in gut microbiota compositions may cause different dietary fiber effects.

4.2 Introduction

The human gut microbiota, comprised of thousands of different microbial strains, is physiologically important for health and acts like functional organ of human body. In case of symbiosis, colonic bacteria have beneficial health effects such as production of short chain fatty acids (SCFAs) that provide energy to the epithelium (Vinolo et al., 2011), prevention of colonization of pathogenic bacteria (Clarke et al., 2010), aid in digestion and absorption of food ingredients (Maslowski and Mackay, 2011), and stimulation of the immune system (Flint., 2012a; Hooper et al., 2012). In the case of dysbiosis, they can conversely have detrimental effects on human health such as production of carcinogens (Zhu et al., 2013) and intestinal putrefaction (Macfarlane and Macfarlane, 2012), and be a causative factor for cardiovascular disease (Wang et al., 2011), obesity (Ridaura et al., 2013), and inflammatory bowel diseases (Macfarlane et al., 2009). Therefore, it is important to maintain the colonic microbiota composition in balance for improved health.

Diet, especially dietary fibers, have been shown to be an important tool that can keep colonic microbiota composition in balance and provide beneficial health outcomes

(Sonnenburg et al., 2016). Moreover, selective consumption of dietary fibers determines which microbial groups are favored in the gut and influence the balance of species in the colon (Koropatkin et al., 2012). This is because dietary fibers differ in their chemical structures and types of linkages, and utilization ability of gut microorganisms depends on their gene content that encode carbohydrases and binding proteins for specific types of fiber. Accordingly, many investigations, both *in vitro* and *in vivo*, have been conducted to identify the novel dietary fibers that specifically modulate the gut microbiota composition by promoting a certain species (i.e. bifidobacteria, lactobacillus), and maximize the SCFA formation and health improvements (Benjamin et al., 2011; Damaskos and Kolios, 2008; Davis et al., 2010; Harmsen et al., 2002a; Hughes et al., 2008; Martinez et al., 2010; Theuwissen et al., 2009; Van den Abbeele et al., 2011a; Worthley et al., 2009; Yang et al., 2013). Most of these studies were conducted using only one type of dietary fiber.

There is some interest in understanding how to deliver fermentable dietary fibers to the distal region of the colon due to a high incidence there of certain diseases such as ulcerative colitis, an inflammatory bowel disease, and colorectal cancer (Bufill, 1990; Cummings, 1997; Rose et al., 2007). Slow colonic fermentation is a natural property of intact plant cell wall fibers, such as found in cereal brans, but soluble fibers rarely ferment slowly and generally are used up in the proximal colon. In the distal colon, oftentimes fermentation of undigested proteins occurs, which results in a production of branched chain fatty acids (BCFA) as well as toxic compounds such as ammonia and sulfur-containing compounds (Szmulowicz and Hull, 2011). Increasing the dietary fiber availability for microorganisms residing in the distal colon promotes beneficial

saccharolytic bacteria (Pompei et al., 2008) and depresses the activity of the proteolytic bacteria, resulting in a decrease in target disease biomarkers. Therefore, one goal of functional fiber development is to identify strategies that deliver the dietary fibers into more distal part of the colon.

Studies using single bacteria strains reveal that when multiple dietary fibers are present together, they prefer to utilize some before others, indicating that degradation of lower prioritized dietary fibers by bacteria species are delayed when they are available along with highly preferred ones. (Lynch and Sonnenburg, 2012; Pudlo et al., 2015; Rogers et al., 2013) (Chapters 2 and 3 of this thesis). We hypothesize here that there exists a hierarchical preference also in the general microbiota that can be measured by individual carbohydrate and, when multiple glycans are present, degradation of some of them are delayed and prolonged saccharolytic activity can be obtained more distally into the colon.

To test our hypothesis, we designed a series of *in vitro* fermentation studies using fecal microbiota obtained from three healthy individuals to compare the degradation profiles of single dietary fibers (AX, CS, GM, PGA, or XG) and then in a mixture containing equal amount (weight basis) of each of them. This might be a logical strategy for delivering particular dietary fibers into distal part of the colon so that the fermentative activity can be stimulated throughout the colon, and beneficial bacteria as well as more SCFA production in the distal colon can be promoted.

4.3 Materials and Methods

4.3.1 Dietary Fibers Used

Polygalacturonic acid from citrus pectin (PGA) [Product Code (PC): P-PGACT], galactomannan from carob (GM) (PC: P-GALML), and xyloglucan from tamarind (XG) (PC: P-XYGLN) were purchased from Megazyme (Megazyme International, Wicklow, Ireland). Fructooligosaccharide from chicory (FOS) (PC: F8052) (used as a positive fast fermenting comparator as discussed detail below) and chondroitin sulphate from bovine trachea (CS) (PC: C9819) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Arabinoxylan (AX) was extracted from sorghum bran (obtained by decorticating sorghum grain, from the Purdue Agronomy Center for Research and Education, using an abrasive dehuller as follows. Bran was defatted by treating with hexane [bran:hexane=1:7 (w/v)] twice for 30 min each, and air dried for overnight. Starch and protein removal from the defatted bran were performed using an enzymatic process (Maes and Delcour, 2002). Alkaline-hydrogen peroxide extraction of AX was then carried out as previously reported (Doner and Hicks, 1997) with the modifications (Rose et al., 2010; Rumpagaporn et al., 2015). For further purification, AX was treated with a simulated upper gastrointestinal digestion procedure described by Lebet et al (Lebet et al., 1998a) and with modification (Rose et al., 2010). These glycans were chosen to be used in this study because each has unique monosaccharide moieties in their structures (Figure 4.S1) that allowed for tracking of their disappearance during bacteria utilization and growth.

The mixture used in this study refers to the mixture of equal amounts (weight basis) of AX, CS, GM, PGA, and XG. Monosaccharide composition of the glycans used in the fermentation study are given in Figure 4.S2.

4.3.2 *In-vitro* Fermentation

In vitro fermentation studies were performed as previously described (Lebet et al., 1998b) with the following modifications to the anaerobic chamber under an 85 % N₂, 5 % CO₂, and 10 % H₂ atmosphere. Carbonate-phosphate buffer was prepared and sterilized by autoclaving at 121 °C for 20 min. The buffer was then cooled down to room temperature, oxygen removed by bubbling with carbon dioxide, and cysteine hydrochloride (0.25 g/L of buffer) was added. The buffer was then immediately placed into the anaerobic chamber overnight. Glycans (50 mg) were weighted into test tubes for each time point (0, 2, 4, 6, 8, 10, 12, and 24 h), and the tubes were then transferred into the anaerobic chamber. All analyses were performed in triplicate.

The next day, 4 ml of carbonate-phosphate buffer was added on each tube. Fecal samples were collected from 3 healthy donors who were consuming their routine diets and had not taken antibiotics for at least 3 months. The fecal samples were tightly sealed in plastic tubes, kept on ice and immediately transferred into the anaerobic chamber, and used within 2 h of collection. Fecal slurries were obtained by homogenizing the fecal samples with carbonate-phosphate buffer [feces:buffer 1:3 (w/v)], followed by filtration through 4 layers of cheese cloth. Filtered fecal slurry (1 ml) was then inoculated in each tube. The tubes were then immediately closed with rubber stoppers, sealed, and incubated

at 37°C in a shaking water bath (150 rpm). Test tubes containing no substrate were used as blanks at each time point.

4.3.3 Total Gas Production and pH Change Measurements, and Sample Collection for SCFA, Carbohydrate Depletion and DNA Analysis

At each time point, total gas production in each tube was measured with a graduated syringe by passing the needle through the rubber stopper (Figure 4.S3), and the tubes were then opened. Two different aliquots were immediately collected from each tube for DNA isolation (1 ml) and SCFA analysis (0.4 ml). Samples collected for DNA isolation were immediately stored at -80°C until further analysis. Samples collected for SCFA analysis were immediately combined with 100 µl of an internal standard mixture [internal standard mixture was prepared by combining 157.5 µl of 4-methylvaleric acid, 1.47 ml of 85% phosphoric acid, 39 mg of copper sulfate pentahydrate. Then the final volume of this mixture was brought to 25 ml with purified water]. The samples were then stored at -80°C until further analysis.

The remaining samples were used for determining the pH values (Figure 4.S4) and then placed in boiling water for 60 min to inactivate the microorganisms followed by storage at - 80 °C for further analysis of glycan disappearance at each time point.

4.3.4 SCFA Analysis

The frozen samples for SCFA measurements were thawed at room temperature and centrifuged at 13,000 rpm for 10 min. Supernatants (4 µl) were injected into a gas chromatograph equipped with a fused silica capillary column (Nukon™, Supelco No:

40369-03A, Bellefonte, PA) and a flame ionization detector (GC-FID 7890A, Agilent Technologies, Inc., Santa Clara, CA) under the following conditions: injector temperature, 230 °C; initial oven temperature, 100 °C; temperature program, 8 C/min to 200 °C with hold for 3 min at final temperature. Helium was used as the carrier gas at 0.75 ml/min. A short chain fatty acid standard mix (Supelco, Bellefonte, PA) was used as the external standards. Quantification was carried out by measuring the peak areas for acetate, propionate, and butyrate relative to 4-methylvaleric acid.

4.3.5 Measuring the Glycan Disappearance

Samples collected for the measurement of the glycan disappearance were cleaned before derivatization for gas chromatography analysis, because our preliminary analysis showed that the compounds found in the carbonate-phosphate buffer and/or the metabolites produced by bacteria inhibit the derivatization of the glycans for gas chromatography analysis (data not shown). For this purpose, samples were first treated with a simulated upper gastrointestinal digestion procedure as described above followed by dialysis (1000 Da cutoff) and lyophilization. Neutral and acidic monosaccharides of the samples were determined on a weight basis as their trimethylsilyl (TMS) derivatives using a gas chromatography coupled with mass spectroscopy (GC-MS) on a DB-5 capillary column (Doco et al., 2001).

Throughout the fermentation studies, the remaining AX, CS, GM, PGA, and XG percentages relative to their initial amounts were calculated by measuring the arabinose, glucuronic acid, mannose, galacturonic acid and glucose amounts in the samples. Each of

the targeted monosaccharides are unique to each of the corresponding glycans (Figure 4.S1).

FOS was included in this study as a positive fast fermenting comparator. Therefore, FOS disappearance was not measured during the *in vitro* fermentation experiments.

Monosaccharide content was also measured in the fecal slurry blank at time 0 (before addition of substrates) as well as throughout the fermentation studies (Figure 4.S5).

4.3.6 DNA Extraction

DNA extractions from the samples were carried out using FastDNA SPIN® kit for feces (PC: 116570200) (MP Biomedical, Santa Ana, USA) according to the manufacturer's instructions with the following modifications. Frozen, stored samples for DNA extraction were thawed, centrifuged at 13,000 rpm for 10 min, and the supernatants were discarded. The pellets were homogenized with phosphate buffer and transferred into the lysing matrix E tube. Manufacturer's instructions were then followed.

4.3.7 16S rRNA Gene Sequencing

The V4 region of the 16S rRNA gene was amplified for each sample as previously described (Kozich et al., 2013). Sequencing was performed on the Illumina MiSeq platform using a MiSeq Reagent Kit V2 500 cycles (PC: MS-102-2003) according to the manufacturer's instructions (Illumina, Inc. San Diego, CA). Analyses were

performed at the Center for Microbial Systems at University of Michigan Medical School (Ann Arbor, MI).

