AN ABSTRACT OF THE THESIS OF

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Title: The Preparation of Common Prebiotic Oligosaccharides with Defined Degree of Polymerization

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Prebiotics are a subset of dietary fiber that is growing in demand within the food industry. The health benefits of prebiotics have been well established, leading to the increase in its incorporation into various food products. Given the importance of prebiotics as functional ingredients, it is important to understand their sensory properties. However, such knowledge is not well established because commercially available prebiotics are a mixture of saccharides with varying degree of polymerization (DP), including mono- and disaccharides. The goals of this research were to develop fractionation methods to prepare common prebiotic oligosaccharides [i.e., fructooligosaccharides (FOS), galacto-oligosaccharides (GOS), and xylooligosaccharides (XOS)] with well-defined DP from commercially available prebiotic oligosaccharides mixtures and to conduct chemical analysis to characterize the fractionated prebiotic oligosaccharides.

To achieve the first goal, column chromatography was performed following principles of adsorption chromatography, whereby the analyte adsorbed to the surface of the stationary phase, then desorb and elute from the column by using a gradient mobile phase. During the chromatography run, cellulose was used as the stationary phase, whereas ethanol/water mixtures were used as the mobile phase. The packing material and solvents were selected based on their Generally Recognized as Safe (GRAS) status. The mobile phase was delivered to the column as a

step gradient within the range of 85%-55% ethanol. The percentage used was determined by the solubility of the oligosaccharide, which were impacted by their chemical structures with heterogenous saccharides being more soluble (i.e., FOS, GOS) than homogenous saccharides (i.e., XOS). The specifics for chromatography conditions (i.e., sample load, volume of solvent, and mobile phase composition) differ based on the class of oligosaccharide and were tailored to best fit the separation capabilities of each oligosaccharide. It was important to find a balance between resolution, which impacted yield, and time taken for the chromatography run. Although the amount produced had relatively low (30-75%) recovery, the study made use of the fact that economical preparation does not require baseline resolution since the commercially available starting materials were relatively inexpensive.

To confirm the identify and purity of the fractionated oligosaccharide preparations (FOS DP3, FOS DP4, GOS DP3, GOS DP4, XOS DP2, XOS DP3, and XOS DP4), various chemical analyses were performed. These included total carbohydrate analysis, moles quantification, nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC). Total carbohydrate analysis found that each prebiotic fraction was approximately 99-100% carbohydrate on a dry weight basis. Results from the moles quantification experiment and NMR analysis confirmed that the DP corresponds with the targeted profile for each oligosaccharide. HPLC results further verified the identity and purity for each oligosaccharide preparation through comparison with commercially available standards.

Overall, this research produced and characterized seven fractions of prebiotic oligosaccharides with distinct chemical structure. This economical method of obtaining purified, fractionated prebiotic oligosaccharide is valuable to researchers interested in studying the properties of prebiotic oligosaccharides with specific chain lengths.

The Preparation of Common Prebiotic Oligosaccharides with Defined Degree of Polymerization

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Megan C.Y. Ooi, Author

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CONTRIBUTION OF AUTHORS

Megan Ooi wrote Chapters 1 and 3. Megan Ooi performed the data collection for Chapter 2. Megan Ooi, Juyun Lim, and Michael Penner were involved with research design for Chapter 2. Chapter 2 was written by Megan Ooi, Juyun Lim, and Michael Penner, with inputs from Christopher Beaudry. Xiaojie Zhang assisted with NMR analyses.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Foods for health

The Greek physician Hippocrates once said, "let food be thy medicine and medicine be thy food." This idea stresses the importance of including foods with beneficial properties into our daily diet for good and balanced health. Research has shown the link between nutrition and prevention of diseases such as osteoporosis (Cashman, 2007; Stránský & Ryšavá, 2009), hypertension (Vasdev et al., 2001), gastrointestinal diseases (Ford et al., 2014; Tuohy et al., 2005), cardiovascular diseases (Ignarro et al., 2007; Sala-Vila et al., 2015), and vitamin deficiency related disorders (Mozos & Marginean, 2015; Sommer, 2008). Demand for healthy foods has been increasing over the past few decades as consumers become more conscious about the food they consume (Feng & Chern, 2000). This can be seen with the increasing number of functional foods appearing on the market and new functional food products being launched.

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) stated:

> "A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of diseases. Functional foods must remain foods, mostly composed of bulk ingredients and they must demonstrate their effects in amounts that can be expected

for normal consumption patterns: they are not pills or capsules, but part of a normal diet." (Contor, 2001)

It is estimated that more than half of the adults in the United States make food choices for health purposes (Tee et al., 2004). Older individuals tend to make their selection based on potential disease risk reduction, whereas younger individuals opt for foods that improve mental and physical performance (Milner, 2002; Powell et al., 2019). The global market for functional foods was estimated to be valued at $173 billion in 2019 and projected to hit $309 billion by the year 2027 (Precendence Research, 2020), with the largest consumers being Japan, the United States and Europe (Vicentini et al., 2016).

1.2 Functional ingredients

The functional foods can further categorized into five types based on whether the bioactive compound is naturally existing or is introduced into the food product (see table 1). The diversity of food that is considered functional suggests that numerous bioactive compounds exist in nature, with an estimated more than 20,000 unique chemical compounds present within the fruits and vegetables consumed by human (Tee et al., 2004). This suggests that functional components can be extracted from various sources (e.g., plants and animal products) and used as added ingredients within the food industry. Among these chemical compounds, a handful have gained the most attention due to their health-influencing properties. These compounds include carotenoids, flavonoids, curcumin, fermentable fibers, and omega fatty acids (Patil et al., 2009; Tee et al., 2004).

Functional foods must be able to deliver their bioactive compound to the body to observe the health benefits. The portion of the total compound mass absorbed and used per its intended

function is called bioavailability (Price & Patel, 2021). Nutrient absorption (Hollman, 2004; McGhie & Walton, 2007; Sandström, 2001) and the food matrix (Parada & Aguilera, 2007) impact the bioavailability of functional foods. These two factors determine the most effective methods for delivering nutrients to ensure the greatest absorption.

Table 1. Types of functional food

Type of functional food	Example
Naturally contains sufficient amounts of a beneficial component	Beta-glucans in oats
Naturally enhanced through special growing conditions such as new feed composition, genetic manipulation, or other methods	Plant breeding to produce golden rice
Modified formulation that incorporates a functional ingredient	Prebiotics and/or probiotics being added to food products
The nature of one or more components or their bioavailability in humans has been modified through specialized food processing technologies	Fermentation using specific bacteria to yield bioactive peptides
A deleterious component has been removed, reduced or replaced with another substance with beneficial effects	Chewing gum sweetened with xylitol instead of sugar

(Kotilainen et al., 2006; Spence, 2006)

1.3 Prebiotics

Prebiotics are fermentable fibers that affect an individual's health through the microflora community in the gastrointestinal tract. Over the past 30 years, significant research has been carried out on prebiotics and their impact on gastrointestinal health and food applications.

1.3.1 Definition of prebiotics

Prebiotics are dietary fibers that are fermented in the gastrointestinal tract by gut microbiota, producing bioactive compounds such as short-chain fatty acids that are utilized by the host (Cummings et al., 2001; Fung et al., 2017; Silva et al., 2020). The majority of prebiotics are a subset of carbohydrates and are typically oligosaccharides. Oligosaccharides, in turn, are defined as having 3-10 saccharide units (Cummings & Stephen, 2007). According to Wang (2009), the major criteria for a food to fit the prebiotic classification include:

1. Resistant to the upper gastrointestinal tract
2. Able to be fermented by intestinal microbiota
3. Beneficial to the health of the host
4. Selectively proliferates beneficial gut microorganisms
5. Able to withstand food processing treatments

The current definition, developed during the 6th annual meeting by the International Scientific Association of Probiotics and Prebiotics (ISAPP), describes prebiotics as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health" (Gibson et al., 2010). Bindels et al., (2015) provided further insight and specifications by defining prebiotics as "A

nondigestible compound that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus conferring a beneficial physiologic effect on the host."

1.3.2 Types and sources of prebiotics

To date, the most prevalent prebiotic oligosaccharides in terms of weight produced include fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), and soybean oligosaccharides (Al-Sheraji et al., 2013; Wang, 2009). Other types of substances that are not classified as oligosaccharides but fit the prebiotic definition include the disaccharides lactulose and kojibiose; the polysaccharides polydextrose and inulin; and polyols such as isomaltitol and lactitol (Binns, 2013; Carlson et al., 2018; Ruiz-Aceituno et al., 2018). Table 2 shows a list of prebiotic types along with their structure and sources.

Table 2. List of prebiotics with molecular structure and their sources. The types of prebiotic oligosaccharide listed below are examples of those used commercially.

Type of prebiotic	Structure [a]	Examples of prebiotic sources
Fructooligosaccharides	Glu – $(Fru)_n$ n=2-9	Synthesized from sucrose; Hydrolyzed from inulin
Fructans (i.e., inulin, levan)	Glu – $(Fru)_n$ $n \geq 10$	Extracted from chicory root and Jerusalem artichoke
Lactulose	Fru – Gal	Synthesized from lactose (milk)
Lactosucrose	Fru – Glu – Gal	Synthesized from lactose (milk)

Xylooligosaccharides	$(Xyl)_n$	Hydrolyzed from xylans
Soybean oligosaccharides	$Fru – Glu – (Gal)_n$	Extracted from soybean
Raffinose	Fru – Glu – Gal	Extracted from legumes, soybean, chickpeas, and beans
Stachyose	$Fru – Glu – (Gal)_2$	Extracted from legumes, soybean, chickpeas, and beans
Isomaltooligosaccharide	$(Glu)_n$	Processed from starch (rice and tapioca)
Galactooligosaccharide	$Glu – (Gal)_n$	Synthesized from lactose

(Al-Sheraji et al., 2013; Mussatto & Mancilha, 2007; Sako et al., 1999; Wang, 2009)

[a] Glu: glucose; Fru: fructose; Gal: galactose; Xyl: xylose

Prebiotic oligosaccharides can be found naturally in vegetables (e.g., onions, soybeans, asparagus), fruits (e.g., banana, nectarine, watermelon), milk (e.g., human, cow, and goat), and honey. Depending on the food source, the percentage of prebiotic oligosaccharide is typically up to 4% (Jovanovic-Malinovska et al., 2014). However, chicory root and Jerusalem artichokes are exceptions, with prebiotic content of around 14% and 23% (Moshfegh et al., 1999). Table 3 provides average amounts of prebiotic oligosaccharides found in common fruits and vegetables. The recommended serving of dietary prebiotic to observe its benefits is an average of about 6 g/day (Manning & Gibson, 2004; Moshfegh et al., 1999).

