UNIVERSIDADE DE SÃO PAULO

ESCOLA DE ENGENHARIA DE LORENA

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Evaluation of the prebiotic potential of xylooligosaccharides from sugarcane bagasse extracted within the biorefinery concept

Avaliação do potencial prebiótico de xilooligossacarídeos do bagaço de cana-de-açúcar extraído dentro do conceito de biorrefinaria

Lorena

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Tese apresentada à Escola de Engenharia de Lorena da Universidade de São Paulo para obtenção do título de Doutor em Ciências do Programa de Pós-Graduação em Biotecnologia Industrial na área de concentração de Biotecnologia Industrial

Orientador: Prof. Dr. Valdeir Arantes

Versão Corrigida

Lorena

2023

Ficha catalográfica elaborada pelo Sistema Automatizado
da Escola de Engenharia de Lorena,
com os dados fornecidos pelo(a) autor(a)

Marcondes, Wilian Fioreli
Evaluation of the prebiotic potential of xylooligosaccharides from sugarcane bagasse extracted within the biorefinery concept / Wilian Fioreli Marcondes; orientador Valdeir Arantes - Versão Corrigida. - Lorena, 2023.
102 p.

Tese (Doutorado em Ciências - Programa de Pós Graduação em Biotecnologia Industrial na Área de Biotecnologia Industrial) - Escola de Engenharia de Lorena da Universidade de São Paulo. 2023

1. Sugarcane bagasse. 2. Prebiotic. 3. Fecal fermentation. 4. Xylooligosacharides. I. Título. II. Arantes, Valdeir, orient.

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Thesis presented at Escola de Engenharia de Lorena of Universidade de São Paulo to obtain the degree of Doctoral of Science issued by the Programa de Pós-Graduação em Biotecnologia Industrial in the field of Industrial Biotechnology

Advisor: Prof. Dr. Valdeir Arantes

Corrected Version

Lorena

2023

ACKNOWLEDGMENTS

First, I would like to thank this superior force that guides the energy of the universe, for giving us the opportunities to reach our potential.

I thank my family for the support and encouragement to continue in my academic life, especially my father, my son and my wife who were present all these years by my side. My mother and my brother who, even though they are not present in the body, are present in the soul.

To my advisor Prof. Dr. Valdeir Arantes for the time invested in me, for the trust and orientation for a better execution of the work and for the academic and personal training.

To the employees of the secretary, the postgraduate coordination and the library in helping to resolve all bureaucratic issues with agility and speed.

To the technicians of the biotechnology department (DEBIQ) in helping to carry out experiments. To my laboratory colleagues for their strength, patience, help in technical and personal matters.

This research used pilot plant of Brazilian Biorenewables National Laboratory (LNBR), part of the Brazilian Center for Research in Energy and Materials (CNPEM), a private non-profit organization under the supervision of the Brazilian Ministry for Science, Technology, and Innovations (MCTI). The Dr. Carlos Alberto de Oliveira Filho, Pilot Plant Coordinator for Process Development (PPDP) is acknowledged for the assistance during the experiments PPDP-MP-25524.

RESUMO

MARCONDES, W. F. **Avaliação do potencial prebiótico de xilooligossacarídeos do bagaço de cana-de-açúcar extraído dentro de conceito de biorrefinaria**. 2023. 102p. – Tese (Doutorado em Ciências) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2023

O desenvolvimento de processos mais sustentáveis vai de encontro em obter produtos de alto valor agregado a partir de biomassa vegetal, sendo o bagaço de cana-de-açúcar (BCA) um resíduo agroindustrial de grande potencial de exploração quando nos referimos ao cenário brasileiro e mundial. Dentre estes produtos, destaca-se os xilooligossacarídeos (XOS), um composto que pode ser extraído da fração hemicelulósica do BCA e apresenta grande destaque como composto prebiótico. Recentemente foi desenvolvido dentro do conceito de biorrefinaria dois processos integrados para extração de XOS, um em escala de bancada e outra em escala piloto. Em ambos os cenários o XOS foi extraído por processo hidrotérmico em meio ácido ou autohidrólise, gerando hidrolisados que contém XOS com características estruturais distintas, como grau de polimerização (GP), além de outros produtos derivados da degradação de carboidratos e lignina, como furfural e polifenóis respectivamente. Neste sentido, o objetivo do presente trabalho foi analisar o potencial prebiótico do XOS obtido do BCA, tanto em escala de bancada como piloto, comparado com XOS comercial e verificando a necessidade de etapas de purificação. Para isto foram realizadas fermentações anaeróbicas utilizando XOS como fonte de carbono tanto com cepas isoladas de *bifidobacterium* como em uma comunidade de microrganismo que representa melhor a microbiota intestinal humana através de fermentação fecal humana. Durante a fermentação por cepas isoladas de *bifidobacterium* foi possível verificar que XOS com GP até 6 geraram melhores resultados como composto prebióticos, como decréscimo de pH, aumento de liberação de ácidos graxos de cadeia curta (AGCC) e crescimento microbiano quando comparados com XOS com GP maior que seis. Na fermentação fecal, XOS com diferentes estruturas, mas todas dentro da faixa de GP 2-6, não resultaram em diferença significativa quanto a capacidade de ser metabolizado pela população microbiana. Outro aspecto estudado foi a capacidade do XOS de atenuar os efeitos adversos de uma alta carga de proteínas e derivados no distal colón, como aumento de pH e concentração de amônia. Para isto foi feito uma simulação da digestão de proteínas e posterior fermentação fecal, com e sem adição de XOS, observando que a presença do oligossacarídeo foi benéfica para manter o baixo pH e concentração de amônia no meio fecal. XOS oriundo do BCA também apresentou melhores resultado de toxicidade celular em relação a dano na parede celular, podendo isto ser atribuído a sua estrutura ou a presença de outros compostos benéfico no hidrolisado hemicelulósico. Com foi possível concluir que o XOS extraído do BCA, dentro das condições estudadas que resultem em GP até seis, apresenta propriedades prebióticas e que etapas de purificação não foram necessárias.

Palavras-chave: Bagaço de cana-de-açúcar. Xilooligossacarídeos. Prebióticos. Fermentação fecal.