4.3.8 Bioinformatics

Illumina-generated sequencing data were analyzed using Qiime version 1.9.1 as previously described (Caporaso et al., 2010). After confirming reproducibility, treatment replicate sequence runs were combined into a single file for each sample (biological replicates were not combined). Quality filtering of sequencing data were done as previously described and data were rarefied to the lowest number of reads (Bokulich et al., 2013). OTUs were selected using the “pick open otus” script in QIIME and the Greengenes database (version 13_8) with a 97 % similarity threshold using UCLUST (Edgar, 2010). α -Diversity of the samples was measured by Shannon’s index. β -Diversity measures were calculated using UniFrac on rarefied OTU tables (Lozupone and Knight, 2005). Three dimensional PCoA plots of the β -diversity measures were visualized using Emperor (Vazquez-Baeza et al., 2013).

4.4 Results and Discussions

This study asked the question whether degradation of dietary fibers can be delayed when they are presented as a mixture. It was conducted using microbiota obtained from different individuals to account for inter-individual differences in composition (Lozupone et al., 2012), which can significantly affect dietary fiber degradation profiles (Conlon and Bird, 2015), as well as the type and amount of the SCFA produced (Bourriaud et al., 2005)

4.4.1 Microbiota Composition of Fecal Samples Before Fermentation

Taxonomy based comparisons of the initial fecal microbiota compositions of the three donors at phylum level showed all to be Firmicutes dominant, although differences were apparent particularly in levels of Bacteroidetes and Actinobacteria (Figure 4.1a). There were eight different phyla detected in the fecal sample of Donor 1 (Euryarchaeota, Actinobacteria, Bacteroidetes, Firmicutes, Lentisphaerae, Proteobacteria, Tenericutes, Verrucomicrobiota), while two of these phyla, Euryarchaeota (belonging to the kingdom of Archaea) and Lentisphaerae were absent in Donors 2 and 3 (Figure 4.1a). The relative abundance of Proteobacteria, Tenericutes, and Verrucimicrobiota in Donors 2 and 3 were less than 1%; whereas in donor 1, they were 7.6, 1.1., and 2.9%, respectively. The relative abundance of Actinobacteria in Donors 2 and 3 was much higher (17.1 and 9.2%, respectively) than that in Donor 1 (2.4%).

More than 85 % of the fecal microbiota composition of the Donor 1 was comprised of members of 20 different genera (Figure 4.1b). In Donors 2 and 3, four of these genera (*Succinivibrio* from the Proteobacteria phylum, *Oscillospira* from the Firmucutes phylum, and *Metahnobrevibacter* and *Methanosphaera* from the Euryarchaeota phylum) were absent, and the remaining 16 genera comprised more than 85% of the total population. Obvious differences in the abundances of shared genera among donors were observed. For example, relative abundance of the members of *Bifidobacterium* in the Donor 2 was about 8 and 2 times higher than in Donors 1 and 3, respectively. The bacteria belonging to this genus is physiologically important for host health since they produce significant amount of lactate from dietary fiber fermentation,

that is then converted into butyrate by butyrogenic bacteria including *Anaerostipes caccae*, *Eubacterium hallii*, and the members of Clostridium cluster XIVa group (Belenguer et al., 2006; Bourriaud et al., 2005; Duncan et al., 2004). Similarly, the relative abundance of the family of Lachnospiraceae in Donors 2 and 3 was almost 2 times higher than that in Donor 1. Several members of this family including *Eubacterium* spp., *Butyrivibrio* spp., *Roseburia* spp., and *Anaerostipes* spp. are known as butyrate producers (Rajilic-Stojanovic and de Vos, 2014). Conversely, the fecal microbiota of the Donor 1 had relatively higher proportions of the members of *Prevotella* and *Bacteroides* genera. Some members of these genera are well recognized for their ability to utilize a broad range of dietary fibers (Flint et al., 2012a; Koropatkin et al., 2012; Martens et al., 2011). Because of these differences in the fecal microbial compositions of the donors, it was not unexpected that their responses to the dietary fibers were different.

4.4.2 Delay of Degradation of Some Dietary Fibers When in a Mixture

The dietary fibers AX, CS, GM, PGA, XG were chosen because they have monosaccharide units unique to each of their structures (Figure 4.S1) which allowed for tracking of their disappearance during the time course experiments. Depletion of dietary fibers in the media containing the mixture of the fibers (Figure 4.2; solid lines) were then compared with single dietary fibers (Figure 4.2; dashed lines).

Degradation profiles of individual dietary fibers by fecal microbiota were affected by the presence of other types of dietary fibers, with delay in some of them when presented in a mixture (Figure 4.2). For example, CS was effectively utilized by the colonic bacteria when it was singly present, though in the fiber mixture, its degradation

rate was slowed down. Moreover, CS in the mixture was not utilized as effectively as when presented singly in the media. In Donor 1, for example, at 12 h, the percentage of remaining CS in the media was less than 5% when presented singly as the only carbon source, whereas at the same time point more than 30% of CS in the mixture remained unfermented. That there was no further change in CS after this time point This might be attributed to the fact that after 12 h time points, the pH of the media containing mix as a carbon source dropped below 5.9 (Figure 4.S4), where majority of the bacteria may not show fermentative activity on dietary fibers; thus, no more CS degradation was observed after this point in the mix. Similar trend was also observed in the media containing the fecal microbiota of donor 2 or 3, but the CS amount in the mix inoculated with any of these fecal samples reached plateau earlier (after 6 h), where the pH of the media dropped below 6.0 (Figure 4.S4).

Similar to CS, PGA degradation by fecal microbiota, in all cases, was rapid when it was present singly in the medium with the majority of the PGA utilized within the first 4 h (76% in media containing fecal microbiota of donor 1, 94% for donor 2, and 88% for donor 3 were depleted within 4 h). In the mixture, however, PGA degradation rate was slowed down, and it was not utilized as effectively. Overall, in the mixture, at the end of the fermentation period, almost 40% of PGA remained unfermented.

XG in the mixture was degraded by the fecal microbiota obtained from Donors 1 and 3 at a slower rate than when presented singly. In Donor 2; the degradation rate of XG presented singly in the media was similar to that in the mixture. Probably Donor 2 microbiota was better adapted for degradation of XG, so that the presence of alternative dietary fibers does not affect its degradation.

Contrary to CS, PGA, and XG, degradation rate of AX when presented in the mixture (Figure 4.2; solid lines in top three graphs) was faster than when presented singly (Figure 4.2; dashed lines in top three graphs). Similar to AX, GM degradation by the fecal microbiota was very rapid. Degradation rates of GM in the mixture were either equal to (Donor 2) or faster (Donors 1 and 3) than when presented singly. AX and GM were among the most preferred dietary fibers.

Thus, we conclude that degradation of some of the less preferred dietary fibers by fecal microbiota is delayed when they are present as a mixture. This might be a reasonable strategy for slowing fermentation of particular dietary fibers and delivering them into the distal part of the colon, and accordingly to increase fermentation and SCFA production throughout the colon.

Degradation profiles of dietary fibers were donor dependent. Most notable was that the fecal microbiota of Donor 1 degraded the given dietary fibers slower than those of Donors 2 and 3. For example, when AX was the sole carbon source (singly present in the media), its utilization by the microbiota obtained from Donors 2 and 3 was very rapid and more than 90% was degraded within 6 h of incubation. For Donor 1, degradation of AX occurred more slowly with more than 50% still present at 6 h (Figure 4.2; dashed lines in top three graphs). Differences in degradation profiles of AX are related to the microbial composition of the donors. Compared to Donor 1, the presence of a higher proportion of *Bifidobacterium* in Donor 2 and of *Bacteroides* in Donor 3 may have caused rapid degradation of AX, because members of *Bifidobacterium* and *Bacteroides* genera have been shown to be capable of AX degradation (Despres et al., 2016; Dodd et al., 2011; Rogowski et al., 2015; Truchado et al., 2015; Van den Abbeele et al., 2011a;

Van den Abbeele et al., 2013; Van Laere et al., 1999). These results are also partially in agreement with a previous study from our laboratory, which showed that AX obtained from sorghum bran is rapidly fermented by fecal microbiota due to its simple structural feature (Rumpagaporn et al., 2015). However, the current study further adds to this that fast fermenting property of sorghum AX is tightly tuned to the fecal microbiota compositions of donors.

4.4.3 SCFA Production During *In Vitro* Fermentation

SCFAs, namely acetate, butyrate, and propionate, are the main metabolites produced by colonic microbiota as a result of dietary fiber fermentation. They are physiologically important for host health for such reasons as they inhibit the growth of pathogenic bacteria by reducing the pH of the colon (Slavin, 2013), improve bone health by increasing the mineral absorption by the colonic epithelial cells (Scholz-Ahrens et al., 2007), and protect against development of allergies, asthma and autoimmunity disease by stimulating the immune system (Maslowski and Mackay, 2011). Recent evidences also revealed that acetate (Frost et al., 2014; Murugesan et al., 2015), butyrate and propionate (Lin et al., 2012) have protective effects on diet-induced obesity. Butyrate, which is thought to be the main energy source for the colonic epithelial cells (Hague et al., 1996), has also been shown to have an anti-inflammatory effect (Mathew et al., 2014).

SCFA production from *in vitro* fermentation of fibers was higher for Donors 2 and 3 in all cases, producing 1.5-2 times higher total SCFA than Donor 1 (Figure 4.3). The higher total SCFA production in Donors 2 and 3 than Donor 1 ($P < 0.05$) was contributed by higher production of butyrate from the substrates, except CS. For

butyrogenic FOS (Kaur et al., 2011), Donor 1 produced 3-3.5 times less amount of butyrate than the other two donors. . Compared to Donor 1, Donor 2 had a much higher proportion of members of the *Bifidobacterium* genus which promote butyrate through lactate (Bourriaud et al., 2005), and Donor 3 had elevated Lachnospiraceae family which contains a number of butyrogenic species (Rajilic-Stojanovic and de Vos, 2014).

In all donors for total SCFA, the mixture was among the few dietary fibers that caused rapid SCFA production, even though some of its constituents including XG, GM, and CS generated SCFA at slow initial rates when presented singly. The fiber mixture contains different types of dietary fibers conceptually favoring different bacteria, so it seems reasonable that a number of fiber types together may promote a broader range of bacteria growth, while certain single fibers would promote a more limited number of bacteria.

Highest acetate production was observed with the fiber mixture, PGA, and FOS were used as substrates. Common trends for butyrate were low production with CS, medium production for PGA and the mixture, and high production for FOS. Other fibers varied in butyrate production between Donors 2 and 3 (Donor 1 had relatively low production for all tested fibers); Donor 2 produced high butyrate for XG, GM, and AX, and Donor 3 had only fairly high production for GM. These results agree with recent work done in our laboratory (Chen et al., unpublished data) that fermentation rate and fermentation-end-products obtained from a particular dietary fiber are dependent on the initial microbiota composition.

Although sorghum AX was reported as rapidly fermented by fecal microbiota and produced a high amount of total SCFAs as well as butyrate (Rumpagaporn et al., 2015),

only Donor 2 showed this effect here (Figure 4.3). This again highlights the importance of the initial microbiota composition in determining fermentation rate as well as the SCFAs obtained from a particular dietary fiber or mixture.

Notably, for Donor 3, fibers, except CS and PGA, caused increase in propionate production after 12 h, even though there was no substrate degradation activity seen after this time period. This might have occurred due to the cross-feeding of the lactate metabolite produced up until this time, and then afterwards microbial activity by propionate producers. Supporting evidence for this explanation comes from the investigation of Bourriaud et al. (Bourriaud et al., 2005) showing that some members of the human colonic microbiota have ability to produce propionate from microbial metabolites, especially from lactate. A recent report detailed that some members of *Megasphaera* spp. and Lachnospiraceae family are mainly responsible for lactate to propionate production (Reichardt et al., 2014).