Table 3. Amount of prebiotic oligosaccharides naturally present in various plant material

Fruit/Vegetable	Average amount of prebiotic oligosaccharide (gram oligosaccharide per 100 g fresh weight)
Nectarine	0.9
Pear	0.6
Watermelon	0.8
Jerusalem Artichoke	13.5
Broccoli	0.8
Garlic	5.0
Scallion	4.1
Chicory Root	22.9

(Jovanovic-Malinovska et al., 2014; Moshfegh et al., 1999)

1.3.3 Health Benefits of prebiotics

Prebiotics are substrates that help with the growth of indigenous microbial communities. Consumption of prebiotics allows for the stimulation of these bacteria in the gut. Research has shown that introducing prebiotics in the diet has a significant impact on gut microorganisms that improves the health of the host (Jung et al., 2015; Monteagudo-Mera et al., 2016; Neyrinck et al., 2011). Among the clinical studies performed, the most used prebiotics are fructooligosaccharides and galactooligosaccharides. Many microbial species can utilize these prebiotics for growth and proliferation, such as Bifidobacteria and Lactobacilli. These gut microorganisms produce B-vitamins and digestive enzymes, such as casein phosphatase and lysozyme (Teitelbaum & Walker, 2002). Addtionally, proliferation of beneficial bacteria was found to inhibit the growth and prevent

the spread of pathogenic microorganisms. Apart from that, individuals with lactose intolerance may benefit from *Lactobacillus* s*pp* proliferation, as this microorganism aids in the digestion of lactose (Mussatto & Mancilha, 2007; Teitelbaum & Walker, 2002).

Furthermore, prebiotics also provide many other health benefits such as boosting the immune system (Frei et al., 2015; Shokryazdan et al., 2017), improving calcium absorption (Cashman, 2003; Scholz-Ahrens et al., 2001), decreasing the risk of colorectal cancer (Ambalam et al., 2016; Lim et al., 2005; Raman et al., 2013), and reducing occurrences of irritable bowel syndrome (IBS) (Ford et al., 2014; Ooi et al., 2019; Spiller, 2008). These benefits show the importance of prebiotics to gut health and the significance of gut health to the individual's overall well-being.

1.3.4 Functional properties of size-defined prebiotics

In the past, studies have investigated functional properties of prebiotic oligosaccharides composed of different chain lengths within the same class of oligosaccharide (Fonteles & Rodrigues, 2018). Only recently have researchers investigated the qualitative characteristics of oligosaccharides with specific degrees of polymerization (DP). Recent studies reported kestose (fructooligosaccharide with DP3) proliferating the growth of bifidobacteria and *F. prausnitzii*, a next-generation probiotic strain (Tochio et al., 2018). Among the common XOS, xylotriose was best at bifidogenic proliferation, followed by xylobiose and xylotetraose (Gullón et al., 2008).

Nystose (a fructooligosaccharide with DP4) was found to be low-cariogenic compared to sucrose, meaning it is less likely to cause tooth decay (Ikeda et al., 1990). Furthermore, nystose does not break down into its monomeric unit (glucose and fructose) making it a potential alternative for diabetic patients (Bharti et al., 2015). From an industry perspective, removing simple sugars

from prebiotic mixtures will lower the cariogenicity and caloric value, allowing diabetic patients to consume them.

The plant-derived disaccharide cellobiose was able to repress the pathogenicity of *Listeria monocytogenes* by suppressing crucial virulence factors (Park & Kroll, 1993). These findings suggest expanding the prebiotic concept to include substances able to reduce microorganism pathogenicity.

Prebiotic oligosaccharides are well established in terms of their health benefits, leading to the increase in its incorporation in various food products. This raises the question as to how their incorporation would affect the sensory and structural properties of food.

1.4 Prebiotics in the food industry

1.4.1 Global Market of prebiotics

Prebiotic oligosaccharides have been garnering worldwide interest ever since their beneficial and functional properties were established (see 1.3). Prebiotics have an estimated global market value of $8.95 billion as of 2020 and are forecasted to soar even more in the coming years (Insights, 2021). This value is likely to continuously grow with the increasing awareness from consumers regarding its health benefits and application in various fields.

1.4.2 Food Industry Applications of prebiotics

Non-digestible oligosaccharides are used in confectionery, bakery, and brewing industries as dietary fibers, sweeteners, weight-controlling agents, and humectants (moisture retention). Prebiotics are often applied in foods for a double benefit – improving mouthfeel and providing

enhanced nutrition (Franck, 2002). Prebiotics have been incorporated into various foods such as yogurts, beverages, baked goods, chocolates, and baby food (Wang, 2009). Recent studies have looked at the sensory impacts of adding prebiotic oligosaccharides into cheese (Belsito et al., 2017; Ferrão et al., 2018), chocolates (Cardarelli et al., 2008; Konar et al., 2016), and fruit juices (Fonteles & Rodrigues, 2018).

1.4.3. Sensory properties of prebiotics

The type of prebiotic is dependent on the food system involved. For example, oligofructose was described to have technological properties closely related to sugar (i.e., bulking properties), making it a great sugar substitute. FOS are mildly sweet, approximately 0.3-0.6 times as sweet as sucrose (Crittenden & Playne, 1996; Franck, 2002; Mussatto & Mancilha, 2007). FOS can also produce a gel at high aqueous concentrations, described as having a creamy fat-like texture resulting in fat mimetic properties (Huber & BeMiller, 2017). FOS is potentially suitable for beverages or meat product applications as it is mildly sweet-tasting and has fat-like properties. GOS are widely used in dairy foods such as infant formula and yogurt due to their stability and prebiotic properties. XOS is about 0.3 times as sweet as sucrose, non-cariogenic, low in calories and stable over a wide range of pH (2.5-8.0) and temperature (up to 100˚C) making it a great candidate for confectionery and bakery applications (de Freitas et al., 2019; Vázquez et al., 2000).

An interesting discovery recently showed that humans are able to taste starch-derived maltooligosaccharides (MOS) (Lapis et al., 2016; Pullicin et al., 2017). Furthermore, MOS DP3 was stated described as sweet, whereas MOS DP4 and above were detected independent of the sweet taste receptor (Pullicin et al., 2017). In a recent study on the sensory properties of commercial prebiotic oligosaccharides, they were found to have a mildly sweet and clean taste

profile (Ruiz-Aceituno et al., 2018). In contrast, a recent study showed that oligofructose mixtures (average DP 10) were able to be tasted independent of the sweet taste receptor at low concentrations (Low et al., 2017). These findings imply that prebiotic oligosaccharides elicit a taste response, but further studies are necessary to confirm the qualitative properties of these responses.

It is important to understand the sensory properties of prebiotic oligosaccharides given their importance as functional ingredients in food, and the impact of different chemical structures. However, evaluating the psychophysical properties of specific prebiotic oligosaccharides is challenging due to a lack of food grade, DP-defined product available. Often, the prebiotic oligosaccharides available commercially are mixtures of saccharides with varying DP, including mono- and disaccharides. Fractionating oligosaccharides would also allow for more in-depth studies on the functionalities of specific DP oligosaccharides and how they compare to digestible oligosaccharides.

1.5 Production and purification methods of prebiotic oligosaccharides.

1.5.1 Production

The production of prebiotic oligosaccharides can be performed through physical, enzymatic, or chemical methods.

1.5.1.1 Physical methods

One of the methods to produce oligosaccharides is through physical processes, such as aqueous extraction. This method is accomplished by first cooking the raw food item, followed by the extraction process. Extraction is most commonly used on soybeans to obtain raffinose family oligosaccharides and soybean oligosaccharides (Ku et al., 1976). Another physical method recently

investigated used ultrasound to assist the extraction of oligosaccharides from fruits and vegetables. Although this method produces a higher amount of product compared to conventional extraction methods, it requires specialized ultrasound water bath equipment which might not be available in most labs (Jovanovic-Malinovska et al., 2015).

1.5.1.2 Enzymatic Synthesis

Enzymatic synthesis of prebiotics are sometime difficult due to the sporadic and unpredictable nature of the enzymes involved in production (Chen, 2018; Rabelo et al., 2006). On top of that, due to the reactive hydroxyl groups present within the carbohydrates, the chemical synthesis route is less ideal, and often large-scale production opts for the enzymatic approach (Vera et al., 2021). Enzymatic synthesis of oligosaccharides uses sugars (most commonly lactose and sucrose) as the starting material and utilizes the enzymatic activity of glycosyltransferases (e.g., fructosyltransferase) to produce oligosaccharides.

Industrial level production of FOS can be achieved through transfructosylation of sucrose using β-fructofuranosidases (Singh & Singh, 2010). Transfructosylation is the process of transferring a fructose moiety from a fructose donor to a suitable acceptor. This process results in the donor losing one fructosyl unit and the acceptor gaining one (Herrera-González et al., 2017; Zambelli et al., 2014). Synthesis using sucrose as the starting material is common for FOS production as sucrose is inexpensive. The final products have a smaller DP range (up to 5) and all molecules have a glucose moiety at the terminal unit. The most prevalent FOS produced from this method are 1-kestose (DP3), nystose (DP4), and fructosylnystose (DP5) (Hidaka et al., 1990; Yun, 1996). However, this production method often includes unreacted sucrose and residual glucose, which require some cleaning up to remove the sugars.