ABSTRACT

MARCONDES, W. F. **Evaluation of the prebiotic potential of xylooligosaccharides from sugarcane bagasse extracted within the biorefinery concept**. 2023. 102p. – Thesis (Doctoral of Science) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2023

The development of more sustainable processes is aimed to obtain high value products from vegetal biomass, being sugarcane bagasse (SCB) an agro-industrial residue with great exploitation potential when referring to the Brazilian and world scenarios. Among these products, xylooligosaccharides (XOS) stand out as a compound that can be extracted from the hemicellulosic fraction of SCB and has great prominence as a prebiotic compound. Recently, within the concept of a biorefinery, two integrated processes for extracting XOS were developed, one on a bench scale and the other on a pilot scale. In both scenarios, XOS was extracted by hydrothermal process in acidic medium or autohydrolysis, generating hydrolysates that contained XOS with distinct structural characteristics, such as degree of polymerization (DP), in addition to other products derived from the degradation of carbohydrates and lignin, such as furfural and polyphenols, respectively. In this context, the objective of the present work was to analyze the prebiotic potential of XOS obtained from SCB, both in bench and pilot scale, compared with commercial XOS and verifying the need for purification steps. For this, anaerobic fermentations were carried out using XOS as a carbon source both with isolated strains of *Bifidobacterium* and in a microorganism community that better represents the human intestinal microbiota through human fecal fermentation. During fermentation by isolated strains of *Bifidobacterium*, it was possible to verify that XOS with DP up to 6, even in the presence of high levels of xylose, generated better results as a prebiotic compound, such as a decrease in pH, an increase in the release of short-chain fatty acids (SCFA) and microbial growth when compared to XOS with DP higher than six. In fecal fermentation, XOS with different structures, but all within the GP 2-6 range, did not result in a significant difference in the ability to be metabolized by the microbial population. Another aspect studied was the ability of XOS to attenuate the adverse effects of a high load of proteins and derivatives in the distal colon, such as increased pH and ammonia concentration. For this, a simulation of protein digestion and subsequent fecal fermentation, with and without addition of XOS, was performed, noting that the presence of oligosaccharide was beneficial to maintain low pH and ammonia concentration in the fecal medium. XOS from BCA also showed better results in cellular toxicity in relation to cell wall damage, which can be attributed to its structure or the presence of other beneficial compounds in the hemicellulosic hydrolysate. Therefore, it was possible to conclude that the XOS extracted from BCA, within the studied conditions that result in GP up to 6, has prebiotic properties and that purification steps were not necessary.

Keywords: Sugarcane bagasse. Xylooligosaccharides. Prebiotics. Fecal fermentation.

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SUMMARY

1 INTRODUCTION

Lignocellulosic materials (LCM) such as wood species, grasses, agro-industrial and forestry residues considered the raw material for the development of a sustainable economy, both regional and worldwide, and the most abundant renewable raw material in nature (Nygaard et al., 2016).

In Brazil, sugarcane bagasse (SCB), a residue from the sugar-alcohol industry, is considered the main raw material to produce second-generation ethanol (2G). The concept of a biorefinery using bagasse as a raw material has been studied for a long time, always seeking to add high value to its fractions, which are mainly composed of cellulose, lignin and hemicellulose. From the hemicellulosic fraction, it is possible to extract xylooligosaccharides, which among its many uses stands out for being a compound with prebiotic properties.

Prebiotics are food compounds that influence the regulation of the microbiota in order to generate health benefits for those who ingest them (Gibson and Roberfroid, 1995). Among the most famous prebiotics, we highlight those found in breast milk, due to its great importance because the human microbiota is strongly defined in the first years of children's lives, and dietary fibers, such as galactooligosaccharides (GOS) and fructooligosaccharides. (FOS) and which are more used as a food supplement (Gibson et al., 2017). However, XOS has been gaining prominence due to some superior properties compared to commercial prebiotics, such as the need for lower daily doses and greater selectivity for *Bifidobacterium*, a known probiotic health bacterium, than for other bacteria found in the intestinal microbiota (Mäkeläinen et al., 2010a, 2010b).

Divided into chapters, this thesis is organized to lead the reader in a logical sequence for the study of the prebiotic potential of XOS extracted from sugarcane bagasse, not only by the classic benefits as selectivity for *Bifidobacterium* and SCFA production, but also as a component that can attenuate undesired effect of a high protein fecal fermentation.

The first chapter brings a succinct bibliographic review to provide the basis for the justification of the work and an introduction to the topics that will be addressed in the following chapters.

The second and third chapters bring the studies of extraction of XOS from SCB, in bench scale and pilot scale, and the study of their prebiotic potential using anaerobic fermentation with isolated strains of *Bifidobacterim*.

The fourth chapter brings a more advanced study of the potential prebiotic capacity of XOS extracted from SCB through human fecal fermentation. The XOS was fractionated into short and long chain fractions and used to better understand the influence of XOS' DP on the prebiotic propriety.

The fifth and final chapter presents an exploratory study on the possible beneficial effects of XOS on human health when it is administered together with a high-protein diet, also using human fecal fermentation for this evaluation.

2 OBJECTIVES

To analyze the potential of XOS extracted from sugarcane bagasse through hydrothermal process within a Biorefinery concept as a prebiotic compound.

2.1 SPECIFIC OBJECTIVES

a) Chemically characterize the hemicellulose hydrolysates obtained through hydrothermal treatment of sugarcane bagasse in relation to the structural characteristics of XOS, such as degree of polymerization (DP) and substitution (DS) and presence of monomeric carbohydrate degradation compounds (hydroxymethyl furfural and furfural);
b) Evaluate the hemicellulosic hydrolysates as a carbon source for the metabolism of isolated strains of *Bifidobacterium*;
c) Evaluate the impact of purification of the hemicellulose hydrolysates on their use as a carbon source in human fecal fermentation;
d) Evaluate the potential benefits that the use of hemicellulose hydrolysates could cause to human health.

CHAPTER 1: LITERATURE REVIEW

1.1 LIGNOCELLULOSIC AS RAW MATERIAL

More than 200 years ago, coal was the main source of global energy, being replaced by crude oil in the last 60 years. Currently, plant biomass has been gaining prominence as an alternative raw material for energy generation (Cheng and Zhu, 2009). As in the case of petroleum where the raw material is treated in a refinery to obtain, in addition to energy, products with high added value, the use of vegetal biomass has great potential to fit into a biorefinery concept since its fractions may have market potential (Vinoth Kumar et al., 2016).