4.4.4 Composition of Fecal Microbiota Community After *in Vitro* Fermentation of Dietary Fibers

4.4.4.1 Effects of *in vitro* fecal fermentation of dietary fibers on α -diversity of microbial communities

α -Diversity is a measurement of the diversity within a single community or habitat, and it is calculated by accounting for the total number of species (richness) and/or the relative abundances of the species detected in a given community (evenness) (Lozupone and Knight, 2008). α -Diversity of the communities in this study was calculated by measuring Shannon's index calculator (Figure 4.4). Since the Shannon

index calculator accounts for both species richness and evenness, a community will have high Shannon index value when high number of species are detected and their abundances are similar.

The α -diversity of the fecal microbiota communities of the three donors responded differently to the fiber treatments (Figure 4.4). Initial α -diversity was greatest in Donor 1 fecal microbiota, and approximately the same in Donors 2 and 3. Shannon index values of the microbiota communities of Donors 1 and 3 significantly decreased ($P < 0.05$) after *in vitro* fecal fermentation for all fiber treatments, though the trends were different between the two donors. Donor 2 fecal microbiota showed no significant changes in Shannon Index α -diversity for any of the fiber treatments (two tailed student's t test, $P < 0.05$), possibly indicating a highly redundant bacterial community for utilization of fibers with ample ability to cross-feed and that with a better ability to adapt to dietary fibers. For Donor 1, AX and FOS caused the largest loss in α -diversity and the fiber mixture caused the least loss, the latter providing substrate for a variety of different groups of bacteria. The significant loss in α -diversity in Donor 3 fecal microbiota when fed any of the fibers or the fiber mixture is hard to explain, particularly since they generally produced high levels of the individual and total SCFAs.

4.4.4.2 Effects of *in vitro* fecal fermentation of dietary fibers on β -diversity of microbial communities

β -Diversity is a measurement of the diversities of the two or more communities and evaluates how communities differ than each other (Lozupone and Knight, 2008). The comparison of the overall structures of the fecal microbiota communities of all samples

were conducted using the Bray-Curtis dissimilarity test based on the relative abundances of OTUs at 97% similarity level. The results showed that the donor had a definitive effect on microbial community composition, since the microbial communities of each sample were clustered well based on the donor, and the donor accounted for 70 % of the total variations (Figure 4.5a). We also observed similar results when other analysis methods were used including Jaccard, Euclidean, weighted- and unweighted-UniFrac (data not shown).

In addition, fiber type and time had effects on microbial communities (Figure 4.5b). Following the PCoA analysis, statistical analysis (Adonis and Permanova tests) showed significant effects of fiber type and time on microbial communities ($P < 0.001$, for both tests), further confirming the importance of dietary fibers on microbial community structures.

4.4.4.3 Changes in microbiota composition after *in vitro* fermentation of given dietary fibers

Changes in microbiota composition throughout *in vitro* fecal fermentation at phylum, family, and genus levels are shown in Figures 4.6, 4.S6, and 4.7, respectively. At first glance, the obvious changes in the microbiota compositions were seen in the abundances of Bacteroidetes members that dramatic increased in relative abundance in all fiber treatments. These increases were mainly contributed to by dramatic increases in the abundance of bacteria species belonging to the *Bacteroides* genus (Figure 4.7). This may be attributed to the fact that the members of this genus are well equipped for the degradation of dietary fibers (Flint et al., 2012a; Koropatkin et al., 2012), and perhaps

better ability to compete for soluble fibers. For example, *B. cellulosilyticus* and *B. thetaiotaomicron* have the ability to secrete a total of 424 and 304 different enzymes, respectively, that are responsible for the degradation of a broad range of dietary fibers (Martens et al., 2008; McNulty et al., 2013). In contrast, the relative abundances of Firmicutes decreased with fiber treatments, except that its abundance in the samples containing GM were similar to that at the starting point (time 0). This also explains why high butyrate production was found in the samples containing GM (Figure 4.4). The majority of the butyrate producing bacteria residing in our gut belong to the Firmicutes phylum (Barcenilla et al., 2000; Duncan et al., 2002; Flint et al., 2012a; Louis and Flint, 2009; Louis et al., 2010).

Specific types of dietary fiber were associated with an increase in the relative abundances of specific bacteria at the genus level, in addition to promoting *Bacteroides* members. For instance, AX fermentation by the fecal microbiota of Donors 1 and 2 resulted in approximately 2-fold increase in the relative abundance of the *Prevotella* genus (Figure 4.7). This can be attributed that some members of this genus effectively use soluble xylans (Miyazaki et al., 1997). In contrast, this effect of AX on the *Prevotella* group was not observed in Donor 3 as *Prevotella* was initially absent in its fecal microbiota (Figure 4.7). Similarly, PGA fermentation by fecal microbiota of any of the donors resulted in a dramatic increase in the relative abundance of members of *Lachnospira* (at least a 6-fold increase) (Figure 4.7). This can be attributed to the fact that some members of this genus is well equipped for the degradation of pectin (Duskova and Marounek, 2001), of which PGA forms the basis. Pectin fermentation by *Lachnospira* members occurs very rapidly and results in production of high amount of acetate

(Duskova and Marounek, 2001), and this was confirmed in the present study (Figure 4.3). CS fermentation resulted in an increase in the relative abundance of Parabacteroides members in all donor groups. XG fermentation by the fecal microbiotas, in all fiber treatments, resulted in at least a 1.5-fold increase in the relative abundance of the members of the Lachnospiraceae family (Figure 8). This is, perhaps, the reason why a relatively high amount of butyrate was observed as a result of XG fermentation (Figure 4.3), because the members of Lachnospiraceae including *Butyrivibrio* and *Roseburia* are known to be butyrate producers (Cotta and Forster, 2006; Meehan and Beiko, 2014). In addition to this family group, at the order level, XG fermentation by fecal microbiota of Donor 2 also promoted the members (other than the members of *Lachnospira*, *Feacalibacterium*, *Blautia*, and *Ruminococcus*) of the Clostridiales group, which harbors butyrate-producing bacteria species as well (Vital et al., 2014); its relative abundance was increased almost 1.5 fold within the 6 h of incubation (Figure 4.7). This explains why XG fermentation by the fecal microbiota of Donor 2 (but not that of Donors 1 or 3) produced the highest amount of butyrate (Figure 4.3).

In the fiber mixture-containing samples, we hypothesized that all of the bacterial groups, which are favored by the specific dietary fibers as discussed above, would be promoted, because the mixture consisted of the equal amount of each of the tested dietary fibers. Fecal microbiota of the Donor 1 was the only group to do so. The relative abundances of members of *Lachnospira*, *Prevotella*, *Parabacteroides*, and *Bacteroides* genera and the members of Lachnospiraceae family (other than the members of *Lachnospira* genus) residing in Donor 1 fecal community were promoted when the mixture was used as substrate. Same trends were not observed when the fecal microbiota

of Donors 2 or 3 was used; instead, only certain bacteria groups (mainly the members of *Parabacteroides*, *Bacteroides*, and Lachnospiraceae) were promoted. This parallels recent work in our laboratory (Chen et al., unpublished data), showing that the effects of a particular dietary fiber on colonic microbiota composition is tightly dependent on the initial microbiota community of individuals.

4.5 Conclusion

Insufficient saccharolytic activity and increased proteolytic activity in the distal part of the colon have been thought as a main reason why the majority of the colonic diseases such as cancer and inflammatory bowel diseases occur in this region. Therefore, there has been an increasing interest in developing strategies to deliver dietary fibers into the distal part of the colon, for the purpose of decreasing disease related biomarkers by increasing the saccharolytic activity and amount of SCFA formed. Data obtained here supports the idea that degradation of some dietary fibers can be delayed when presented in a mixture, so that this might be a reasonable strategy for delivering particular dietary fibers into distal part of the colon. Notably, the study shows that hierarchical preference to carbohydrate substrates translates from single bacteria and simple competitive model systems to the entire fecal microbial community.

Additionally, our data support recent work from our group (Chen et al. unpublished data) that initial microbiota composition is a crucial factor which affects the functional outcomes of dietary fibers. For example, fecal microbiota of Donor 2 produced higher amount of butyrate from XG, compared to GM; however, the opposite trend was observed when fecal microbiota of Donor 3 was used. Similarly, fecal microbiota of

Donor 3 produced high amount of propionate from AX; however, the large propiogenic effect of AX was not observed when fecal microbiota of Donor 2 was used. These results collectively demonstrate that there is no specific dietary fiber structure that can produce the same functional outcomes in all individuals, further suggesting that personalized dietary fibers need to be consumed in order to optimize their benefits for host health.

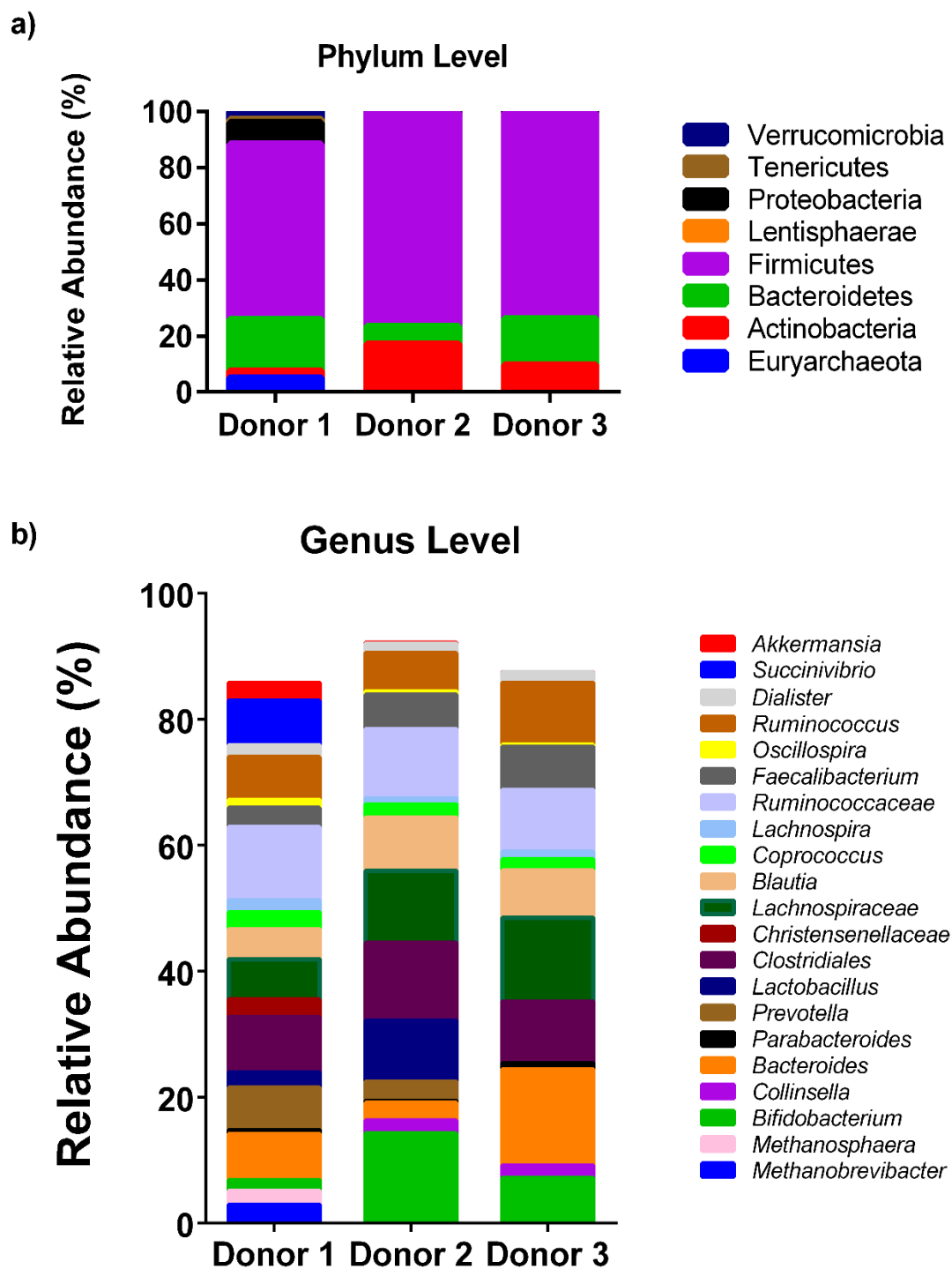


Figure 4.1 Initial microbiota compositions of fecal samples collected from different donors **a)** at phylum level, **b)** at genus level.