GOS production, on the other hand, is performed exclusively through the transgalactosylation of lactose using a β-galactosidase (Lamsal, 2012). Transgalactosylation is the process whereby the B-galactosidase enzyme uses lactose as a galactosyl-donor and transfers the galactosyl unit onto a suitable acceptor. In the case of GOS production, lactose is used as the galactosyl-acceptor through the activity of B-galactosidase (Guo et al., 2018; Otieno, 2010). Lactose is one of the most easily accessible raw materials for producing prebiotic oligosaccharides, as it is available in whey from cheese production. The enzymatic production uses lactose as the galactosyl donor through the activities of β-galactosidases. The yield and types of GOS formed are dependent on the reaction conditions and types of enzymes used (Lamsal, 2012). GOS structure is unique in that it has β(1→2), β(1→3), β(1→4), or β(1→6) glycosidic linkages, though β(1→2) and β(1→3) occur less frequently (Austin et al., 2014). The source of enzyme influences the type of glycosidic linkages formed, subsequently the saccharide conformation (Chen & Gänzle, 2017; Gosling et al., 2010; Torres et al. 2010). Higher DP GOS production requires enzymes with a preference to catalyze the transgalactosylation reaction of lactose rather than the hydrolysis of GOS as the same enzyme is responsible for both processes (Mahoney, 1998). Torres et al., (2010) described an extensive list of different microbial sources of galactosidases along with the optimum conditions required to catalyze the reaction. Similar to FOS production through synthesis, GOS production almost always includes residual lactose and requires additional steps for removal. Another prebiotic produced through enzymatic synthesis is lactulose, an isomer of lactose (Panesar & Kumari, 2011).

1.5.1.3 Enzymatic Hydrolysis

Enzymatic hydrolysis produces oligosaccharides by cleaving glycosidic bonds within polysaccharides to produce shorter chain molecules. The enzyme family used during this process is glycoside hydrolases, such as inulinase, xylanase, and arabinohydrolase.

FOS can be produced through inulin hydrolysis using endo-inulinases (Singh & Singh, 2010). FOS production from the hydrolysis of inulin had a higher yield and higher DP range (up to 10) compared to the enzymatic synthesis method (Singh & Singh, 2010). The products from this method of production are two types of FOS: FOS made of fructosyl moieties with terminal fructose, or FOS made of fructosyl moieties linked together with terminal glucose, which are naturally present at the terminus location on inulin. Enzymatic hydrolysis can also be used to make XOS from xylan. The polysaccharide xylan is available in large amounts as by-products from agriculture, forest, wood, and paper industries. The structure of xylan is a linear backbone made of xylose units with β(1→4) linkages. Xylan is one of the main polysaccharides in hemicellulose. Xylan extraction from hemicellulose is performed by an acid and alkaline treatment (Vázquez et al., 2000). The extracted xylan then undergoes enzymatic hydrolysis using endo-xylanase to produce low DP XOS (Moure et al., 2006; Zhu et al., 2015), with additional auxiliary enzymes to hydrolyze any branching (de Freitas et al., 2019). There are two families of endo-xylanase involved in the hydrolysis of XOS, the GH 10 and GH 11 families. Both differ in their substrate specificity, with the GH 11 targeting xylans while the GH 10 occasionally targets XOS. The product from GH 10 endo-xylanase hydrolysis is lower DP XOS. Hydrolyzed XOS typically are up to DP4, with all XOS comprised of xylosyl units linked to a terminal xylose.

1.5.1.4 Chemical Processes

Aside from enzymatic processes, chemical processes are another route for prebiotic oligosaccharide production. This method is less common than the enzymatic approach but nonetheless still a potential outlet. The synthetic prebiotic disaccharide lactulose is produced through the chemical isomerization process of lactose (Aider & Halleux, 2007; Gänzle, 2011; Panesar & Kumari, 2011). Other studies have been successful in chemically synthesizing various human milk oligosaccharides (HMO) (Bandara et al., 2019, 2020).

These production methods show that there are residual sugars present in the final product and the chemical makeup of each type of prebiotic oligosaccharide is also different. There has yet to be a study performed on the impact of the chemical structure differences on qualitative (i.e., psychophysical) properties of the prebiotics.

1.5.2 Purification

Most of the commercially available prebiotic saccharide products have residual sugars. To study the effects of chemical structure on taste, we must first isolate the prebiotics of interest, presenting another set of hurdles. Pure and structurally defined prebiotic oligosaccharides are critical for proper psychophysical studies. Various methods have been described to clean up and remove sugars, including enzyme bioreactors on FOS (Fan et al., 2020), silica gel column chromatography (Zhu et al., 2015) and membrane processing (Gullón et al., 2008) on XOS, crossflow nanofiltration on GOS (Goulas et al., 2002), and crystallization with ethanol on lactulose (Panesar & Kumari, 2011). Recently, methods for starch-derived maltooligosaccharide and maltopolysaccharide fractionation were demonstrated by Balto et al. (2016) and Pullicin et al.

(2018) using food-grade materials. However, there is not a method for the fractionation of prebiotic oligosaccharides that specifically uses all food-grade materials.

1.6 Research objectives

The overall goal of this study was to produce DP-defined prebiotic oligosaccharides that can be used in human research. To achieve this, the study was divided into two objectives: 1) fractionation methods to prepare common prebiotic oligosaccharides with well-defined DP from commercially available prebiotic oligosaccharides mixtures, and 2) to conduct chemical analysis to characterize the fractionated prebiotic oligosaccharides.

The first objective was to develop simple, cost-effective fractionation methods for the preparation of FOS, GOS, and XOS for use in human foods and/or human testing. This was accomplished by using commercially available prebiotic oligosaccharide as the starting material and food-grade stationary and mobile phases for chromatography. Chapter 2 describes in detail the differences and optimization for fractionating each oligosaccharide preparation (See section 2.4.1). The commercial prebiotics were selected based on the relatively low sugar present. It was important to prepare these fractions in such a way that impurities (i.e., sugars and residual solvents) would not end up in the final preparation. In this instance, sugars would be considered impurities as their properties are already well established.

The second objective was to characterize each fractionated oligosaccharide preparation, and to address the difficulty when working with different classes of oligosaccharides differing with respect to chemical makeup. Various methods of analysis were conducted to determine the purity and identity of each oligosaccharide preparation. Chapter 2 also describes in detail the methods of

chemical analysis to characterize the prebiotic through spectrophotometric and chromatographic processes (see section 2.4.2).

Overall, chapter 2 provides an inexpensive approach to fractionate on average 40-270 mg (per run) of isolated, food-grade prebiotic oligosaccharides that are suitable for use in future human sensory work along with methods for verifying the characteristics of the prebiotic oligosaccharide preparation

CHAPTER 2

Chromatographic preparation of food grade prebiotic oligosaccharide with defined degree of polymerization

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Submitted
Food Chemistry

Chromatographic preparation of food-grade prebiotic oligosaccharides with defined degree of polymerization

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Key words: prebiotics, oligosaccharides, size-fractionation, adsorption chromatography, separation

2.1 Abstract

Prebiotic oligosaccharides are of widespread interest in the food industry due to their potential health benefits. This has triggered a need for research into their functional and sensory properties. Such research is currently limited due to the lack of available well-defined food-grade oligosaccharide preparations. The aim of this study was to develop economical approaches for the preparation and characterization of prebiotic oligosaccharides differing with respect to composition and degree of polymerization (DP). Such preparations were prepared by chromatographic fractionation of commercially available prebiotic mixtures using microcrystalline cellulose stationary phases and aqueous ethanol mobile phases. This approach is shown to work for the preparation of food-grade fructooligosaccharides of DP 3 and 4, galactooligosaccharides of DP 3 and 4, and xylooligosaccharides of DP 2-4. Methods for the characterization of the different classes of oligosaccharides are also presented including those addressing purity, identity, total carbohydrate content, moles per unit mass, and DP.

2.2 Introduction

Prebiotics are currently defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). This definition encompasses commercially available prebiotic oligosaccharide products, which are mixtures containing different-sized oligosaccharides. In recent years, the interest in prebiotic ingredients continues to expand due to their beneficial impact on human health and the related marketing value. Potential health benefits include improving digestion and gastrointestinal health and cardiovascular function, reducing adherence of pathogenic bacteria to intestinal epithelial cells, and reducing the risk of colorectal cancer (Davani-Davari et al., 2019). Dietary prebiotics are also incorporated into foods for their organoleptic effects (Guimarães et al., 2020; Wang, 2009), such as reduced-calorie fat replacers or bulking agents.

For a food ingredient to be classified as a prebiotic, it must: 1) be able to withstand food processing treatments such as high temperatures and low pH, 2) be able to withstand digestive processes before reaching the colon, 3) be selectively fermented by beneficial bacteria in the colon, 4) promote growth and proliferation of beneficial bacteria, and 5) provide benefit to the host's well-being and health (Gibson et al., 2004; Wang, 2009). Although not all prebiotics are carbohydrates (e.g., flavonols), the majority of prebiotics are oligosaccharides, a subset of carbohydrates (Davani-Davari et al., 2019). Oligosaccharides, in turn, are often defined as having 3-10 units (Cummings & Stephen, 2007). Herein, short-chain prebiotic oligosaccharides refer to short-chain carbohydrates containing 3-4 monomeric units.

Prebiotics are obtained in the diet through a variety of natural sources, including fruits (e.g., banana, nectarine, watermelon), vegetables (e.g., onion, soybeans, asparagus, wheat, garlic), honey, and maternal milk (for types and sources of prebiotics, see Al-Sheraji et al., 2013; Gänzle, 2011;

Jovanovic-Malinovska et al., 2014). However, the quantity of prebiotic oligosaccharides present in most natural sources is low relative to the amounts thought necessary to elicit their beneficial effects (Davani-Davari et al., 2019), although there are some exceptions (e.g., chicory root, Jerusalem artichoke). As such, there is a growing market for prebiotic oligosaccharide-fortified food products (Fonteles & Rodrigues, 2018; Manning & Gibson, 2004). The most prevalent prebiotic oligosaccharide ingredients in food products are fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (Al-Sheraji et al), with xylooligosaccharides (XOS) gaining in popularity over the past few years (Vázquez et al., 2000). These prebiotic oligosaccharides are also sold and consumed in a wide range of supplements (Carlson et al., 2018).