Lignocellulosic materials (LCM), which include grass species, wood, agro-industrial residues and forests, are considered the raw material for a sustainable economy, being the most abundant renewable biomass source in Nature (Nygaard et al., 2016). Corn cob, wheat straw and sugarcane bagasse (SCB) are examples of agro-industrial residues with great potential to generate high value-added products, which would probably improve the sustainability of the processes where they are generated.

Brazil is the world's largest producer of sugarcane, with a production of 757 million ton/year, which represents 40% of world production (Statista, 2022). Considering that a ton of sugarcane results in 300 kg of bagasse (Nikodinovic-Runic et al., 2013), there is an annual generation of this residue of 227 million tons, a very abundant source of renewable biomass. The major components of SCB are lignin, cellulose, and hemicellulose, three components that has a variety of application and potential to obtain high value products.

1.2 HEMICELLULOSE AND XYLOOLIGOSACHARIDES

Hemicellulose is one of the main components of the cell walls in LCM and presents differences in its composition and structure depending on its source. It is a branched heteropolymer, and its main chain may consist of one or two types of sugars, hexoses (glucose, mannose, galactose) or pentoses (xylose and arabinose), and its branches are generally composed of uronic acids, such as 4-O -methyl-D-glucuronic and D-galacturonic acid. Some main chain sugars may also contain acetyl groups, mostly attached to the C2 carbons. The presence of galacto-glucomannan-type hemicellulose is predominant in softwoods with small amounts of arabino-glucoxylan, while hardwoods have a predominance of glucoxylan-type

hemicelluloses with small amounts of glucomann (Chen, 2014; Fengel and Wegener, 1989; Hon, 1995).

SCB, classified as grass, has hemicellulose of the arabinoxylan type, with the main chain formed by xylose units joined by β-1,4 bonds and the branch chains by α-1,3 bonds (Figure 1)(Hon, 1995). About 87% of SCB hemicellulose is composed of xylose, which has a ratio of 12:1 for arabinose and 8.8:1 for acetyl groups (Hon, 1995; Rocha et al., 2015) as illustrated in Figure 1.

The abundance of SCB together with its xylan-rich hemicellulose makes this LCM a potential source to obtain xylose and/or xylo-oligosaccharide (XOS). Xylose, a monomeric unit derived from xylan, can be used in (bio)chemical processes to obtain products such as xylitol and ethanol (Felipe et al., 1997; Gírio et al., 2010). XOS can be used in several areas such as the paper industry, production of biofilms with oxygen barrier properties, emulsifiers and adhesives (Du et al., 2018; Lima et al., 2003; Kamm, B and Kamm, M, 2004; Vázquez et al., 2000). However, its use as a food additive compound is growing attention due to its prebiotic properties. This is due to XOS being a selective substrate for certain groups of *bifidobacterium*, making it an emerging prebiotic in the scenario of functional dietary fibers (Gobinath et al., 2010; Mäkeläinen et al., 2010b).

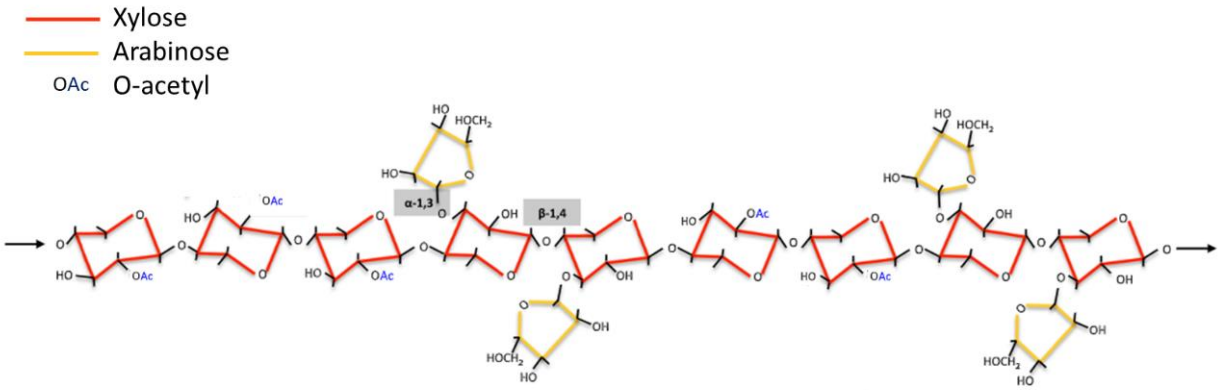

Figure 1. Structure of the xylan present in the SCB, adapted from (Buckeridge and de Souza, 2014)

1.2.1 XOS EXTRACTION

XOS can be extracted from LCMs mainly through chemical or chemical/enzymatic processes (Figure 2).

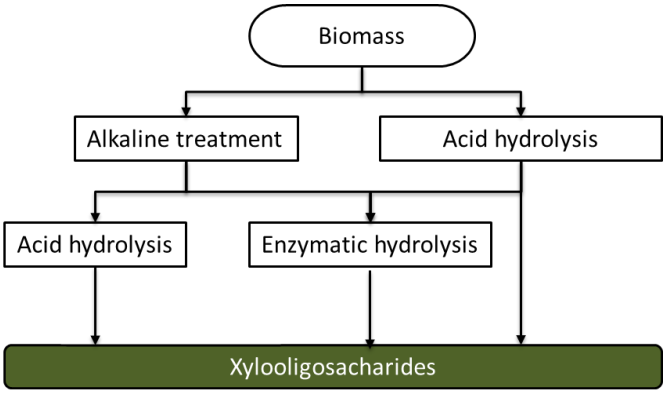

Figure 2. Methods of xylooligosaccharides extraction from biomass rich in xylan

1.2.1.1 CHEMICAL METHODS

The chemical process for extraction of XOS focuses on the solubilization of the hemicellulose fraction of the xylan-rich LCM through reactions in acid or alkaline medium, depending on the desired strategy. On hemicellulose, alkaline processes result in saponification of the side groups of the main chain, thus generating a material that is more soluble at neutral pH and without ramifications, but with a high degree of polymerization (Qing et al., 2013). In order to obtain a prebiotic compound, this xylan chain needs to undergo a breaking process, which can occur chemically or enzymatically (van Craeyveld et al., 2008).