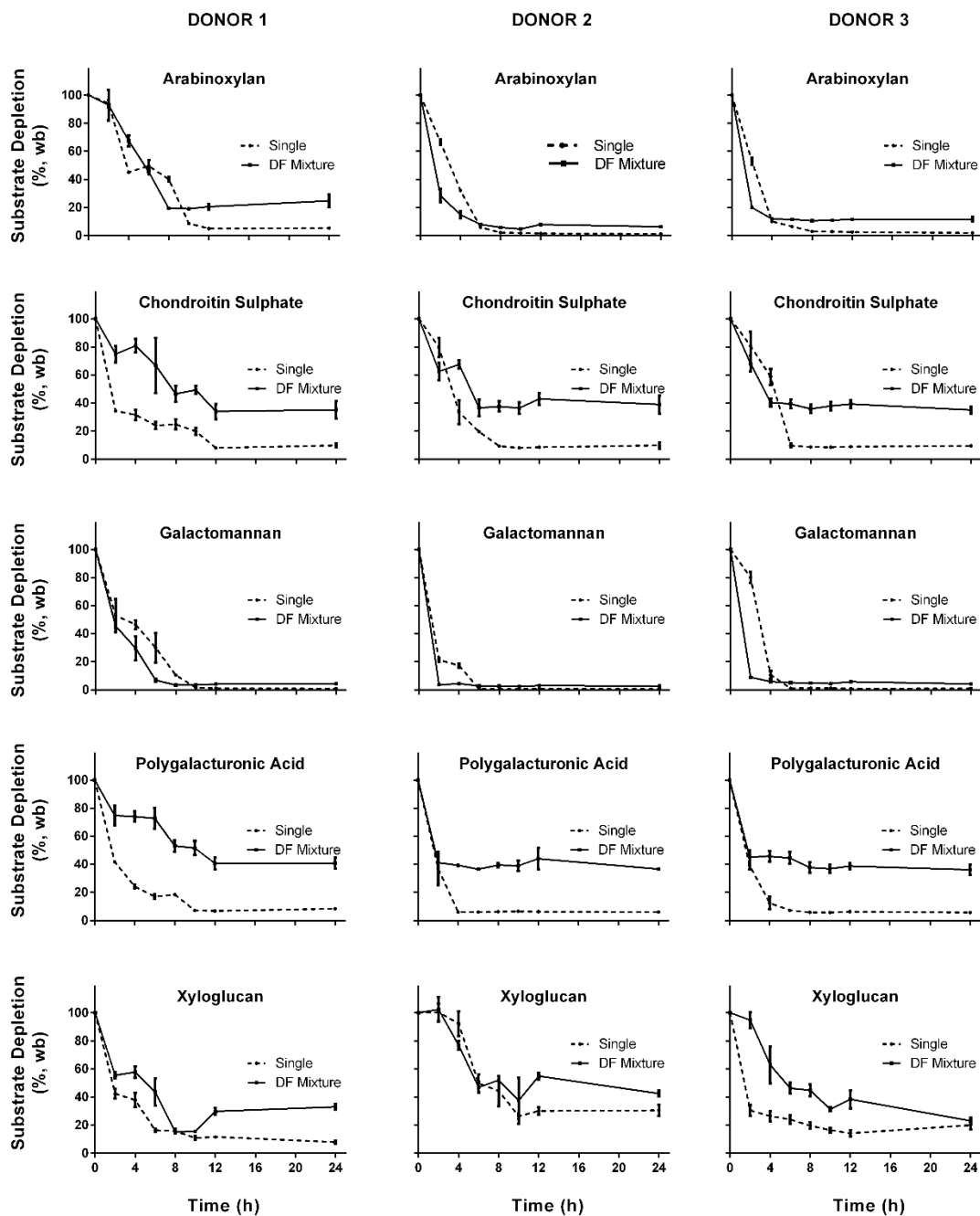


Figure 4.2 Substrate depletions by fecal microbiota obtained from different donors during a time course *in vitro* fermentation. Solid lines indicate depletion of the corresponding dietary fiber (DF) presented in the mixture, while dashed lines represent the depletion of the DF when they were presented as a single substrate. Error bars represent the standard error of the mean of three separate replicates.

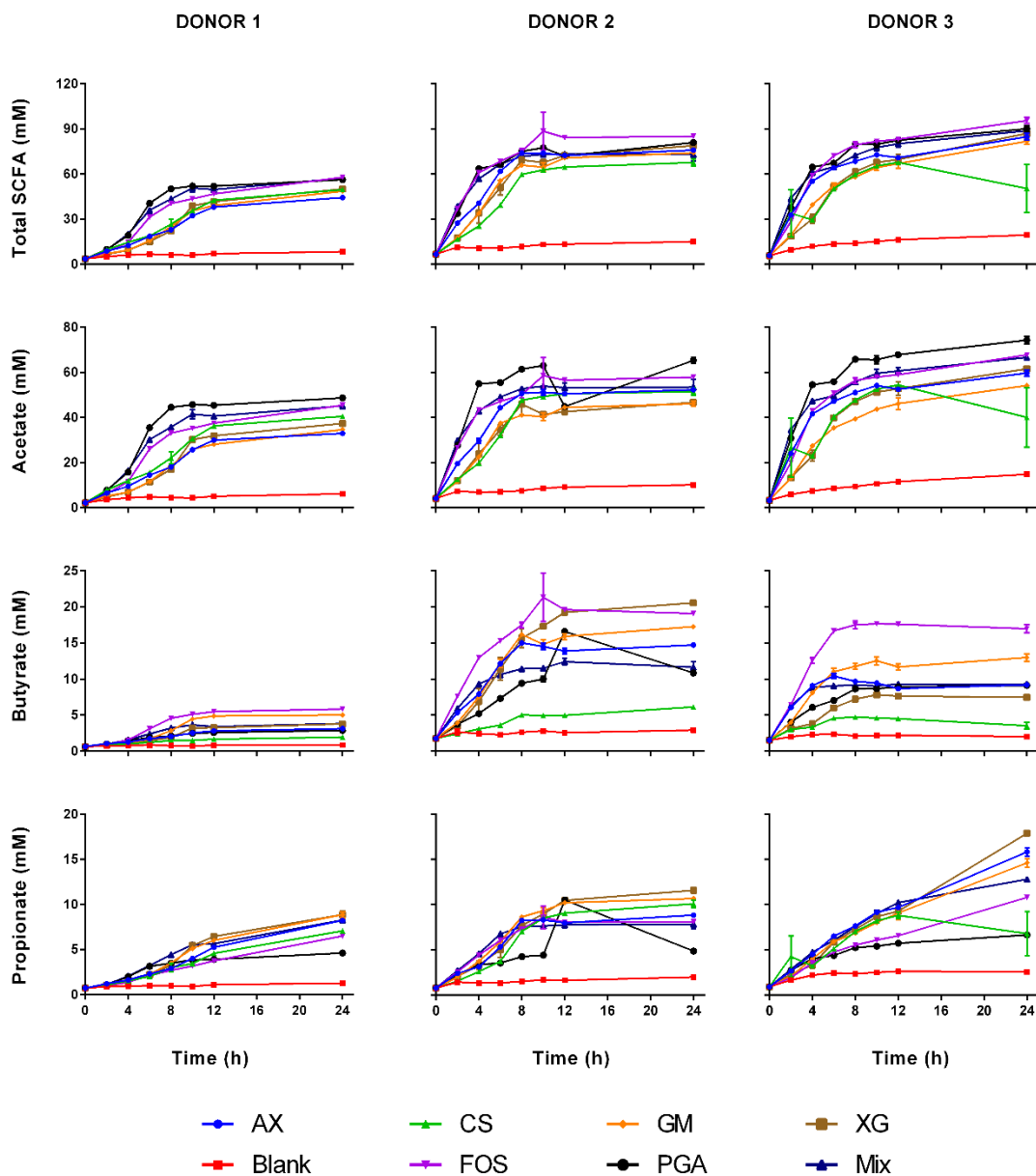


Figure 4.3 Short chain fatty acid (SCFA) production by fecal microbiota obtained from three different donors during a time course *in vitro* fermentation. AX: arabinoxylan (sorghum), CS: chondroitin sulfate, GM: galactomannan, XG: xyloglucan, PGA: polygalacturonic acid, Mix: mixture containing the equal amounts (weight basis) of dietary fibers of AX, CS, GM, XG, and PGA. FOS: Fructooligosaccharide, was used as a fast fermenting comparator. The blank did not contain any substrate. Error bars represent the standard error of the mean of three separate replicates.

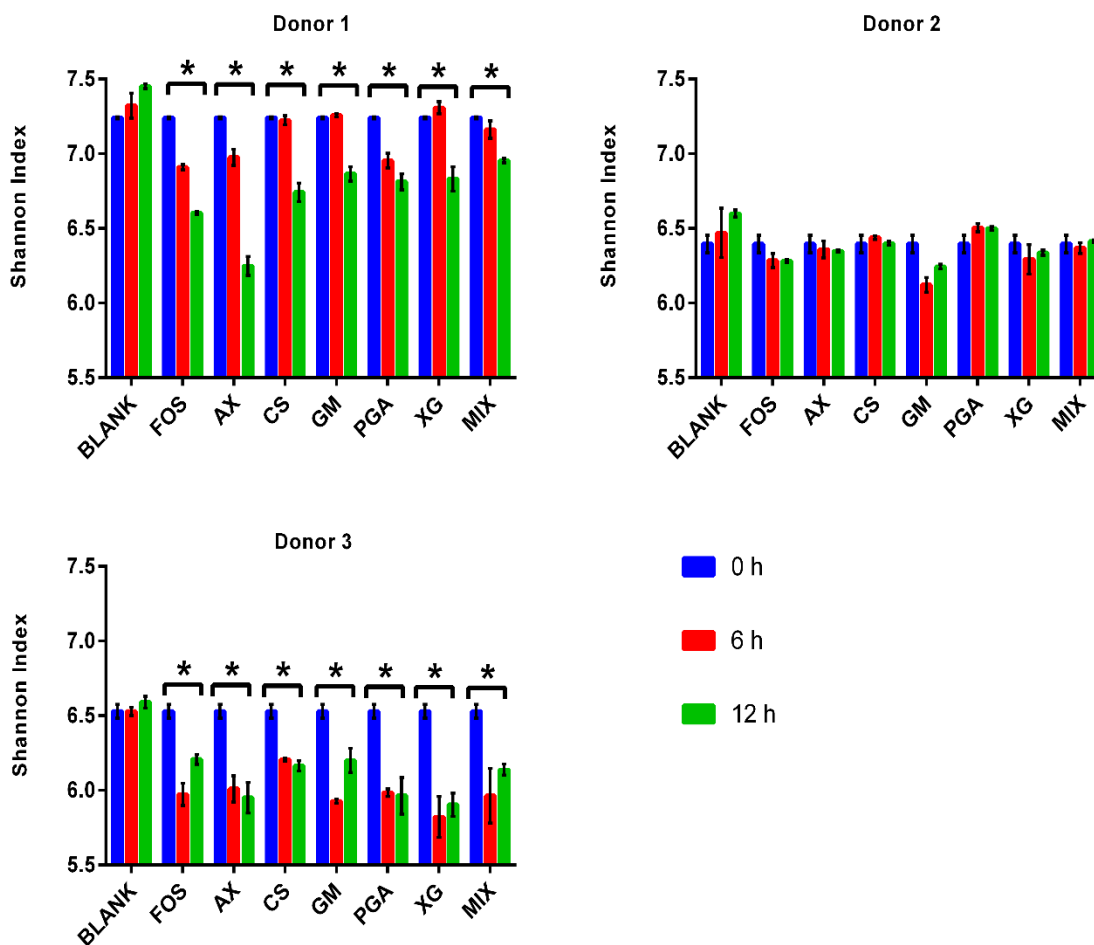


Figure 4.4 Changes in α -diversity of the fecal microbiota communities during a time course *in vitro* fermentation of the dietary fibers, as measured by Shannon's Index Calculator. AX: arabinoxylan (sorghum), CS: chondroitin sulfate, GM: galactomannan, XG: xyloglucan, PGA: polygalacturonic acid, Mix: mixture containing the equal amounts (weight basis) of dietary fibers of AX, CS, GM, XG, and PGA. FOS: Fructooligosaccharide, was used as a fast fermenting comparator. The blank did not contain any substrate. * Significantly different (two tailed student's t test $P < 0.05$).