Prebiotic oligosaccharides differ from one another with respect to chemical structure (see **Fig 2.1**). Structural differences include their unique monomeric units (glucose, fructose, galactose, and xylose), glycosidic linkages [β(1→2), β(1→3), β(1→4), or β(1→6)], and degree of heterogeneity. FOS refers to oligosaccharides of D-fructose residues linked by β(2→1) bonds with a terminal sucrose (fructose-α(2→1)-glucose) (Hidaka et al., 1990; Loo et al., 1999; Yun, 1996). GOS, on the other hand, are oligosaccharides made up of D-galactose linked through β(1→2), β(1→3), β(1→4), or β(1→6) bonds with a terminal lactose (galactose-β(1→4)-glucose) (Gänzle, 2011; Splechtna et al., 2006). Due to the nature of the synthetic process for the production of GOS, which involves β-galactosidase-catalyzed transgalactosylation, the resulting GOS is a heterogeneous mixture comprised of GOS differing with respect to glycosidic linkages and chain lengths; essentially all of the constituent GOS contain lactose (galactose-glucose) at the reducing end (Nauta et al., 2009). XOS are made up of D-Xylose linked through β(1→4) bonds (Loo et al., 1999). Within each class of prebiotic oligosaccharide, the number of monomeric units making up the chains can differ, resulting in homologs with different degrees of polymerization (DP).

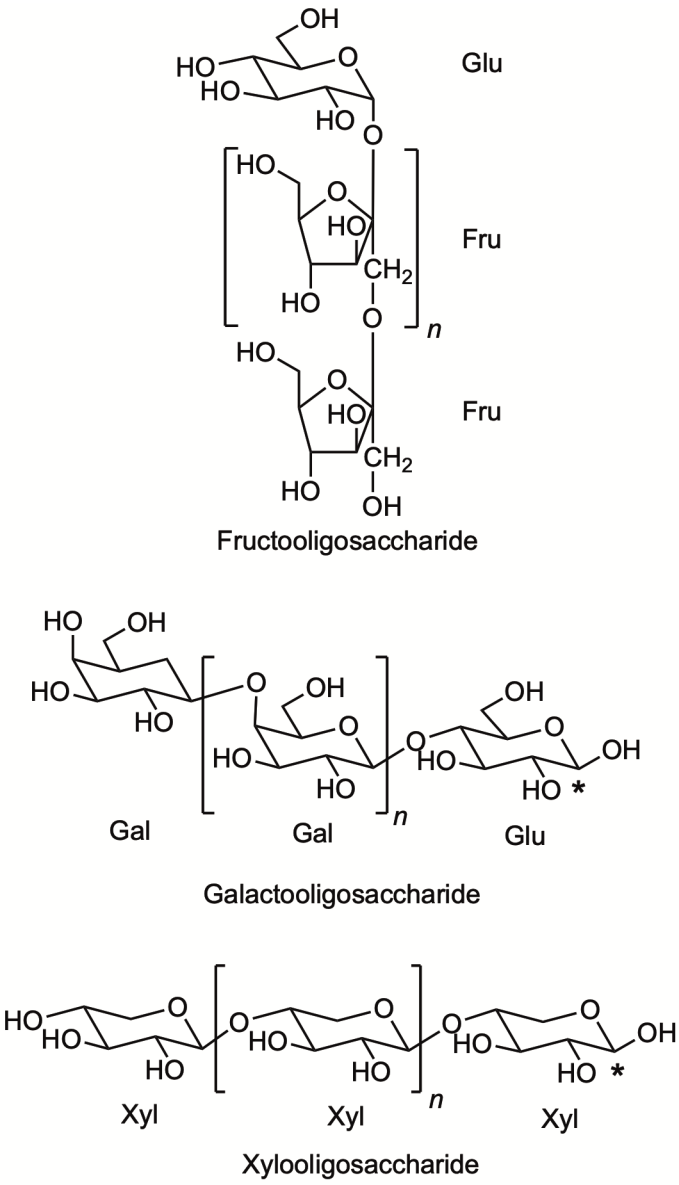


Fig 2.1. Structures of common prebiotic oligosaccharides. The following terms refer to the respective monosaccharides; Fru: fructose, Glu: glucose, Gal: galactose, Xyl: xylose.* denotes the reducing end of the oligosaccharide.

The functional properties of prebiotic oligosaccharides are becoming of greater importance due to their increased prevalence in foods. For example, it is relevant to understand sensory

properties of different prebiotic oligosaccharides, and how these sensory properties differ with chain length and other chemical makeup, particularly given recent findings that humans can taste oligosaccharides derived from starch (Lapis et al., 2014, 2016; Pullicin et al., 2017). Moreover, prebiotic oligosaccharides with different structural properties could confer different health benefits (Belorkar & Gupta, 2016; Davani-Davari et al., 2019). Studying the sensory and functional properties of specific prebiotic oligosaccharides has been challenging because prebiotic oligosaccharides are commonly sold as a mixture of oligosaccharides differing with respect to DP and also including mono- and disaccharides (e.g., glucose, sucrose, xylose). Therefore, the fractionation of prebiotic oligosaccharides based on size is necessary to investigate the relationships between the DP of a prebiotic oligosaccharide and its sensorial and functional properties.

Balto et al. (2016) recently fractionated food-grade maltopolysaccharides (MPS) and maltooligo-saccharides (MOS) from starch and corn syrup solids based on their differential solubilities in aqueous ethanol solutions. That approach was subsequently improved to enable the preparation of food-grade MOS preparations with reduced DP heterogeneity. This was accomplished by incorporating a chromatographic fractionation step based on the interaction of MOS with cellulose-based stationary phases (Pullicin et al., 2018). The method developed by Pullicin et al. (2018) was adapted in this study to allow the preparation of lower molecular weight FOS, GOS, and XOS oligosaccharides of defined DP. Cellulose was used as the chromatographic stationary phase and aqueous ethanol as the mobile phase. Both the stationary and mobile phases can be obtained as food grade materials and thus the method is appropriate for the preparation of prebiotic oligosaccharides suitable for human testing.

The study reported in this paper had two objectives. The first was to develop fractionation methods for the preparation of research-ready, food-safe, DP-defined FOS, GOS, and XOS for use in human foods and/or human testing. The second objective was to develop a series of relatively

straight-forward analytical methods for the characterization of oligosaccharide preparations. This second objective addresses the difficulty that arises when working with oligosaccharides differing with respect to chemical makeup.

2.3 Materials and Methods

2.3.1 Materials

Prebiotic oligosaccharide starting materials used in this study were NUTRAFLORA® P-95 (FOS; Ingredion Inc., Bridgewater, NJ), BIOLIGO™ GL-5700 IMF (GOS; Ingredion Inc. Bridgewater, NJ), and PreticX 95 (XOS; AIDP Inc., City of Industry, CA). Saccharide analytical standards were glucose monohydrate and maltose monohydrate from Spectrum Chemical (Gardena, CA); 1-kestose, nystose, D-galactose, D-lactose, D-xylose, D-cellobiose, sucrose, and D-fructose from Sigma-Aldrich (St. Louis, MO); and xylobiose, xylotriose, and xylotetraose from Megazyme (Bray, Ireland). Solvents were ACS/USP-grade 100% ethanol from Pharmco Aaper (Shelbyville, KT), butanol (n-butanol ≥99%, FCC, FG) from Sigma-Aldrich (St. Louis, MO), HPLC/ACS-grade acetonitrile (CAS 75-05-8) from Fischer Scientific (Fair lawn NJ), deionized (DI) water (18.2 Ω, produced using a Millipore Direct-Q® 5 UV-R water purification system), and deuterium oxide 99.96% (Cambridge Isotope Laboratories, Tewksbury, MA). Chemical reagents included 1-naphthol (ReagentPlus® ≥99%), L-serine (ReagentPlus® ≥99% HPLC) (CAS 56-45-1), ACS-grade calcium carbonate (CAS 471-34-1), and thiourea (CAS 62-56-6) from Sigma-Aldrich (St. Louis, MO); sodium carbonate (CAS 497-19-8), ACS-grade sodium bicarbonate (CAS 144-55-8), and disodium 2,2'-bicinchoninate Pierce BCA solids from Thermoscientific (Rockford, IL); ACS-grade copper (II) sulphate pentahydrate (CAS 7758-99-8) from Avantor (Center Valley PA); ACS-grade anthrone (CAS 90-44-8) from Alfa Aesar (Ward Hill, MA); and ACS-grade sulfuric

acid (CAS 7664-93-9) from EMD Millipore (Billerica, MA). Other materials used include microcrystalline cellulose (Avicel PH-101) from UPI Chem (Somerset, NJ), and TLC silica gel 60 plates from EMD Millipore (Billerica, MA).

2.3.2 Methods

Column chromatography was done using a column with 73 mm I.D x 305 mm length with 1 L reservoir and fritted disc (Synthware, Pleasant Prairie, WI).

2.3.2.1 Fractionation of FOS

Column ready sample was prepared by adding 350 mg of FOS powder to 5 ml 85 % aqueous ethanol solution and stirring at 400 rpm and 30 ˚C until a clear solution was achieved. The stationary phase was prepared using 300 g of microcrystalline cellulose (Avicel PH-101; UPI Chem, Somerset, NJ) mixed with 70% aqueous ethanol and carefully poured down the previously wetted walls of the column to prevent splashing. The column was then rinsed with 70% ethanol using compressed air at ~2 psi until the elute turned from yellow to clear and colorless. Final column height was about 20 cm. The column was equilibrated with 100 ml 85% ethanol and allowed to drain until the solvent reached the top of the packing, before the sample was carefully loaded onto the column using a pipette. A one-step gradient was used for the mobile phase. The initial eluent was 1.9 L of 85% aqueous ethanol; the second eluent was 1.5 L of 80% ethanol. The first 1000 ml of eluate typically consisted of glucose, fructose, and sucrose, which were discarded; subsequent eluate was collected in 15 ml fractions.