To extract XOS, acid hydrolysis occurs under mild conditions to decrease the release of monomers and carbohydrate degradation products, such as hydroxy-methylfurfural (HMF) and furfural (Hu and Ragauskas, 2012). These degradation products are considered impurities and inhibitors for many fermentation microorganisms and, if present, a purification step would be required (Zabed et al., 2016). Compared to the process in alkaline medium, a hydrothermal treatment in dilute acid medium shows the advantage of not needing a post-treatment to decrease DP of the XOS, in addition to obtaining a branched XOS, which has the potential to have distinct activity as a prebiotic agent (Kabel et al., 2002).

1.2.1.2 CHEMICAL-ENZYMATIC METHOD

The enzymatic process is more focused on the enzymatic cleavage of xylan chains extracted by chemical treatment, usually alkaline method. Therefore, xylan is extracted from LCM by chemical treatment and then hydrolyzed to oligomers using enzymes of the endo-xylanase class (EC 3.2.1.8) (Brienzo, 2010; Carvalho et al., 2013; Jayapal et al., 2013; Qing et al., 2013; Zhang et al., 2011).

Endo-xylanases can act on the xylan chain in several ways. They are specific for the decrease of xylan DP, preferentially acting on the β-1,4 bonds of the xylan chain. When necessary, auxiliary enzymes can be used for better hydrolysis of the xylan. In xylans obtained by treatment in an acid medium, which probably have substituent groups in their hydroxyl groups, auxiliary enzymes should be used, such as acetyl-xylan esterase that can specifically remove the substituent acetyl groups from the xylan backbone (Zhang et al., 2011).

In relation to chemical processes, the production of XOS by enzymatic hydrolysis has the advantages of being able to work with milder process conditions, such as temperature, in addition to acting specifically on xylan chains without the formation of sugar degradation products, such as furfural, thus facilitating the purification process (Carvalho et al., 2013; Qing et al., 2013). As disadvantages, the enzymatic production of XOS has a longer reaction time and the use of enzymes, which potentially generates higher costs (Qing et al., 2013). However, it is important to point out that the XOS obtained from these two processes may have different structural characteristics, which may be favorable or unfavorable for their use.

1.2.2 BIOREFINERY FOR XOS EXTRACTION

As an LCM, SCB is constitute of three major components: hemicellulose, lignin and cellulose. The extraction of XOS from the hemicellulose fraction results in a material rich in lignin and cellulose (cellulolignin), which is a potential source to obtain other high value products. Cellulose is commonly used in the pulp and paper industry, however other processes can use this material, such as cellulose derivatives like cellulose acetate, carboxymethyl cellulose (Svetlov, 2019), or products from nanocellulose, such as nanocomposites, films for food packaging, development of matrices for medicine, among others (Kiziltas et al., 2013; Lee et al., 2014; Lin and Dufresne, 2014; Liu et al., 2019; Siró and Plackett, 2010). Lignin is usutally burned to generate heat due to its high calorific value, however there are the possibility to obtain products with high value such as films with ultraviolet blocking properties, polymers and nanocomposites (Tian et al., 2017).

The XOS fractions used in this thesis originate from two works that explored the extraction of XOS by hydrothermal process within a biorefinery concept, using all major SCB fractions to generate high value products or fuel ((Marcondes, 2018; Pereira et al., 2021). The first work investigated the integration of a two-stage hydrothermal treatment for production of xylooligosaccharides (XOS), a high-value product, into the isolation process of cellulose

nanofibrils (CNF) from sugarcane bagasse (Figure 3). For this, a design of experiment was carried out varying the process conditions (time, temperature and acid concentration) in a bench scale pressure vessel reactor (Parr 2 L). The optimized condition for the highest yield of XOS was obtained. Within the concept of biorefinery, the other fractions of SCB were used to generate co-products such as CNF (Marcondes, 2018). In the second work, an integrated biorefining strategy was applied to fractionate SCB into its major constituents, cellulose, lignin and hemicellulose. A hemicellulosic hydrolysate was obtained from a steam explosion process in a pilot scale and the cellulignin residue was used to obtain lignin nanoparticles, ethanol and CNF (Figure 4).

The hemicellulosic fractions from both studies were used to evaluate the prebiotic potential, either by isolated strains of *bifidobacterium* or by fecal fermentation.

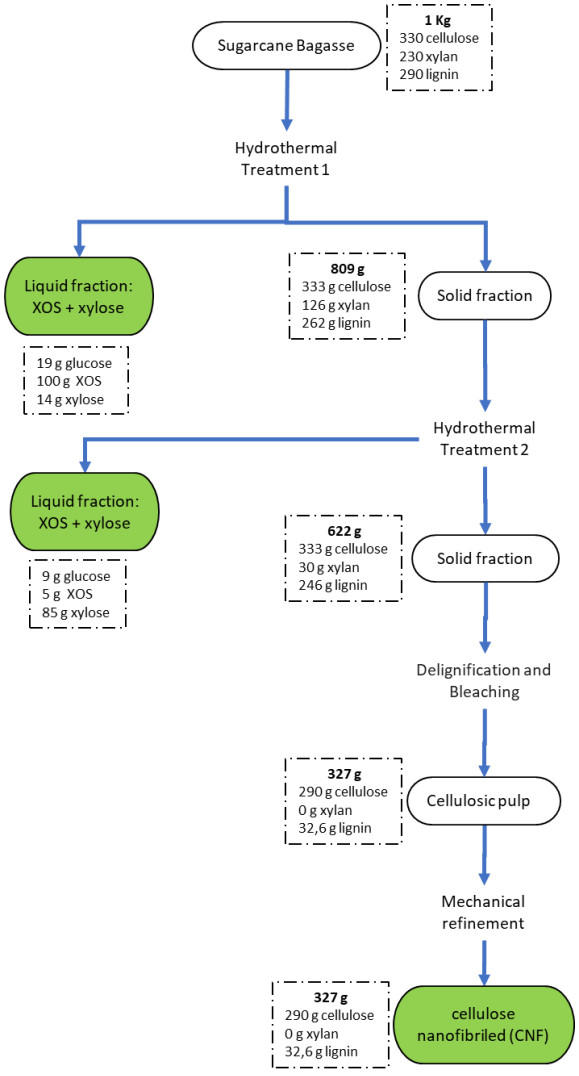

Figure 3. Overall mass balance for the proposed integrated process for production of hemicellulose-derived high-value products (XOS and xylose) to the isolation process of CNF from sugarcane bagasse, with an initial dry mass of 1 Kg. The process also allowed the recovery of lignin (Marcondes et al., 2020)