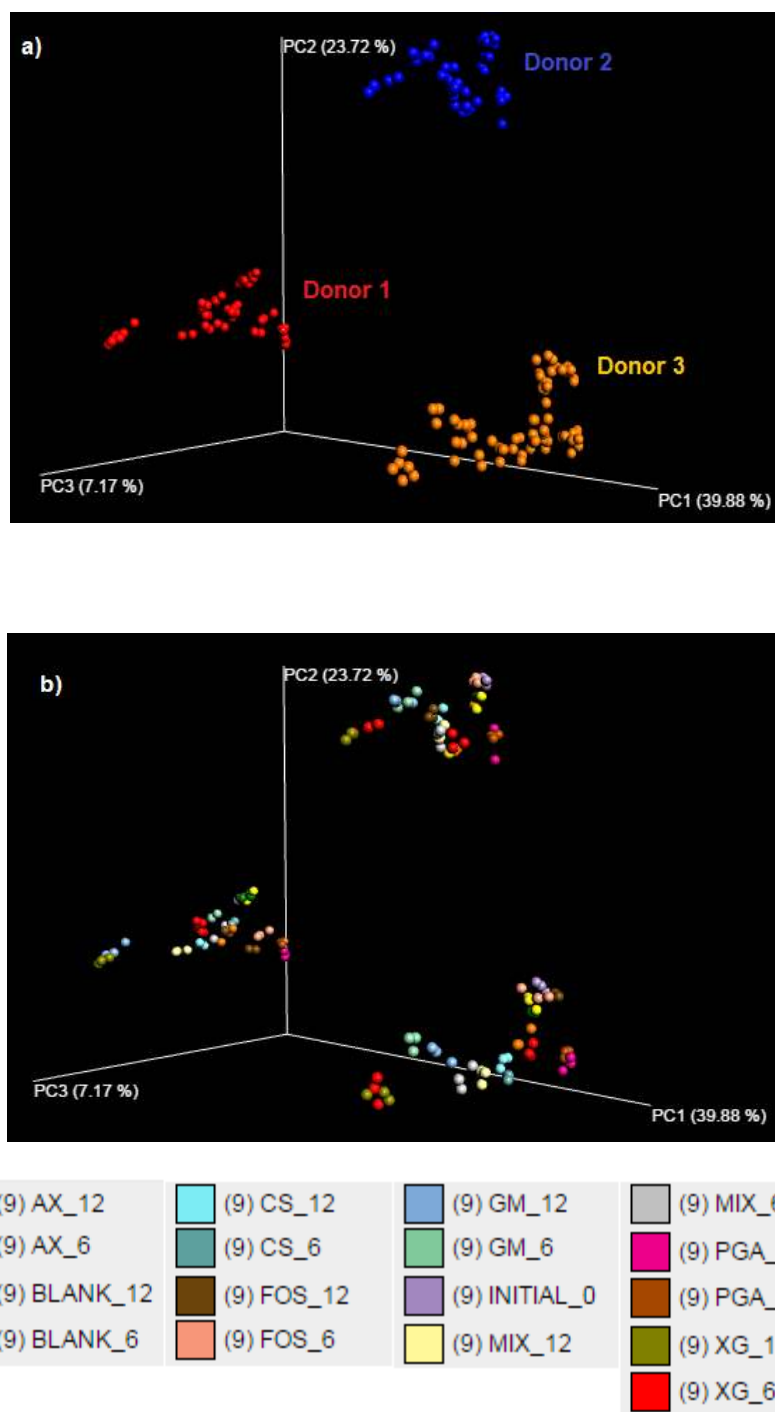


Figure 4.5 Bray-Curtis dissimilarity of fecal microbial communities based on the relative abundances of OTUs at 97% similarity level after *in vitro* fermentation for 0, 6, and 12 h with dietary fibers. **a)** The samples were well clustered based on the donor. **b)** Dietary fiber and time also had effects on microbiota community structures.

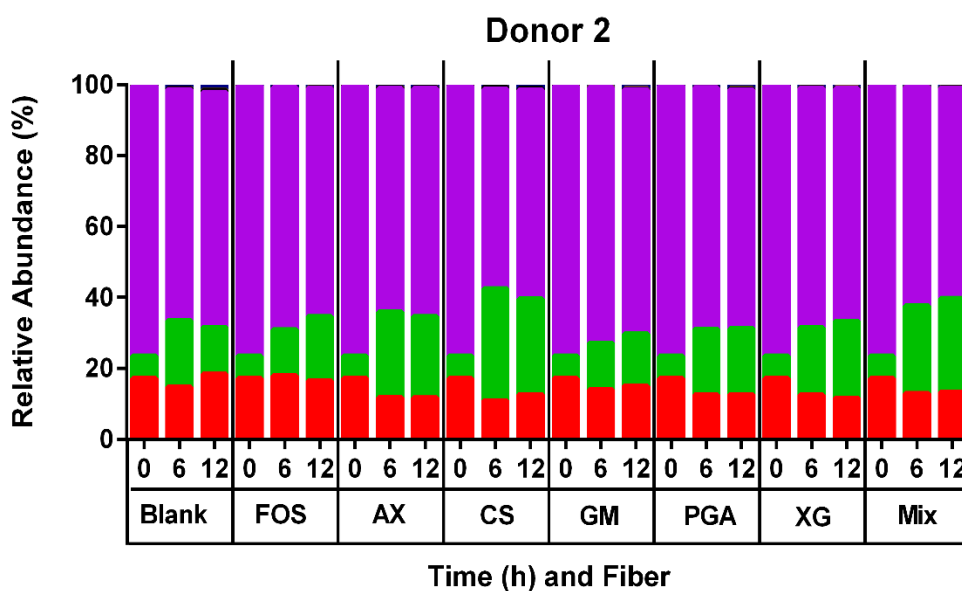
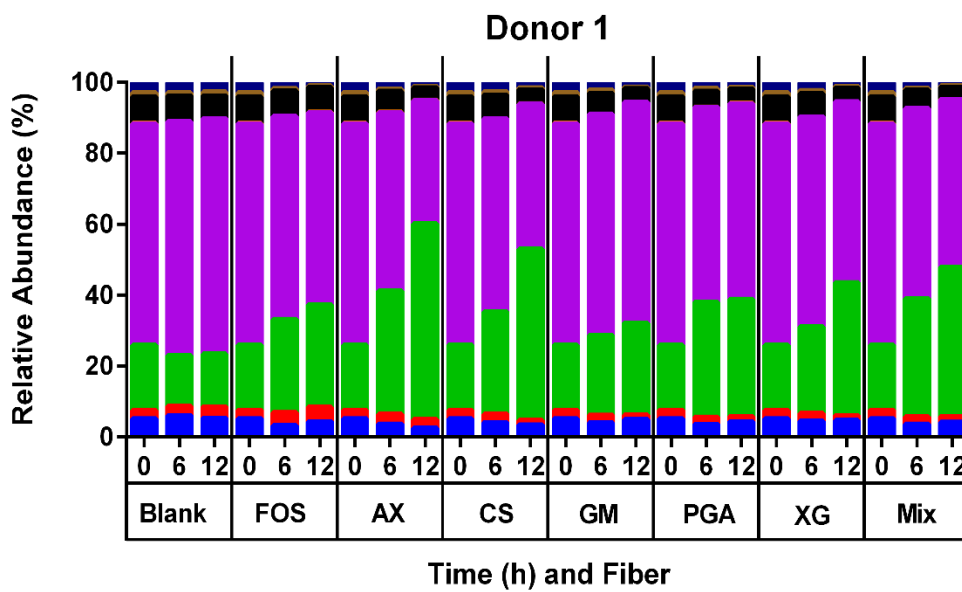


Figure 4.6 Phylum level changes in microbiota composition of the fecal samples after the *in vitro* fermentation for 0, 6, and 12 h with dietary fibers.

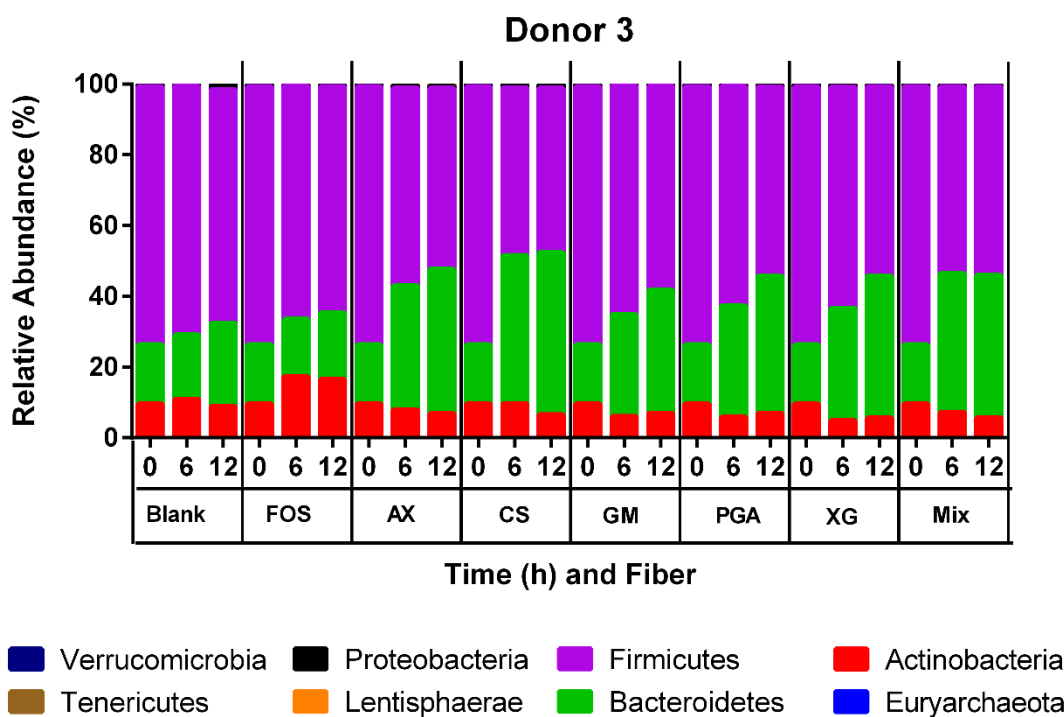


Figure 4.6 Continued. AX: arabinoxylan (sorghum), CS: chondroitin sulfate, GM: galactomannan, XG: xyloglucan, PGA: polygalacturonic acid, Mix: mixture containing the equal amounts (weight basis) of dietary fibers of AX, CS, GM, XG, and PGA. FOS: fructooligosaccharide, was used as a fast fermenting comparator. The blank did not contain any substrate.