2.3.2.2 Fractionation of GOS

Column ready sample was prepared by dissolving 675 mg of GOS syrup (74% solids) in 5 ml 80 % aqueous ethanol solution and stirring at 400 rpm at a temperature of 30 ˚C until a clear solution was achieved. The stationary phase was prepared using 250 g of microcrystalline cellulose. Method for column preparation was similar to FOS column preparation (see 2.2.1). Final column height was about 17 cm. The column was equilibrated with 100 ml 85% ethanol, before the sample was carefully loaded onto the column. The initial eluent was 0.9 L of 85% aqueous ethanol; the second eluent was 1.5 L of 80% ethanol. The first 1200 ml of eluate typically consisted of glucose, galactose, and lactose, which were discarded; subsequent eluate was collected in 15 ml fractions.

2.3.2.3 Fractionation of XOS

Column ready sample was prepared by dissolving 1 g of XOS powder in 5 ml 70 % aqueous ethanol solution and stirred at 400 rpm at a temperature of 30 ˚C until it became a clear liquid. The stationary phase was prepared using 250 g of microcrystalline cellulose. Method for column preparation was similar to FOS column preparation (see 2.2.1). The column was equilibrated with 100 ml 75% ethanol, before the sample was carefully loaded onto the column. The initial eluent was 0.9 L of 75% ethanol; followed by 1.5 L of 65% ethanol; and lastly 0.5 L of 55% ethanol. The first 300 ml of eluate consisted of xylose and was discarded; subsequent eluate was collected in 15 ml fractions.

2.3.2.4 Solvent removal and drying

Ethanol was removed from samples using a rotary evaporator (Büchi Rotovapor R-205, Büchi Labortechnik AG) equipped with a water bath set at 55 °C (Buchi B-490) and a vacuum pump (Chemglass Scientific Apparatus/10 Torr). Samples were initially concentrated to a thick syrup, then washed by resuspending the preparation in additional water and then re-concentrating. This washing process was done twice in order to achieve the desired ethanol removal (Balto et al., 2016; Pullicin et al., 2018). The resulting concentrated samples were stored at -23 ˚C until being lyophilized (Labconco Freezone Freeze Dryer, Hampton, NH).

2.3.3. Chemical Analysis

2.3.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was used for initial verification of the chromatographic resolution of oligosaccharide fractions. A capillary spotter was used to deliver eluate onto TLC plates; concentrated spots were obtained by spotting each sample 3 times on a single location. Plates were thoroughly dried before being placed in the solvent chamber. Mobile phases were mixtures of ethanol, butanol, water (ratios were dependent upon the nature of the oligosaccharides). The mobile phase for FOS was 69:14:17 (ethanol, butanol, water; Robyt & Mukerjea, 1994); XOS was 5:3:2 (ethanol, butanol, water; López-Hernández et al., 2018); and GOS was 3:5:2 (ethanol, butanol, water; Rabiu et al., 2001). The staining solution used for FOS and XOS was 5% H_2SO_4 in ethanol with 0.5% a-Naphthol based on the staining solution used for maltodextrins (Robyt & Mukerjea, 1994). However, the staining solution was found to leave very faint and indistinguishable coloring for GOS. Hence, the staining solution for GOS was 35% H_2SO_4 in ethanol with 0.5% a-Naphtol (Manucci, 2009). In all cases, staining solution was applied by

immersion and color development occurred as a result of heating prior dipped plates using a heat gun (General Lab Supply Co., Wayne, NJ) (Manucci, 2009; Rabiu et al., 2001; Tanriseven & Doğan, 2002). TLC plates were analyzed using JustQuantify online software (Sweday, Sodra Sandy, Sweden).

2.3.3.2 High Performance Liquid Chromatography – Evaporative Light Scattering Detector (HPLC-ELSD)

The purity and identity of the oligosaccharide fractions were evaluated via High Performance Liquid Chromatography (HPLC) equipped with evaporative light scattering detection (ELSD). Lyophilized samples were initially dissolved in DI water and then acetonitrile was added to make an oligosaccharide in 60% acetonitrile/40% water solution. Analyses was performed using Prominence UFLC-HPLC system (Shimadzu, Columbia, MD) equipped with a system controller (CMB-20A), degasser (DGU-20A), solvent delivery module (LC-20AD), autosampler (SIL-10A), column oven (CT20-A), and evaporative light scattering detector (ELSD-LT II; kept at 60 °C with nitrogen gas pressure of 350 kPa) on a HILICpak VN-50 4D analytical column and a HILICpak VN-50G 4A guard column (Shodex, New York, NY) for analysis of all samples. The column oven was set to 30˚C for the analysis of FOS; and 60˚C for analysis of GOS and XOS. Standard curves were prepared using commercially available XOS DP 1-4, FOS DP 1-4, GOS DP 1-3. Peak integrations were done using the manufacturer's LC-solution software (Shimadzu, Kyoto, Japan).

2.3.3.3 Reducing Ends Assay

Reducing end assays were done to determine the moles of reducing ends present per given amount of XOS preparations; that data in turn was used to calculate average DP. Reducing ends per unit

weight XOS preparation were quantified using the BCA/copper-based assay as described by Kongruang et al., (2004). BCA working reagent was prepared with equal amounts of solution A and solution B. Solution A contained 54.28 g/L (512 mM) Na_2CO_3, 24.2 g/L (288 mM) of $NaHCO_3$, and 1.942 g/L (5 mM) of disodium 2,2'-bicinchoninate in DI water. Solution B contained 1.248 g/L (5 mM) $CuSO_4$ * $5H_2O$ and 1.262 g/L (12 mM) of L-serine in DI water. Solutions A and B were kept refrigerated in amber bottles until ready for use. Assays were initiated by adding 0.5 mL of BCA working reagent to test tubes containing 1 mL of aqueous carbohydrate preparation. Tubes were immediately topped with a glass marbles, vortexed, and placed into 100 ˚C water bath for 15 minutes. Tubes were then immersed in an ambient temperature water bath to be brought to room temperature. Solutions were then transferred into cuvettes and the absorbance measured at 560 nm using a Shimadzu 160 UV-Vis spectrophotometer. Calibration curves were produced using known concentrations of xylose standard. Assays were done in triplicate. New BCA working reagent was prepared fresh each day.

2.3.3.4 Glucose Assay

Moles of FOS and GOS per given amount of preparations were determined by quantifying the number of glucose molecules present following acid-catalyzed hydrolysis of the oligosaccharide preparations. The assay is based on FOS and GOS having a single glucose moiety per molecule. Oligosaccharide preparations were hydrolyzed as 1 mg/ml solutions in 1% H_2SO_4 (FOS; Nguyen et al., 2009) and 2% H_2SO_4 (GOS; Sophonputtanaphoca et al., 2018) contained in marble-capped test tubes. Hydrolysis tubes were incubated in a boiling water bath for 90 minutes (FOS) and 60 minutes (GOS), followed by immersion in an ice bath for 5 minutes. Samples were then left to equilibrate to room temperature before being neutralized through the addition of calcium carbonate ($CaCO_3$). Neutralized hydrolysates were used for subsequent glucose determination using the

glucose oxidase/peroxidase method as described by the supplier (Sigma Aldrich); the neutralized hydrolysate was also used for chromatographic analyses primarily aimed at verifying complete oligosaccharide hydrolysis. Analytical grade glucose, lactose and sucrose were used as standards. Acid hydrolyses were done in triplicate and glucose assays were done on each hydrolyzed sample.

2.3.3.5. Total Carbohydrate Assay

The carbohydrate content of oligosaccharide preparations was determined using spectrophotometric anthrone/sulfuric acid-based assays (Haldar et al., 2017). Specifics of the assays used for the different oligosaccharide preparations were altered based on the unique reactivities of FOS, GOS, and XOS (see Results and Discussion). In all cases, a 0.1% (w/v) anthrone solution was prepared in 98% ice cold sulfuric acid and allowed to equilibrate for 15-20 minutes before use. Anthrone reagent for XOS also contained 1% (w/v) thiourea for color stabilization. Four ml of anthrone reagent was pipetted into test tubes containing 1.0 ml aqueous carbohydrate solution. Test tubes were immediately capped with marbles and placed in a boiling water bath for 3 minutes. Sample-containing tubes were then placed in an ambient temperature water bath for 10 minutes prior to taking absorbance measurements at 672 nm (FOS and GOS) and 465 nm (XOS) using a Shimadzu 160 UV-Vis spectrophotometer. Calibration curves were produced using aqueous samples of glucose, xylose, fructose, and galactose prepared at 0-1 mg/ml. All samples were assayed in triplicate. Anthrone reagent was prepared fresh on the days of the analyses.

2.3.3.6. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) was used to verify that the spectra of the experimental oligosaccharide preparations matched those of the corresponding analytical standards. NMR was also used to verify removal of residual ethanol by identifying the CH3 group at 1.17 ppm (Fulmer et al., 2010). A Bruker AVIII 400 MHz 2-channel spectrometer with 5 mm dual carbon (DCH) cryoprobe with a z-axis gradient was used to analyze samples at room temperature dissolved in D_2O. Topspin 2.1 computer software was used to acquire spectra.

2.4 Results and Discussions

All prebiotic oligosaccharides were chromatographically fractionated using food-grade, cellulose-based stationary phases and aqueous-ethanol mobile phases. Fractionations within the distinct classes of oligosaccharides (FOS vs. GOS vs. XOS) differed with respect to mobile phase ethanol contents, sample loads, run times, and flow rates. This approach allowed the economic preparation of well-defined prebiotic oligosaccharides suitable for use in human studies. The characterization of the resulting oligosaccharide preparations was based on chemical, chromatographic (HPLC) and spectrophotometric (NMR) methods for the determination of carbohydrate content, number-average DP, chemical identity and solvent removal.