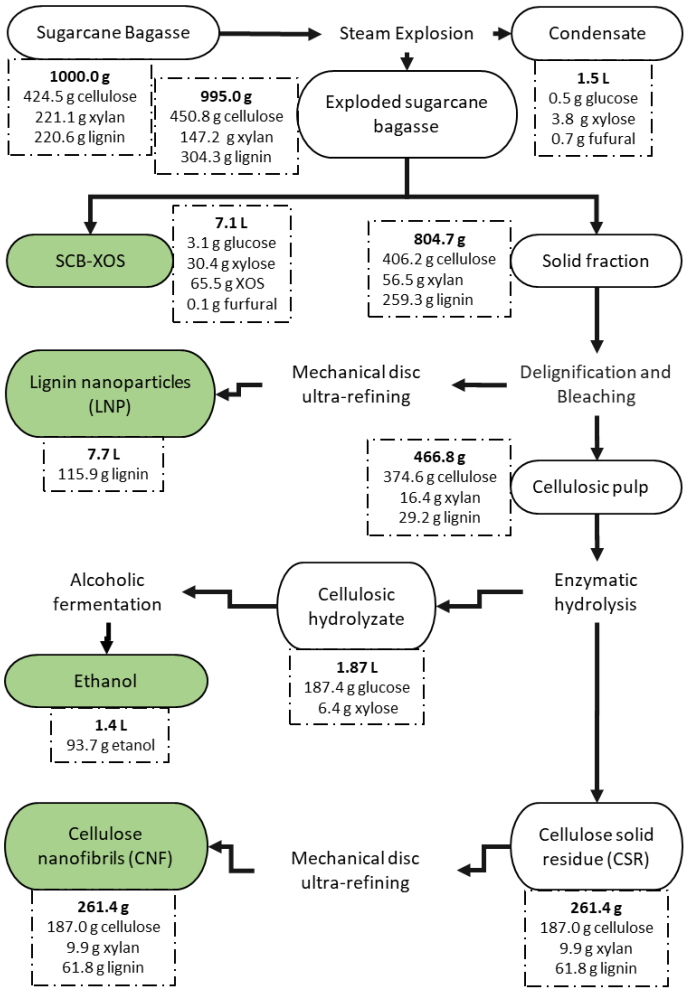

Figure 4. Overall mass balance for the proposed integrated process for production of hemicellulose-derived high-value products (XOS), lignin nanoparticles (LNP), cellulosic ethanol and cellulose nanofibrils (CNF) from sugarcane bagasse, with an initial dry mass of 1 Kg (Pereira et al., 2021).

1.2.3 INDUSTRIAL PRODUCTION

Industrially, XOS is extracted by two main companies, the Chinese Shangdong Longlive Biotechnology and the American Prenexus Health (Santibáñez et al., 2021). The Chinese company uses corn cob as a raw material and uses a chemical treatment to remove the xylan polymer chain and the depolymerization of this chain using enzymes as a XOS extraction strategy, obtaining linear XOS with a degree of polymerization mostly between two and six xylose units (NutraSource, 2013). The American company, on the other hand, uses sugarcane bagasse and a hydrothermal self-hydrolysis process for the extraction of XOS, having as a final XOS product with a degree of polymerization between three and thirteen xylose units (Carlson, 2018). Both the Chinese and American processes result in linear XOS in the form of white powder, high purity (up to 95%) and use malto dextrin as a diluent. However, they present

process steps considered to be expensive, such as enzymatic hydrolysis (in the case of the Chinese company) and purification, clarification, and drying processes (Santibáñez et al., 2021).

1.2.4 MARKET

The value of the XOS market is estimated at $30 million for 2022, with a growth forecast of 1.5% per year reaching a potential of $33 million in 2028 (QYReaseach, 2022). The average value of commercial XOS is in the range of U$ 25-50/Kg, depending on the level of purity (Santibáñez et al., 2021). The animal feed industry represents almost half of this market (49%), followed by medicine and health (25%) and food and beverage (23%) (Ahmad, 2019).

1.3 PREBIOTICS

The term prebiotic was first used in the academic literature by Gibson and Roberfroid, 1995, as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited group of bacteria in the colon, thereby conferring improvement in health" (Gibson and Roberfroid, 1995). However, one of the first studies on the influence of the microbiota by ingestion of carbohydrates was in 1921, which analyzed the change in the microbiota after ingestion of carbohydrates lactose and dextrin together with *Bacterium acidophilus* (Rettger and Cheplin, 1921). Although the largest class of prebiotics are oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), XOS, inulin and its derivatives (Gibson et al., 2017), not all carbohydrates can be classified as prebiotics as they need to follow certain requirements such as:

> 1. It cannot be hydrolyzed or absorbed by the upper part of the gastrointestinal system;
> 2. Be selective for one or a limited group of beneficial bacteria present in the colon, thus stimulating their growth or metabolic activity;
> 3. Be able, through the selective stimulus of bacteria, to change the composition of the microbiota in a way that is healthier for the host;
> 4. Induce luminal or systemic effects beneficial to the health of the host.
>
> (Gibson and Roberfroid, 1995).

By these definitions, carbohydrates such as pectin, celluloses and xylans cannot be classified as prebiotics, since they serve as a substrate for a large group of bacteria in the intestine, having no selectivity (Gibson et al., 2017).

Over the years, the definition and requirements have changed in order to complement and update the term prebiotic. In 2008, the FAO (Food and Agriculture Organization of the United Nations) met to discuss the term prebiotic and how to classify a component as prebiotic or not (Pineiro et al., 2008). From this meeting, it was defined that a prebiotic is a non-viable food that confers a benefit on the health of the host, improvement is associated with modulation of the microbiota, and also defined some terms of this definition:

> 1. Component: not an organism or drug; a substance that can be characterized chemically; in most cases this will be a food grade component.
> 2. Health benefit: measurable and not due to absorption of the component into the bloodstream or due to the component acting alone, and over-riding any adverse effects.
> 3. Modulation: show that the sole presence of the component and the formulation in which it is being delivered changes the composition or activities of the microbiota in the target host. Mechanisms might include fermentation, receptor blockage, or others.
>
> (Pineiro et al., 2008).