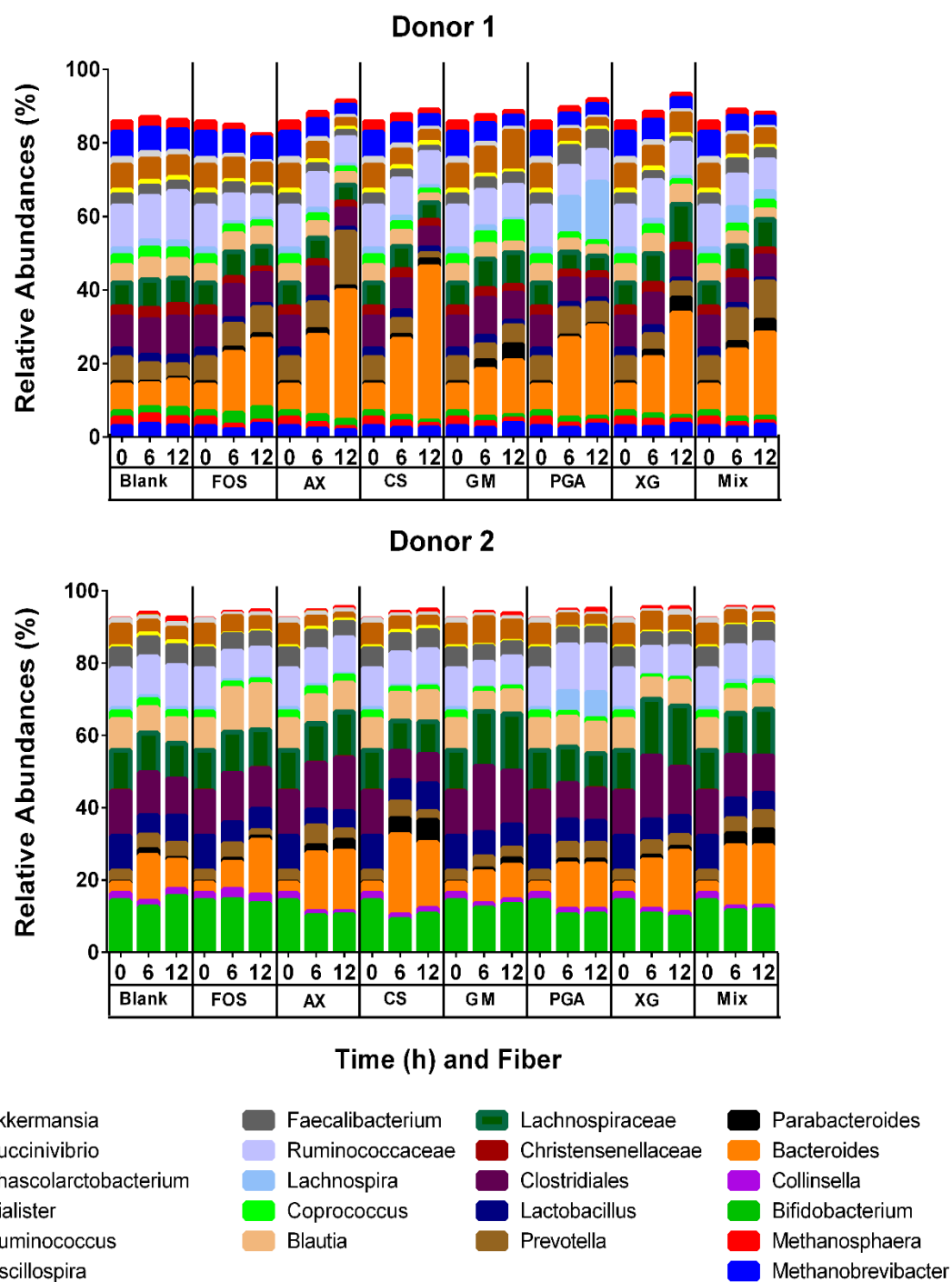


Figure 4.7 Genus level changes in microbiota composition of the fecal samples after the *in vitro* fermentation for 0, 6, and 12 h with dietary fibers.

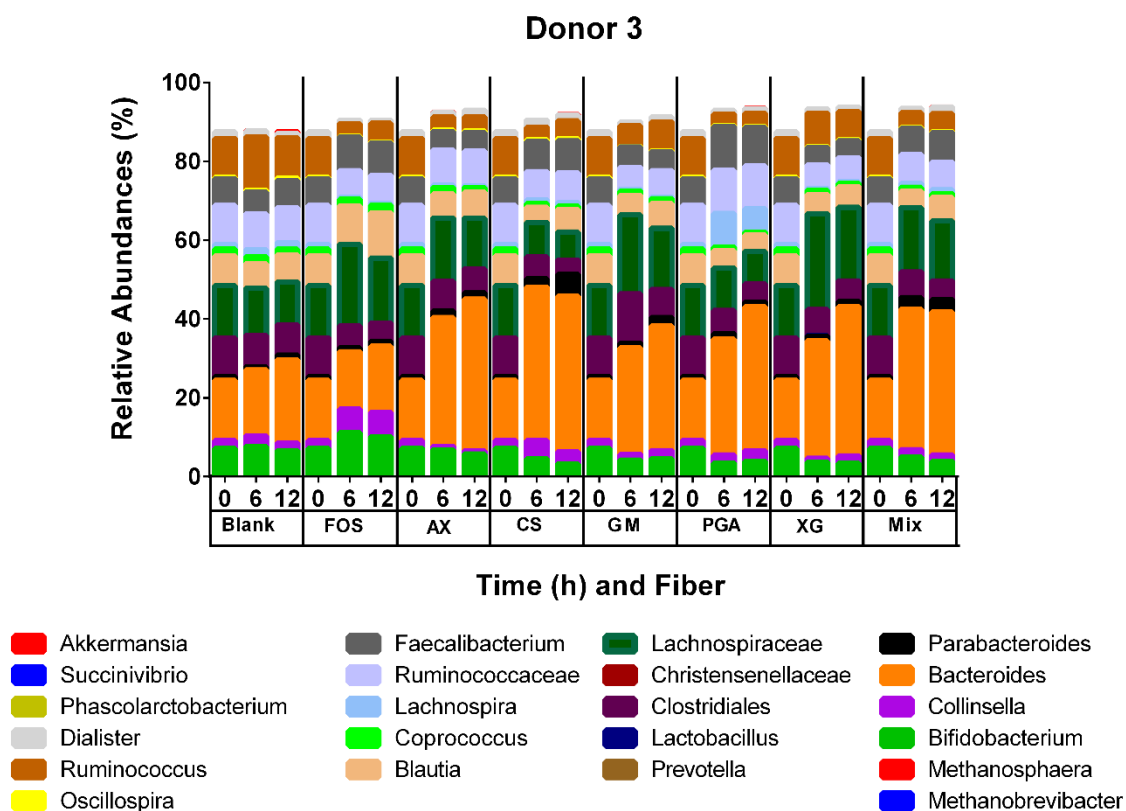


Figure 4.7 Continued. Genus level changes in microbiota composition of the fecal samples after the *in vitro* fermentation for 0, 6, and 12 h with dietary fibers.

4.6 Supplementary Information

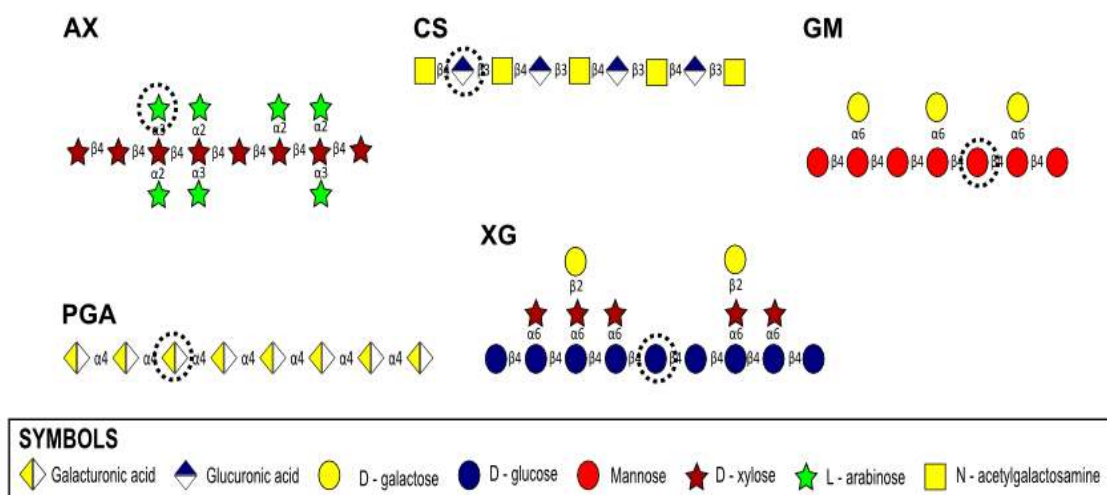


Figure 4.S1 Chemical structures of glycans. Dashed circles indicate the monosaccharides or linkages that were used to measure the remaining amount of corresponding glycan in the media. The remaining AX, CS, GM, PGA, and XG percentages relative to their initial amounts were calculated by measuring the arabinose, glucuronic acid, mannose, galacturonic acid, and glucose amounts in the samples, respectively. AX: arabinoxylan (sorghum), CS: chondroitin sulfate, GM: galactomannan, XG: xyloglucan, PGA: polygalacturonic acid.

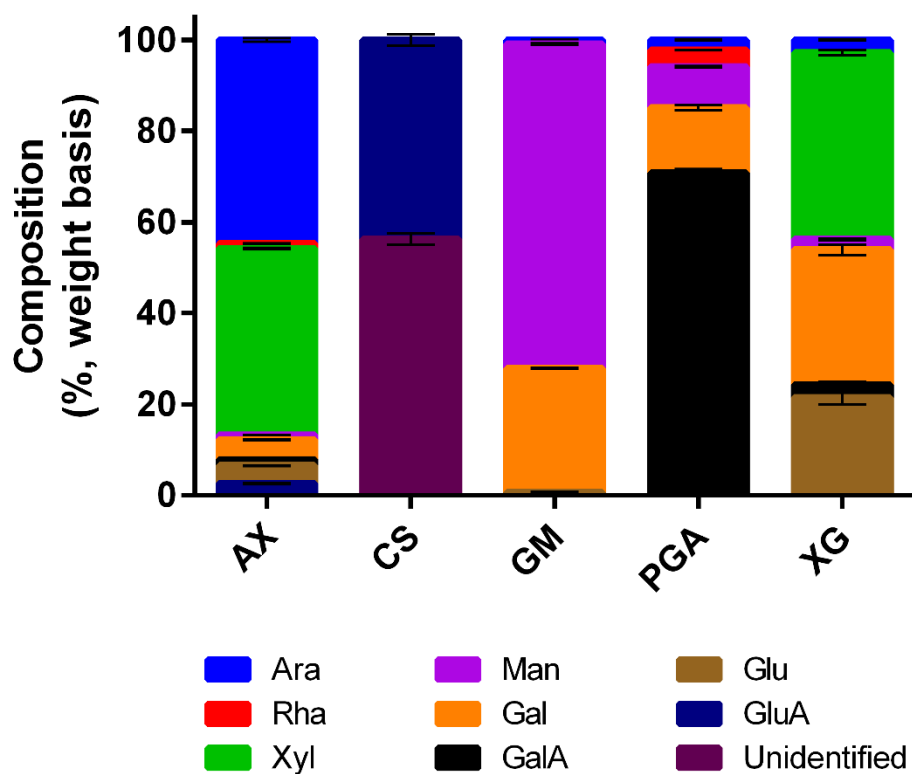


Figure 4.S2 Monosaccharide compositions of the dietary fibers used in this study. Compositions were determined using the TMS derivatization method. Error bars represent the standard error of the mean of two separate replicates. Abbreviations: AX: arabinoxylan (sorghum), CS: chondroitin sulfate, GM: galactomannan, XG: xyloglucan, PGA: polygalacturonic acid, Ara: arabinose, Rha: rhamnose, Xyl: xylose, Man: mannose, Gal: galactose, GalA: galacturonic acid, Glu: glucose, GluA: glucuronic acid.