2.4.1. Fractionation methods for the preparation of prebiotic oligosaccharides

The fractionation of food grade prebiotic oligosaccharides is particularly challenging because of the similarity in the structures of the compounds being fractionated (differing only with respect to DP) and the limited availability of cost-effective food-grade materials for use as chromatographic stationary and mobile phases. Food-/pharmaceutical-grade microcrystalline cellulose was used for the stationary phase and aqueous ethanol for the mobile phase. This study made use of the fact that

the economical preparation of purified prebiotic oligosaccharides does not require baseline resolution since the commercially available starting materials are relatively inexpensive. In the present case, specific oligosaccharide recoveries ranged from 30-75% (see **Table 1**). Lower recovery values reflect greater peak overlap (i.e., lower resolution); the lower resolution was accounted for in this work by collecting relatively small volumes of column eluent as separate fractions and then pooling only those fractions having clean DP profiles [DP profiles of individual fractions were determined by thin layer chromatography (TLC); see Method section for the TLC parameters]. This approach limits recoverable oligosaccharides to those from the center of elution peaks; the better the resolution the greater the center cut of the elution peak available for oligosaccharide recovery (see **Fig. 2.2** for chromatograms and associated fractions recovered). Highest recoveries thus corresponded to those oligosaccharides for which there was the greatest resolution, that being the XOS in the present chromatographic system.

The representative chromatograms depicted in **Fig. 2.2** illustrate the resolution obtained for each of the oligosaccharide preparations. Sample loads for typical chromatograms were 350, 675 and 1000 mg in 5 ml of the noted aqueous-ethanol solutions for FOS, GOS and XOS, respectively. Sample loads were dictated by the required resolution; XOS was chromatographed at the highest sample load (4 mg per g microcrystalline cellulose; **Table 2.1**) because XOS were resolved to the greatest extent in this system. The % ethanol content of the different mobile phases was dictated by the solubility of the oligosaccharides in aqueous-ethanol solutions (relative solubilities in 80% ethanol were FOS > GOS > XOS); increasing the % ethanol content of aqueous solutions corresponded to a decrease in oligosaccharide solubility in all cases. Elution volumes for different oligosaccharides of equivalent DP were similar, but slightly greater for XOS (e.g., compare elution volumes for DP4 components of each oligosaccharide preparation in **Fig 2.2**). The somewhat greater elution volume for XOS is consistent with stronger associations with the

cellulose stationary phase, particularly when noting that the mobile phase used for XOS chromatography was the lowest in % ethanol (i.e., XOS had the weakest mobile phase; that being the mobile phase least likely to promote oligosaccharide-cellulose interactions (Pullicin et al., 2018). Chromatographic run times for the various oligosaccharides were in the range of ten hours, with a general trend of elution times increasing with increasing sample loads and decreasing mobile phase ethanol contents. The amounts of the purified oligosaccharides obtained per chromatographic run are given in **Table 1** along with relevant associated parameters.

Table 2.1. Chromatographic pareters for fractionation of prebiotic oligosaccharides [a]

Targeted Oligosaccharide	Composition of mobile phase gradient [b] (% ethanol)	Sample load [c]	Average recovered oligosaccharide per chromatographic run [d] (mg)	Percent oligosaccharide recovered from loaded sample [e]
FOS DP3	85 → 80	1.2	40	30
FOS DP4	85 → 80	1.2	80	49
GOS DP3	85 → 80	2.7	100	47
GOS DP4	85 → 80	2.7	120	75
XOS DP2	75 → 65 → 55	4.0	270	44
XOS DP3	75 → 65 → 55	4.0	190	76
XOS DP4	75 → 65 → 55	4.0	110	73

[a] Stationary phase was microcrystalline cellulose. Starting materials were commercially available heterogeneous preparations of FOS (NUTRAFLORA® P-95), GOS (BIOLIGO™ GL-5700 IMF), and XOS (PreticX 95). FOS = fructooligosaccharides; GOS = galactooligosaccharides; XOS = xylooligosaccharides.

[b] aqueous ethanol solutions

[c] calculated as mg oligosaccharide preparation loaded onto column divided by grams of stationary phase in column

[d] amount of collected target oligosaccharide for one chromatographic column run in milligrams rounded to the closest ten milligrams

[e] calculated by dividing the recovered grams of targeted oligosaccharide by the estimated amount of targeted oligosaccharide applied to the column and then multiplying by 100

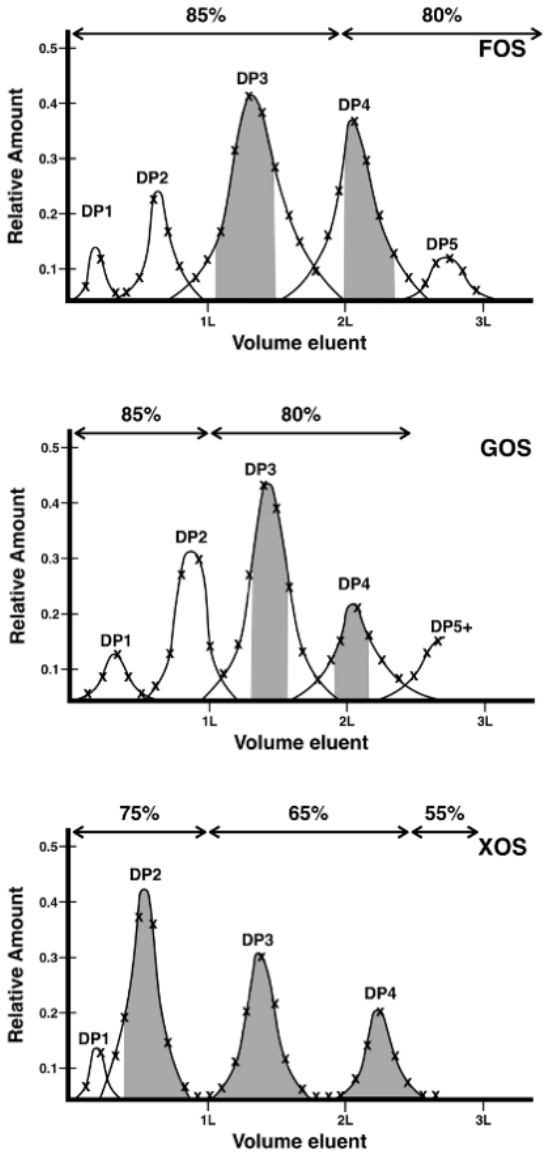


Fig 2.2. Representative chromatogram illustrating the fractionation of fructooligosaccharides (FOS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS). 'Relative amounts' of specific oligosaccharides in the eluent were determined using thin layer chromatography (TLC)/densitometry; every

sixth 15mL fraction was analyzed in this manner (resulting data points are indicated as 'x' in chromatograms). Numerical values for 'Relative Amount' are relative to a 4 mg/ml standard corresponding to the oligosaccharide that was included in each TLC run. Percent values listed across the top x-axis represent mobile phase gradient composition (% ethanol). Purified oligosaccharide preparations were obtained by pooling the fractions within the shaded region of each chromatographic peak.

2.4.2. Analytical methods for the characterization of prebiotic oligosaccharides

The starting materials for the preparation of the individual prebiotic oligosaccharides were commercially available food-grade heterogeneous prebiotic products (heterogeneous with respect to DP). Hence, the analyses performed on the oligosaccharide fractions obtained via chromatography focused on DP, which was ascertained by measuring carbohydrate content, moles per unit weight carbohydrate, HPLC profiles and NMR spectra.

The carbohydrate nature of the purified oligosaccharide preparations (close to 100% carbohydrate in all cases; **Table 2**) was anticipated based on the nature of the starting material. Nevertheless, verification of each preparations' carbohydrate content was necessary to justify subsequent calculations based on these values (i.e., determination of the average DP of oligosaccharides within a given preparation; see below). The quantification of carbohydrate content was done using anthrone/ sulfuric acid-based assays which are themselves based on the reaction of sugar-derived furan derivatives with the anthrone reagent to produce quantifiable colored compounds (Brummer & Cui, 2005). Sugars differ with respect to their reactivities under the assay's reaction conditions. Hence, the assay must be adapted for the different classes of oligosaccharides to which it is applied; this includes the wavelength used for quantification and the applicable calibration standards (**Table 2**). The absorption maxima of the products resulting from the color forming reactions of the pentoses have distinctly shorter wavelengths than those for the hexoses. The wavelength used for XOS quantification in this study was 465 nm, which differs from

that used for the quantification of FOS and GOS (the wavelength corresponding to maximum sensitivity for FOS and GOS was 672 nm). The wavelengths used herein for the quantification of these prebiotic oligosaccharides are analogous to those reported as optimum for the analysis of the corresponding monosaccharides (Haldar et al., 2017). Choosing the appropriate calibration standard is another important aspect of anthrone/sulfuric acid-based assay design. The data depicted in Figure 2.3 illustrate that the different sugars, even those within the same classes (e.g., aldohexoses), have somewhat different color yields under equivalent reaction conditions (this being in general agreement with data of Haldar et al., 2017). Hence, it is prudent to use calibration standards that best reflect the composition of the presumed oligosaccharides in the analyte mixture. In the present case, we know the general structure of the oligosaccharides (**Fig. 2.1**), so we can deduce logical representative monosaccharide mixtures (**Table 2.3**). The importance of this is illustrated in **Fig. 2.3** by comparing the standard curves for glucose and galactose with that of lactose (a disaccharide composed of glucose and galactose); the lactose curve being approximately equidistance between the glucose and galactose curves.