One of the most prominent contests of ISAPP (International scientific association for probiotic and prebiotic) about this new definition of prebiotics is the term related to the selectivity of the compound by bacteria beneficial to health, because it can include compounds such as antibiotics could be classified as prebiotics (Gibson et al., 2010). In order to update the term prebiotic and its use, a ISAPP meeting was held in 2008 in London (Ontario, Canada), and it was defined that a prebiotic is "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2010). However, this definition limited the use of the term prebiotic to intestinal microbiota, leaving out, for example, oral, vaginal, skin and other sites. To redefine this term, another ISAPP meeting was held on 9 December 2016 in London, UK, and prebiotic was defined as "a substrate that is selectively used by the host microorganisms, conferring health benefits", thus encompassing the various sites of the human body (Gibson et al., 2017).

Before characterizing a compound as prebiotic, it is necessary to define who is its host and in which site it is acting. For example, cellulose can be considered a prebiotic if we are referring to ruminants as a host, but the same cannot be said for humans (ben David et al., 2015). Xylitol can be considered a prebiotic in the oral cavity but the same is not true in the intestinal microbiota (Söderling et al., 2011; Uebanso et al., 2017).

Focused more on the intestinal microbiota, the most studied and consolidated prebiotics are fructooligosacharides (FOS), galactooligosacharides (GOS) and inulin derivatives, being responsible for most studies at the most diverse levels, such as *in vitro*, in microbiota simulators, in animals and in humans (Grajek et al., 2005; Rastall and Gibson, 2015). One of least studied prebiotics is XOS, which has been gaining prominence among the oligosaccharides with prebiotic properties. Although most tests with XOS are *in vitro*, there is already evidence that this oligosaccharide has a greater specificity for certain groups of bacteria, such as the group *bifidobacterium*. *In vivo* human trials have shown that doses of 1.4 and 2.8 g per day of XOS significantly increase the population of *bifidobacterium* and bacteroides fragilis when compared to placebo trials, with the population of lactobacillus not being significantly affected (Finegold et al., 2014). In another trial, administration of XOS (8 g/day) resulted in an increase in the population of *bifidobacterium* in the stool, increased mean daily bowel movements and the level of happiness and social behavior of patients (Childs et al., 2014).

1.4 SHOR-CHAIN FATTY ACIDS IMPORTANCE

Prebiotics act on the gut microbiota in the role of selectively stimulating bacteria that result in health improvement. Normally, these bacteria metabolize these compounds and

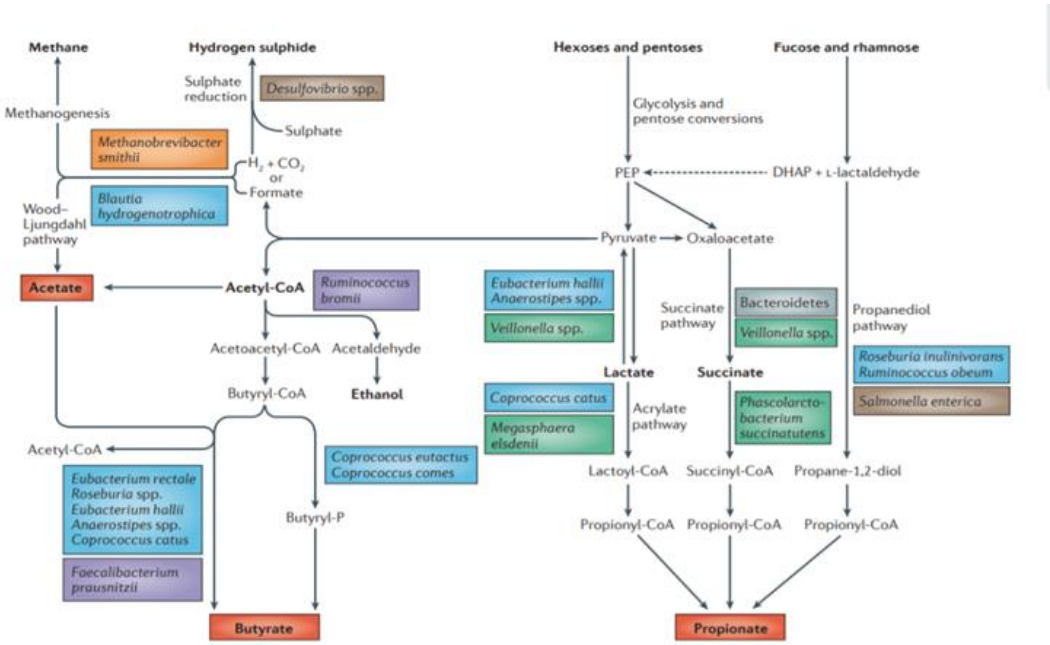

Figure 5. Metabolic pathways responsible for the biosynthesis of the main microbial metabolites resulting from carbohydrate fermentation and cross-feeding of bacteria (Louis et al., 2014).

generate precursors for vitamins, proteins, etc. Most metabolic pathways lead from carbohydrate consumption to short-chain fatty acid (SCFA) release, as illustrated in Figure 5.

One of the most studied genera as a probiotic microorganism, the *bifidobacterium*, has a different metabolic pathway, known as the “fructose-6-phosphate shift” or “bifid shunt” (Figure 6), since bypasses the traditional heterolactic pathway of consuming glucose in the fructose-6-phosphate molecule to generate 25% more ATP molecules. This higher yield in ATP generation allows these bacteria to achieve better energy performance in a carbohydrate-scarce environment (Pokusaeva et al., 2011).