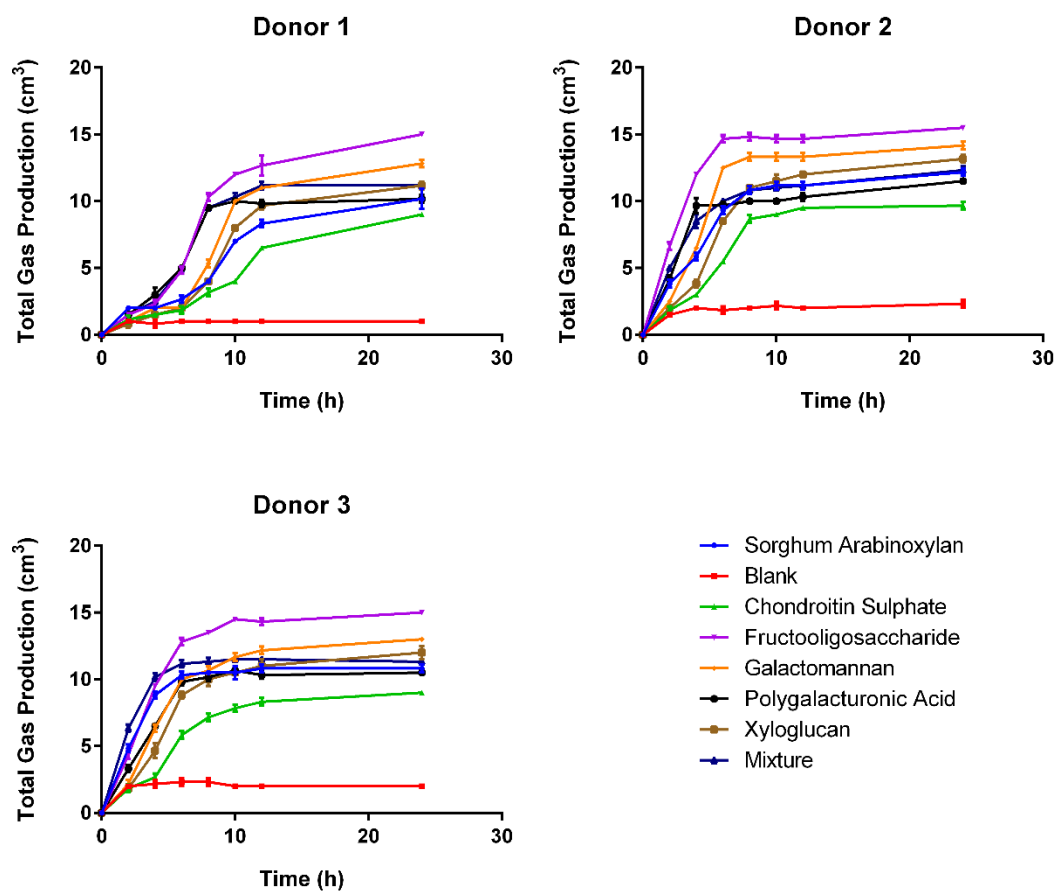


Figure 4.S3 Total gas production by the fecal microbiota as a result of *in vitro* fermentation with dietary fibers. Error bars represent the standard error of the mean of three separate replicates.

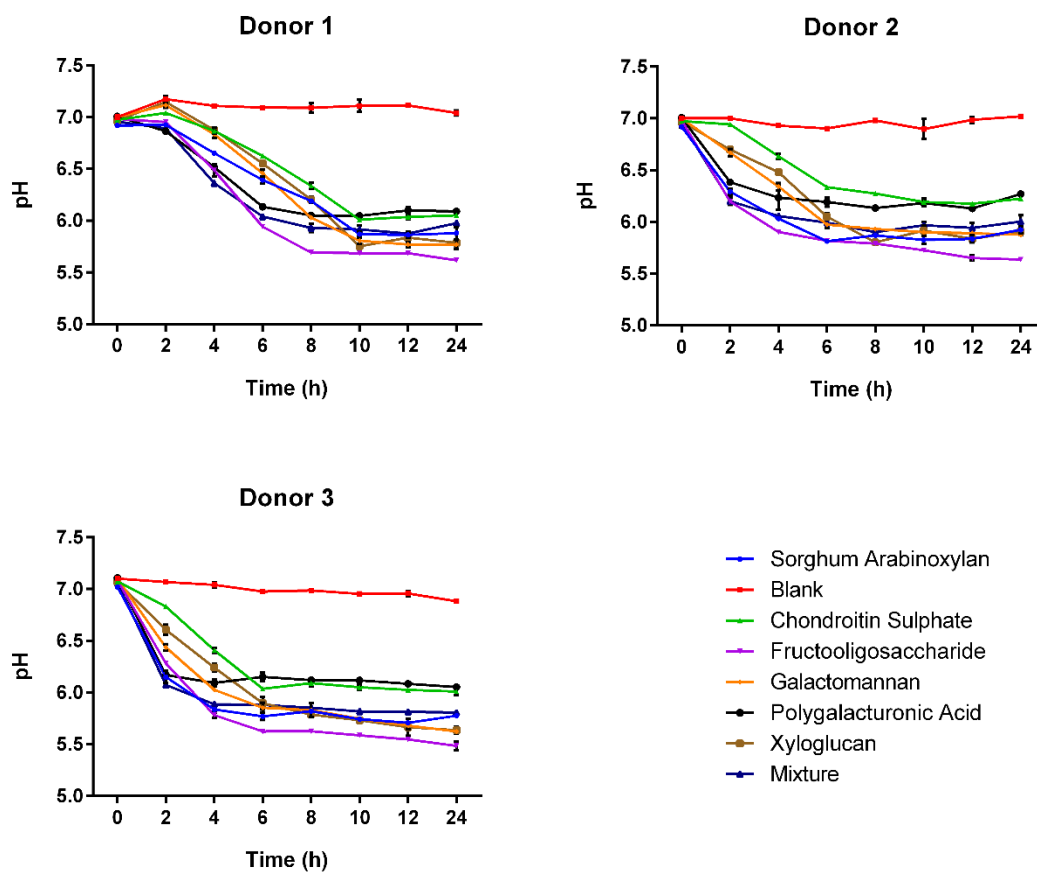


Figure 4.S4 Changes in pH of the media over time as a result of *in vitro* fecal microbiota fermentation with dietary fibers. Error bars represent the standard error of the mean of three separate replicates.

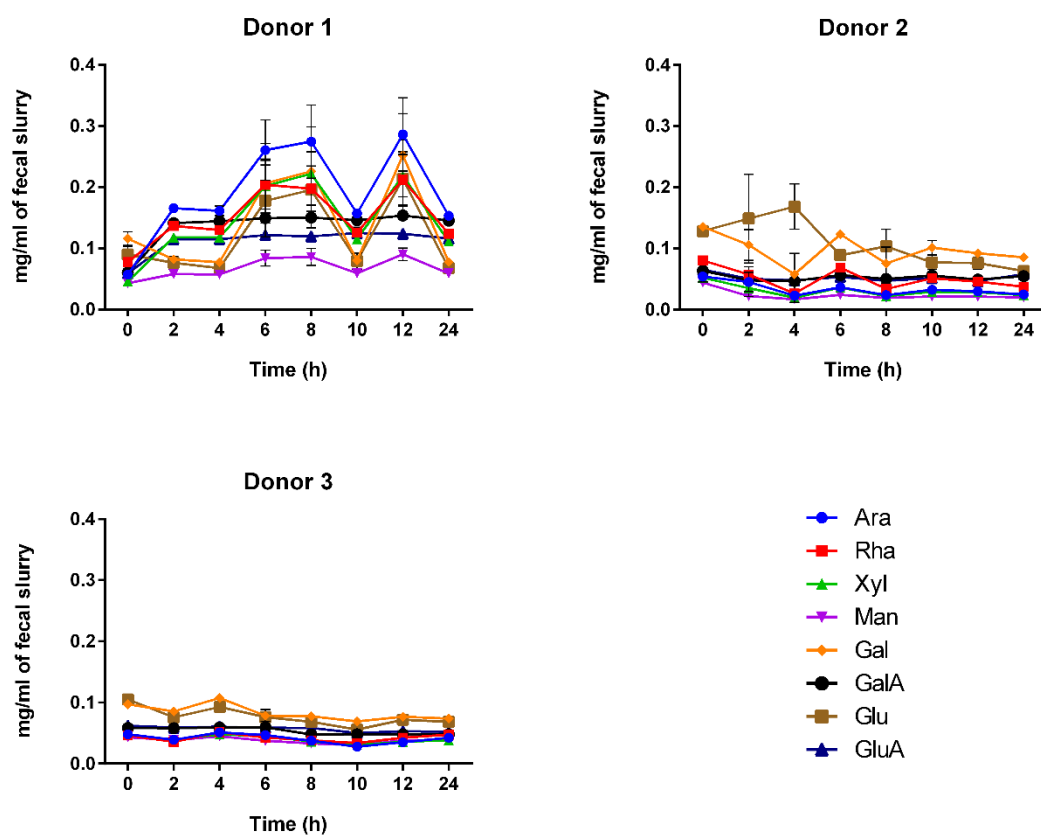


Figure 4.S5 Monosaccharides found in the blank during the time course of the *in vitro* fecal fermentation. Error bars represent the standard error of the mean of three separate replicates. Abbreviations: **Ara**: arabinose, **Rha**: rhamnose, **Xyl**: xylose, **Man**: mannose, **Gal**: galactose, **GalA**: galacturonic acid, **Glu**: glucose, **GluA**: glucuronic acid.