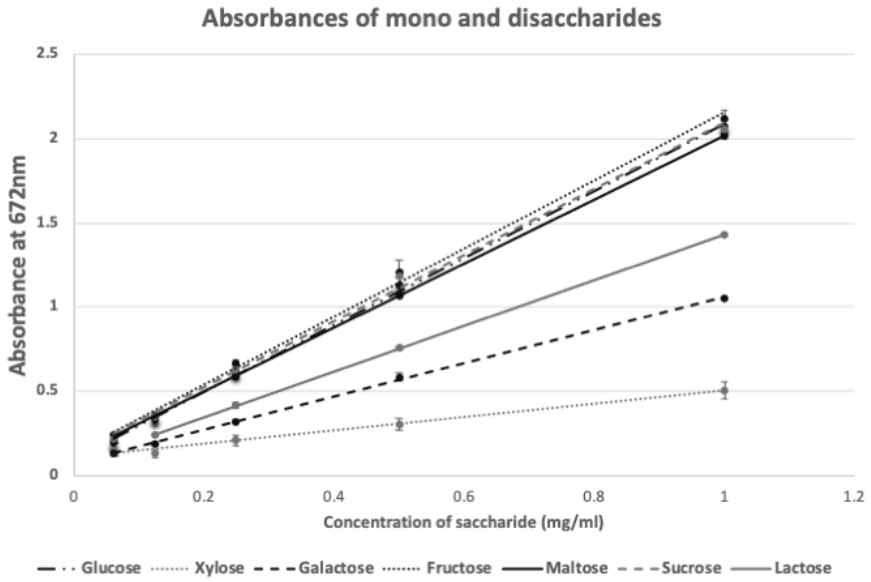


Fig 2.3. Comparison of mono and disaccharides absorbances using the anthrone sulfuric acid method for total carbohydrate determination at 672nm; y-axis shows the absorbances measured using UV spectrometer; x-axis represents the concentration of each corresponding saccharide.

Table 2.2. Total carbohydrate content of oligosaccharide preparations based on anthrone/sulfuric acid-based spectrophotometric quantification

Targeted Oligosaccharide [a]	Percent Carbohydrate [bc]	Assay Parameters	
		Spectrophotometer Wavelength (nm)	Calibration Standard composition [de]
FOS DP3	98.3 ± 1.49	672	1 Gu : 2 Fr
FOS DP4	99.1 ± 0.19	672	1 Gu : 3 Fr
GOS DP3	100.6 ± 0.74	672	1 Gu : 2 Ga
GOS DP4	99.9 ± 1.89	672	1 Gu : 3 Ga
XOS DP2	99.7 ± 0.51	465	Xy
XOS DP3	100.8 ± 1.55	465	Xy
XOS DP4	100.2 ± 0.29	465	Xy

[a] FOS = fructooligosaccharide; GOS = galactooligosaccharides; XOS = xylooligosaccharide

[b] All color-development reaction mixtures contained 1 ml aqueous carbohydrate solution in 4 ml reagent solution (reagent solution: 0.1% (w/v) anthrone in 98% H_2SO_4 with or without added thiourea) and were reacted

[c] Percent carbohydrate values are means per ± SD in triplicate, calculated based on dry weight basis

[d] Calibration standards were made up with the following ratios of monosaccharides.

[e] Gu = Glucose, Ga = Galactose, Fr = Fructose, Xy = Xylose

The moles of oligosaccharides per unit weight purified preparation was determined using two approaches (**Table 2.3**). FOS and GOS both have single terminal glucose residues (**Fig. 2.1**). Thus, the moles of glucose resulting from complete hydrolysis of a unit weight of the parent oligosaccharide is equal to the moles of said oligosaccharide in that amount of preparation. Acid-

catalyzed hydrolysis was used to convert the parent oligosaccharides into their constituent monosaccharides (Nguyen et al., 2009; Sophonputtanaphoca et al., 2018). The glucose content of the resulting solution was then determined using the spectrophotometric glucose oxidase/peroxidase (GOP) assay (Raba & Mottola, 1995). A second approach was used to quantify the moles of XOS per unit weight preparation; this being the quantification of reducing ends. The GOP assay was not considered for the analysis of XOS preparations because they do not contain a defined number of glucose moieties per molecule. Instead, the BCA/copper-based reducing sugar assay was applied using xylose as the calibration standard (Waffenschmidt & Jaenicke, 1987). This approach could have been used for the quantification of the GOS preparations as well since they are reducing oligosaccharides; although in this study those preparations were assayed as specified above using the GOP assay. FOS preparations are not reducing oligosaccharides, so the reducing sugar assay was not applicable for those preparations. The results from the quantification of the moles per unit weight oligosaccharide preparation (**Table 3**) combined with the % carbohydrate content of the different preparations (**Table 2**) were used to calculate the average DP of each fractionated oligosaccharide preparation (**Table 3**).

Table 2.3. Moles of oligosaccharide per unit weight and average degree of polymerization (DP) of purified prebiotic oligosaccharide preparations

Targeted Oligosaccharide [a]	Moles of oligosaccharide per 100 g oligosaccharide preparation [b]	Average molecular weight [c] (g/moles)	Average DP [d]
FOS DP3	0.201 ± 0.003	497.5	3.0
FOS DP4	0.155 ± 0.009	645.2	3.9
GOS DP3	0.198 ± 0.002	505.1	3.0
GOS DP4	0.163 ± 0.003	613.5	3.7
XOS DP2	0.347 ± 0.005	288.2	2.0

XOS DP3	0.247 ± 0.012	404.9	2.9
XOS DP4	0.182 ± 0.020	549.5	4.0

[a] FOS = fructooligosaccharide; GOS = galactooligosaccharides; XOS = xylooligosaccharide

[b] Values are means per ± SD in triplicate, calculated based on dry weight basis based on quantification of terminal residues

[c] Average molecular weight calculated as 100 g of sample divided by the Moles per 100 g oligosaccharide preparation.

[d] The following equations were used to calculate the average DP of the different oligosaccharide preparations:

GOS/FOS: 180 g/mol for a single hexose unit + n (162 g/mol remaining hexose units) = 'Average molecular weight'; 'Average DP' = n + 1

XOS: 150 g/mol for single xylose unit + n (132 g/mol remaining xylose units) = 'Average molecular weight'; 'Average DP' = n + 1

The DP values (**Table 2.3**) obviously provide information as to the size of the oligosaccharides, but they can also be indicative of the purity of the samples. They are only 'indicative' in the sense that a pure preparation of DP4 would give the same average DP as a 1:1 molar mixture of DP3 and DP5. Thus, it is prudent to verify purity using an alternative method. Herein we used chromatography. Representative chromatograms of the different preparations are depicted in **Fig. 2.4**. The dominant single peak for each of the preparations supports the conclusion of sample purity. The one exception to this is the GOS-DP4 sample, which shows a small but notable peak corresponding to GOS-DP3 in the GOS-DP4 preparation. The presence of DP3 contaminant in the GOS-DP4 preparation is in agreement with the measured average DP for the GOS-DP4 preparation being somewhat lower than the theoretical value of 4 (measured value is 3.7; **Table 2.3**). The presence of small amounts of GOS-DP3 in the GOS-DP4 preparation is also in agreement with the collected fractions depicted by the shaded regions in **Fig. 2.2**; the implication being that the amount of GOS-DP3 in the GOS-DP4 preparations can be lowered if narrower bands of eluate are pooled for collection (the trade-off being between purity and yield, as discussed

above). The identity of the purified oligosaccharide preparations was further verified in this work by confirming that the retention times of chromatographed samples agree with those of commercially available analytical standards

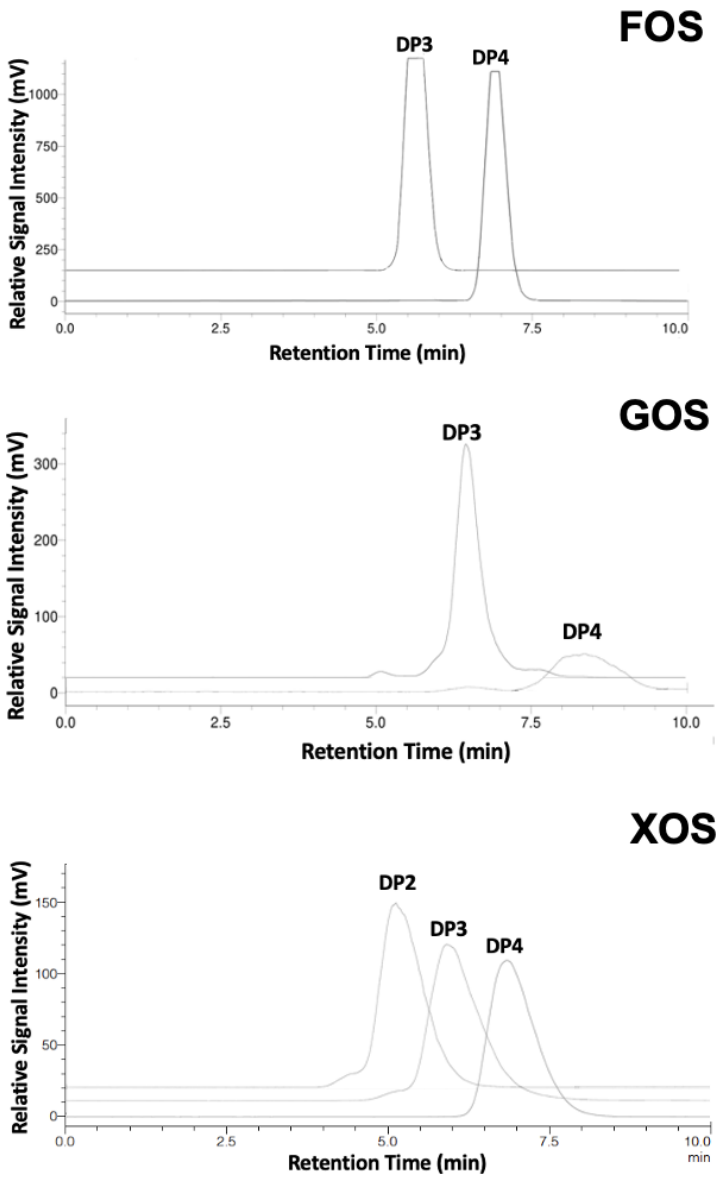
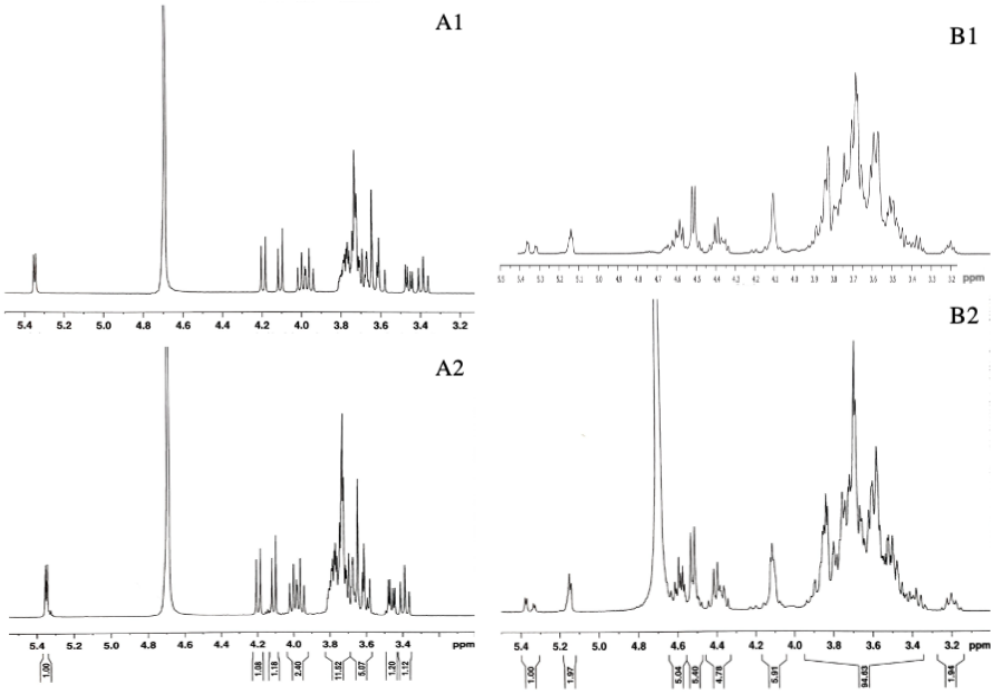