The release of SCFAs into the microbiota affects local microbial composition and metabolism in several ways. First, SCFA released by fermentation by *bifidobacterium* are absorbed by the host system, with an equilibrium formed by the bicarbonate released by conocytes (Figure 7) resulting in a pH value dependent on the efficiency of this equilibrium. The closer to the end of the intestine, the higher the pH found and this difference in pH results in different microbial composition in different parts of the intestine (den Besten et al., 2013). For example, regions with a pH close to 5.5 have an average composition of 20% of bacteria that produce buritrate, while at pH above 6.5 this population is close to being null and the population of bacteria that produce acetate and propionate become dominant (Walker et al.,

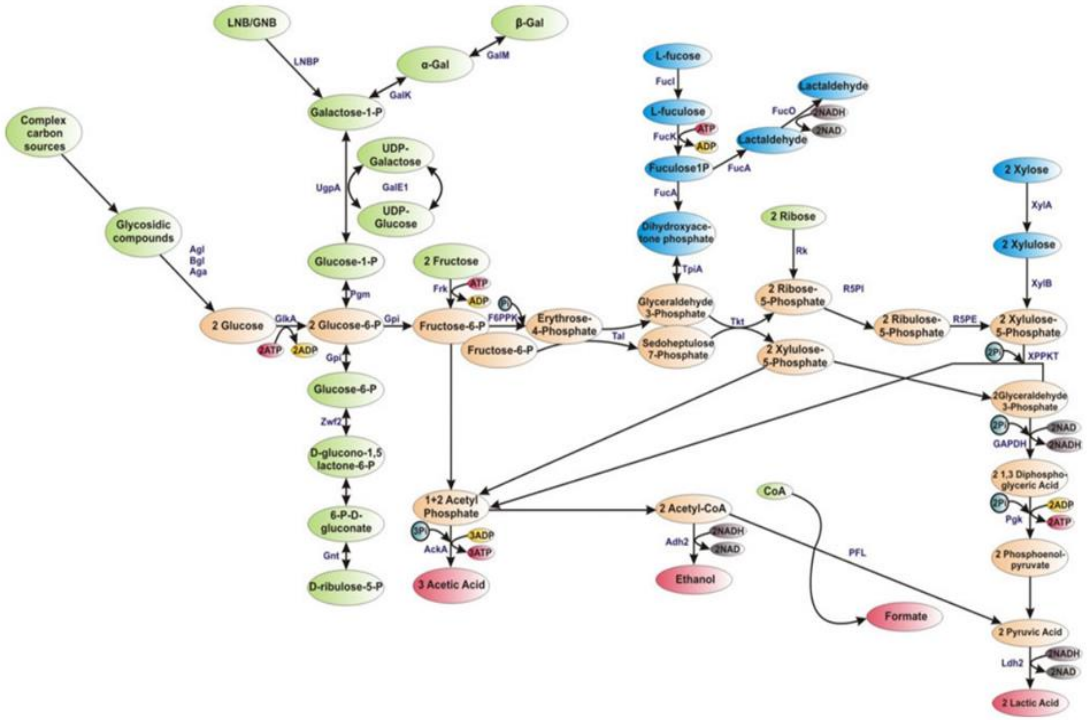

Figure 6. Schematic of the common metabolic pathway of *bifidobacterium*, known as "fructose 6-phosphate shift" or "bifid shift" (Pokusaeva et al., 2011).

2005). Potentially pathogenic bacteria, such as *Escherichia coli K12* and *Salmonella spp*, when subjected to a pH close to 5, generated by propyl acid (a SCFA), had a population reduction of about 90% after 1 h (Cherrington et al., 1991). As the microbial population in the microbiota is vast, SCFA can serve as substrates for other bacteria in a cross-feeding system, which consists of the product of the metabolism of one bacterium serving as a substrate for the metabolism of another bacterium present in the environment (Lee et al., 2018)(Figure 9). *Faecalibacterium prausnitzii*, *Eubacterium and Roseburia* are genera of bacteria that produce buritrate in the human microbiota and have their accentuated growth in the presence of acetate, which can be exogenously inserted or generated by the activity of *bifidobacterium*, demonstrating the cross-feeding system in the microbiota. (Duncan et al., 2004; Rivière et al., 2016).

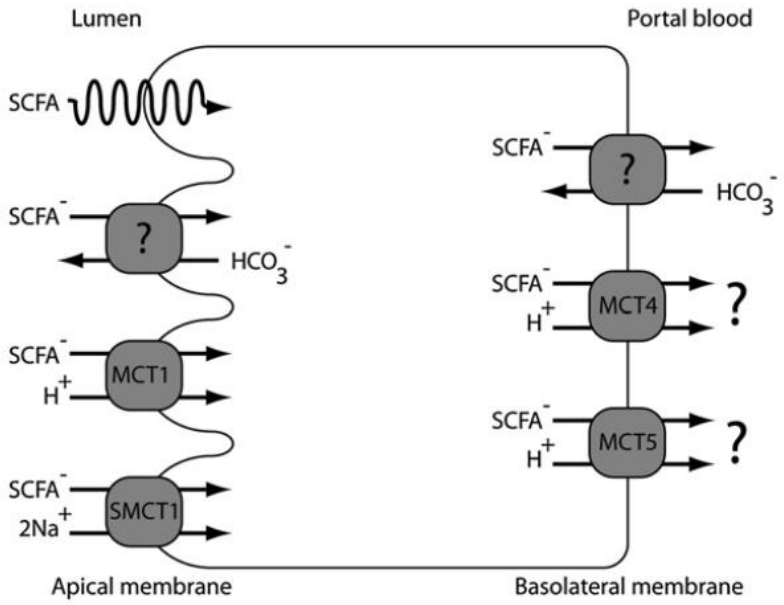

Figure 7. Schematic overview of the proposed transport mechanisms of SCFAs in colonocytes. Across the apical membrane, the major part of SCFAs is transported in the dissociated form by an HCO_3 exchanger of unknown identity (?) or by one of the known symporters, MCT1 or SMCT1.A small part may be transported via passive diffusion (spiral). The part of SCFAs that is not oxidized by the colonocytes is transported across the basolateral membrane. The basolateral transport can be mediated by an unknown HCO 3exchanger, MCT4, or MCT5 (den Besten et al., 2013).

SCFAs also play a signaling role for some metabolism and immune system regulators, resulting in health improvements (Shapiro et al., 2014). For example, some G protein-coupled receptors, which are cellular interacting proteins, are sensitive to the presence of SCFAs, such as GPR41 and GPR43, also known as free fatty acids Ffar3 and Ffar3 (“free fatty acid”). respectively (Poul et al., 2003). The interaction of GPR43 or GPR41 receptors and SCFA potentiates the activation of leukocytes and the inflammatory response of the immune system, in addition to increasing intracellular calcium levels (Maslowski et al., 2009; Poul et al., 2003).