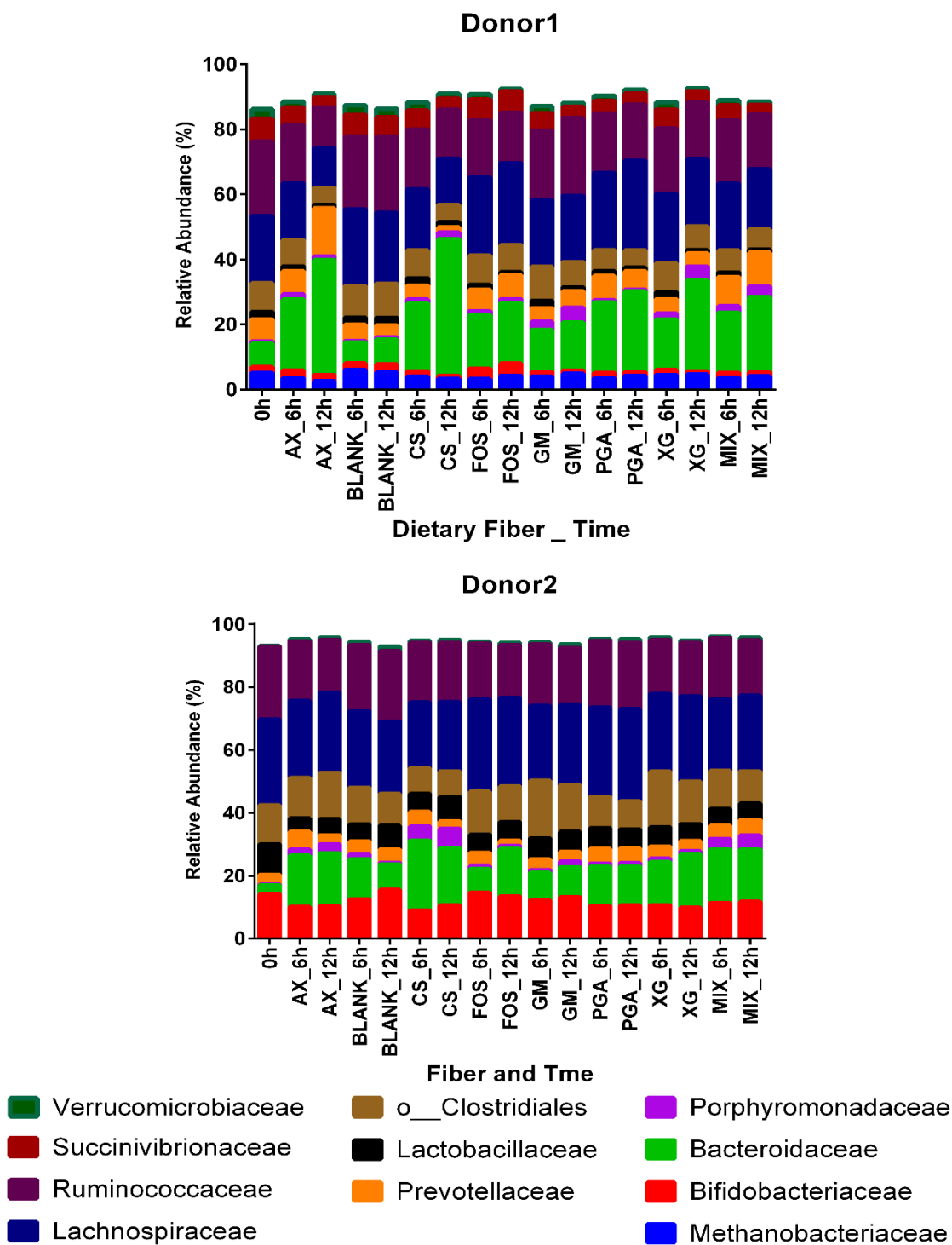


Figure 4.S6 Family level changes in microbiota composition of the fecal samples after the *in vitro* fermentation for 0, 6, and 12 h with dietary fibers.

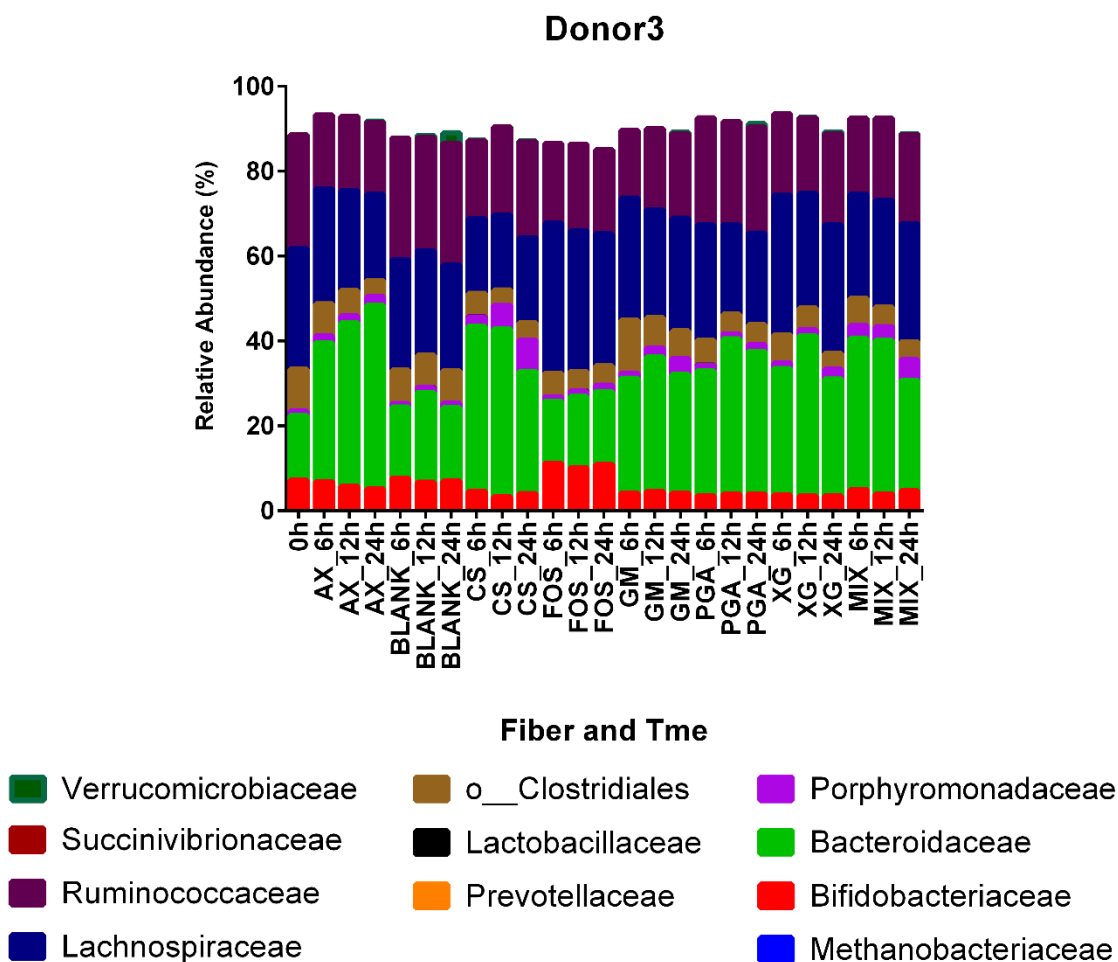


Figure 4.S6 Continued. Family level changes in microbiota composition of the fecal samples after the *in vitro* fermentation for 0, 6, and 12 h with dietary fibers.

OVERALL CONCLUSION

The human colon microbiota composition is significantly affected by its food supply (Hamaker and Tuncil, 2014a; Koropatkin et al., 2012; Martens et al., 2014). Accordingly, there has been an increasing interest to modify its composition via diet, especially via indigestible dietary fibers or glycans, as a way to improve health. This requires advanced understanding of the interactions between glycans and the individual members of our colonic bacteria, and their strategies to utilize these dietary compounds (Hamaker and Tuncil, 2014; Martens et al., 2014). From this point of view, many investigations, both *in vitro* and *in vivo*, have been conducted to investigate these interactions. Many rely on biological aspects of bacteria responses (i.e. transcriptional profiling) to given information on glycan utilization. However, advanced understanding of interactions between glycans and bacteria species is best done through monitoring the changes in glycan amount.

Here, we used carbohydrate depletion analysis in conjunction with transcriptional profiling to understand the responses and strategies of two closely related human gut symbionts, *B. ovatus* and *B. thetaiotaomicron*, to utilize different complex glycans presented as a mixture. Throughout the studies, having these two types of analyses enabled us to thoroughly measure cellular aspects of glycan utilization, and to understand

the relationship between gene expression and actual carbohydrate use by these two organisms.

The results of this thesis work provide new insights into understanding glycan utilization strategies of *Bacteroides* species that likely translates to colonic bacteria. In addition to supporting past work that human gut symbionts show species specific hierarchical preferences to complex glycans presented as a mixture, our results show for the first time that these metabolic hierarchies are protected, when they are in a competitive environment and suggest that bacteria are ‘programmed’ to utilize different glycans in an ordered way. The data also suggests that these reciprocal metabolic programs of different bacteria species, that was first identified by (Pudlo et al., 2015), allow them to maintain their coexistence in the competitive environment. This could be one of the mechanisms that explains how different bacteria species survive in the competitive environment of the colon.

Our results also show that bacterial responses to a particular glycan are tightly tuned to its molecular structure and that molecular structure of a glycan affects its place in the hierarchy. These results further demonstrate that the structure of a glycan is an important factor that govern the glycan-microbiota interactions.

The last study demonstrated that, from the carbohydrate perspective, the hierarchical preferences of carbohydrates also exists in the competitive environment of the colon microbiota, and that the presence of a mixture of glycans causes a delay in the utilization of some of them. This could be an important strategy to target increased fermentation in the distal part of the colon.

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EDUCATION

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- M.S., Food Science and Technology, 2012, Texas A&M University, College Station, Texas, USA
- B.S., Food Engineering, 2008, Ataturk University, Erzurum, Turkey

RESEARCH INTERESTS

- Carbohydrate structures
- Interactions between carbohydrates and other food compounds
- Dietary fibers, gut microbiota, and colonic health

PUBLICATIONS

Peer Reviewed Journals

- Hamaker, B. R., **Tuncil, Y. E.** 2014. A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. *Journal of Molecular Biology*; 426: 3838 - 3850.
- **Tuncil, Y. E.**, Jondiko, T., Tilley, M., Hays, D. B., Awika, J. M. 2016. Combination of null alleles with 7+9 allelic pair at Glu-B1 locus on the long arm of group 1 chromosome improves wheat dough functionality for tortillas. *LWT-Food Science and Technology*; 65: 682 - 688.

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Presentations

- **Tuncil Y.E.**, Xiao Y., Porter N., Reuhs B.L., Martens E.C., and Hamaker B.R. 2016. Human colonic bacteria show substrate dependent hierarchical preference to dietary fibers, with structure determining rank. October 23 – 26, 2016 Savannah, GA.
- **Tuncil Y. E.**, Martens, E. C., Hamaker, B. R. 2015. Growth rate of a human gut symbiont on starch is source dependent. AACCI Annual Meeting. October 18 - 21, 2015 Minneapolis, MN.
- **Tuncil Y.E.**, Xiao Y., Porter N., Martens E.C., and Hamaker B.R. 2015. Dietary fibers as presented in a meal are utilized in a hierarchical order. Institute of Food Technologists (IFT) Annual Meeting, July 11-14, 2015 Chicago, IL.
- **Tuncil Y.E.**, Martens E.C., and Hamaker, B.R. 2013. Dietary Fiber Preferences of Bacteria Strains May be a Useful Tool to Manipulate Colonic Bacteria via Diet. Whistler Center Board Meeting and Technical Conference. May 12 – 13, 2013 West Lafayette, IN.
- **Tuncil, Y.E.**, Fevzioglu, M., Campanella, O.H., and Hamaker, B.R. 2013. Accessible protein body-free sorghum kafirin show better functionality than kafirin in normal sorghum. AACCI Annual Meeting. September 30 – October 3, 2013 Albuquerque, NM.
- **Tuncil, Y.E.**, Jondiko, T.O, Tilley M., Hays, D.B, and Awika, J.M. 2012. Analysis of *Glu-1* Deletion Lines Reveals the Importance of High Molecular Weight Glutenin Sub-unit 7+9 at *Glu-B1* locus on Wheat Flour Tortillas Quality. AACCI Annual Meeting. September 30-October 4, 2012 Hollywood, FL.
- Jondiko, T.O., **Tuncil, Y.E.**, Puerta-Gomez, A.F., Castell-Perez, M.E., and Awika, J.M. 2012. Use of dynamic tests to determine the effects of sodium reduction on dough rheology and its correlation with tortilla quality. AACCI Annual Meeting. September 30-October 4, 2012 Hollywood, FL.