Fig 2.4. Chromatograms from HPLC-ELSD depicting the fractionated oligosaccharides. FOS=fructooligosaccharide, GOS=galactooligosaccharide, XOS=xylooligosaccharide, DP= degree of polymerization.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was also used to verify the nature of the oligosaccharide preparations obtained via chromatography. The approach was to compare the spectra of the prepared samples with either those of commercially available analytical standards in the cases of FOS and XOS, or published chemical shift data for analogous compounds in the case of GOS; recall that glycosidic linkages of GOS starting materials are heterogeneous in nature, while commercially available GOS standards are homogeneous molecules. The spectra of the prepared FOS and XOS samples matched those of the standards for the presumed compounds (see **Fig. 2.5, A** and **C)**. The spectra of the prepared GOS-DP3 samples matched those recently published by van Leeuwen et al. (2014a; 2014b) (see **Fig. 2.5, B**).



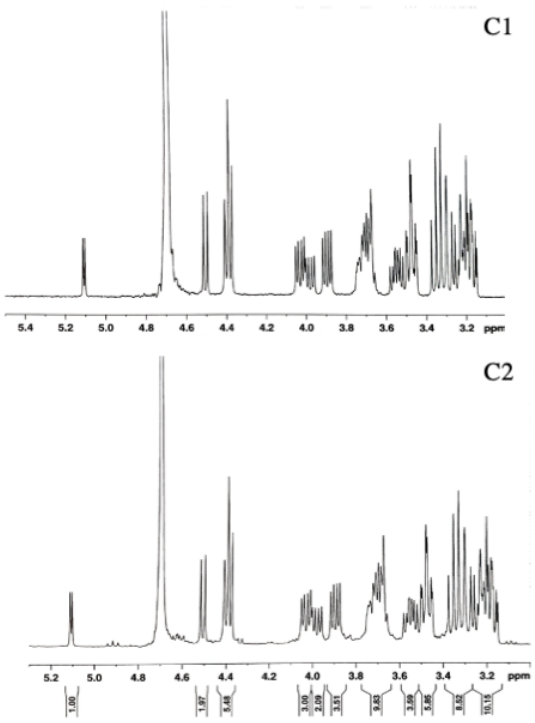


Fig 2.5. ^{1}H NMR spectra of A1) FOS DP3 standard and A2) FOS DP3 fractionated sample; B1) GOS DP3 adapted from (van Leeuwen et al., 2014a) and B2) GOS DP3 fractionated sample; C1) XOS DP3 standard and C2) XOS DP3 fractionated sample. Fractionated samples were dissolved in D_2O, which represents the peak at ~4.7ppm. B1 is reprinted from van Leeuwen et al. 2014a with permission from Elsevier.

2.5 **Conclusion**

This study is the first to address the important issue of obtaining relatively low-cost, size-defined prebiotic oligosaccharides suitable for human testing. Here we show that such oligosaccharides can be obtained via chromatographic fractionation of commercially available food-grade prebiotic oligosaccharide mixtures using microcrystalline cellulose stationary phases and aqueous-ethanol mobile phases. The specifics of productive chromatographic conditions differ depending on the class of oligosaccharides being fractionated. This approach is shown to be successful in obtaining FOS, GOS and XOS of DP3 and DP4. XOS of DP2 (xylobiose) is also readily prepared using the

described method; FOS and GOS of DP2 were not a focus of this study as they are readily available as relatively high purity food-grade products (FOS-DP2 is sucrose; GOS-DP2 is lactose).

The second objective of this study was to develop analytical methods for the characterization of size-defined prebiotic oligosaccharides differing with respect to constituent composition; the focus again being on FOS, GOS and XOS. A series of methods were outlined for measuring the total carbohydrate content, moles per unit weight and DP of each of the aforementioned classes of oligosaccharides. Furthermore, it is shown how HPLC and NMR can be used in a complimentary manner to further establish each preparations' purity and identity. The combined methods presented herein provide an excellent starting point for the economical preparation of size-defined, physiologically relevant, prebiotic oligosaccharides for use in human testing.

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Conflicts of interest statement

The authors declare no competing financial interests.

CHAPTER 3

GENERAL CONCLUSION

The overall objectives of this study were: 1) to develop simple, cost-effective fractionation methods for the preparation of common prebiotic oligosaccharides [fructooligosaccharides (FOS), galactooligo-saccharides (GOS), and xylooligosaccharides (XOS)] with specific degree of polymerization (DP) to be used in human foods and/or human testing, and 2) to develop a series of analytical methods for the characterization of each fractionated oligosaccharide preparation. These goals were sequential, in that success of the first was essential in allowing the second to be achieved.

In the first objective, seven oligosaccharide samples (FOS DP3, FOS DP4, GOS DP3, GOS DP4, XOS DP2, XOS DP3, and XOS DP4) were successfully produced from commercial prebiotic mixtures through chromatographic preparations. This study reported the differences in separation parameters for each class of oligosaccharide. The findings suggests that the mobile phase composition is dependent on the ethanol solubility of the prebiotic oligosaccharide, which was impacted by its chemical structure. This method is valuable to researchers interested in obtaining prebiotic oligosaccharides with specific chain length that are food safe for the purpose of human testing. While the work done showed success in developing a fractionation method, there are recommendations to adapt this method with a possibility to scale up and increase efficiency. A recommendation is to use a significantly longer column to increase the number of plates within the column and ultimately increase efficiency. However, having a long column could result in very long chromatographic run; a pump system attachment may be useful to improve time efficiency.

This recommendation might have the potential for greater yields of product within the same or shorter time frame.

In the second objective, various methods of chemical analysis [i.e., total carbohydrate analysis, moles quantification, High-Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR)] were used as ways to characterize the fractionated oligosaccharide samples. The results confirmed that the prebiotic oligosaccharides obtained from the fractionation process in objective 1 closely matched the targeted profile of the desired oligosaccharide and were of high purity. The findings show that different classes of oligosaccharides having distinct chemical structure can be analyzed differently (i.e., moles quantification for FOS and GOS were performed through terminal glucose quantifications, whereas XOS was through reducing end quantification). Having multiple methods of analysis used in a complementary manner (e.g., number of moles quantification along with comparison of HPLC retention times of the samples with known analytical standards to determine the average DP for the samples) can further establish the characteristics of the products and provide the researcher confidence in the product from the fractionation process.

This research successfully addresses the lack of food-safe prebiotic oligosaccharide with well-defined chain lengths. With these products, further studies can be performed. For example, psychophysical properties of size defined prebiotic oligosaccharides would be of great interest to challenge/address a long-held assumption that only simple sugars can be tasted. In particular, recent studies have shown that digestible saccharides larger than simple sugars [maltooligosaccharides (MOS) with DP3 up to ~DP14] can be tasted by human subjects. Taste quality has been shown to differ, with MOS DP3 having sweet taste, and MOS DP4 and above having a “starchy taste” (Lapis et al., 2016; Pullicin et al., 2017). It would be of great interest to compare the sensory properties of the digestible and non-digestible saccharides at the same chain lengths differing only in chemical

structure (i.e., monomeric constituent, glycosidic linkages, reducing vs non-reducing). Other potential areas of research are to look at the applicability of the produced oligosaccharides in the health and pharmaceutical sectors. These sectors might seek the most benefit from fractionated prebiotic oligosaccharides. Based on future findings, the health and pharmaceutical industries could use size-defined prebiotics to come up with products that cater to individuals with special health needs. Other potential areas include synbiotic products which consist of both prebiotics and probiotics; some studies have shown probiotic strains to have preference for certain size prebiotics over the others (Zhu et al., 2015).

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