When subjected to induced colitis, mice that received a diet with SCFA had a better anti-inflammatory system response caused by the SCFA interaction with the GPR43 receptor that express the T cells responsible for the immune system (Smith et al., 2013).

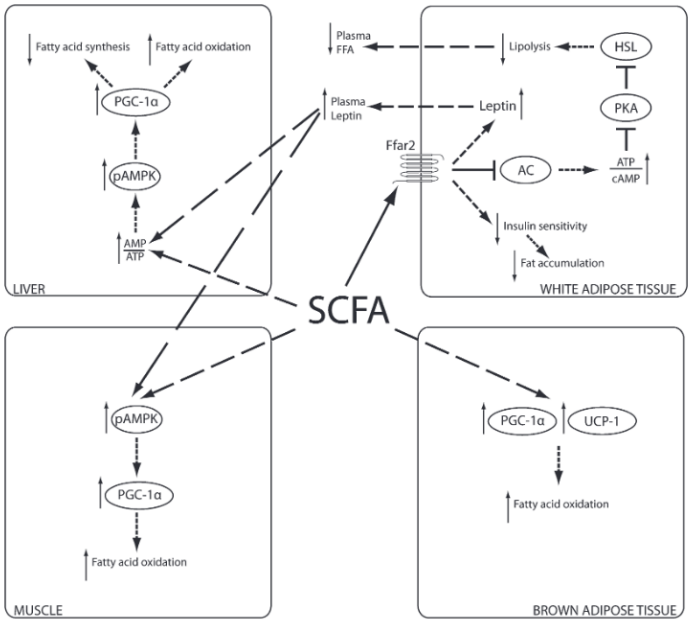

Figure 8. Schematic overview of the proposed mechanisms by which SCFAs increase fatty acid oxidation in liver, muscle, and brown adipose tissue. In muscle and liver, SCFAs phosphorylate and activate AMPK (pAMPK) directly by increasing the AMP/ATP ATP ratio and indirectly via the Ffar2-leptin pathway in white adipose tissue. In white adipose tissue, SCFAs decrease insulin sensitivity via Ffar2 and thereby decrease fat storage. In addi-tion, binding of SCFAs to Ffar2 leads to the release of the G i/o protein, the subsequent inhibition of adeny-late cyclase (AC), and an increase of the ATP/cAMP ratio. This, in turn, leads to the inhibition of PKA and the subsequent inhibition of HSL, leading to a decreased lipolysis and reduced plasma free fatty acids. (den Besten et al., 2013)

SCFA exert a great influence on the accumulation of fat in various organs of the human body, as illustrated in Figure 8, resulting in a mechanism to combat obesity. In liver and muscles, SCFAs increase the expression of AMPK (5' adenosine monophosphate-activated), which triggers the expression of PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), that controls a series of transcription factors, resulting in decreased accumulation of fat in these organs (den Besten et al., 2013). In brown adipose tissue, SCFA increase the expression of PGC-1α and UCP-1 (uncoupling protein), increasing thermogenesis and decreasing fat accumulation (Gao et al., 2009). In white adipose tissue, activation of GPR43 by SCFAs acts on energy metabolism by suppressing insulin signaling in adipocytes, which inhibits fat accumulation in adipose tissue and promotes glucose and unincorporated lipid metabolism (Kimura et al., 2013). Through the GPR43 regulator, SCFA increase the expression of leptin, which in the bloodstream stimulates the oxidation of fats in various organs, such as muscles and liver (den Besten et al., 2013).

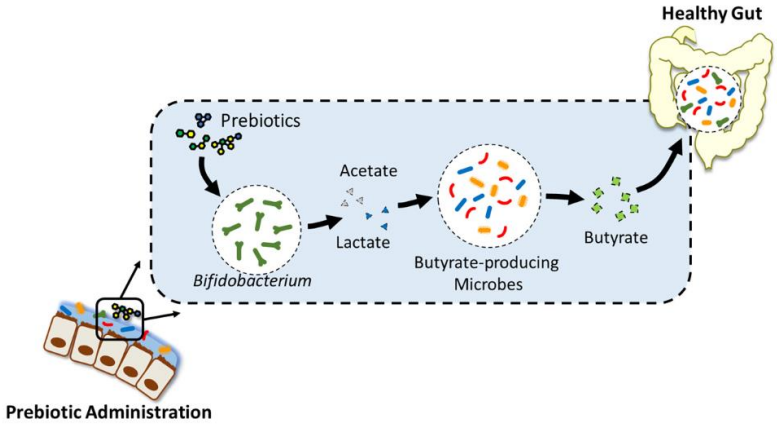

Figure 9. Cross-feeding effect between *Bifidobacterium* and butyrate-producing bacteria. *Bifidobacterium* utilizes supplemented prebiotics, which stimulates their growth. Acetate produced by *Bifidobacterium* becomes a carbon source for butyrate-producing microbe, stimulating their growth and butyrate-producing activities and, in turn, modulating the microbiome function and improving gut health (Lee et al., 2018).

1.5 MECHANISMS OF XOS UTILIZATION BY BIFIDOBACTERIUM

The use of XOS by bacteria of the human intestinal microbiota is practically done by *bifidobacterium*, which has a very specific prebiotic in relation to other bacterial genera, such as lactobacillus (Mäkeläinen et al., 2010b). As previously mentioned, *bifidobacterium* use a specific metabolic pathway, the bifid-shunt, which has the compound xylulose 5P as a gateway to the use of XOS as a prebiotic. XOS is first hydrolyzed to xylose through xylanases, which are synthesized by the bacterium itself, and then the xylose produced is taken to xylulose 5P, which integrates with the bifid shift pathway (O'Callaghan and van Sinderen, 2016).

Transcriptome analyzes show that *B. adolecentis* in the presence of XOS, in relation to the presence of xylose, increases the expression of some genes related to carbohydrate transport and metabolism, such as the ABC transport system, xylosidases, galactosidases, glucosidases and amylases (Yang et al., 2019). A model of XOS consumption by the *bifidobacterium* genus can be based on transcriptome analyzes (Figure 10). The presence of XOS increases the expression of the genes responsible for transporting the carbohydrate into the cell, where it will undergo the actions of xylanases, which also have their expression increased, and will result in xylose. This xylose will be converted to 5P xylulose and will enter the bifid bypass pathway (Andersen et al., 2013; Gilad et al., 2010).

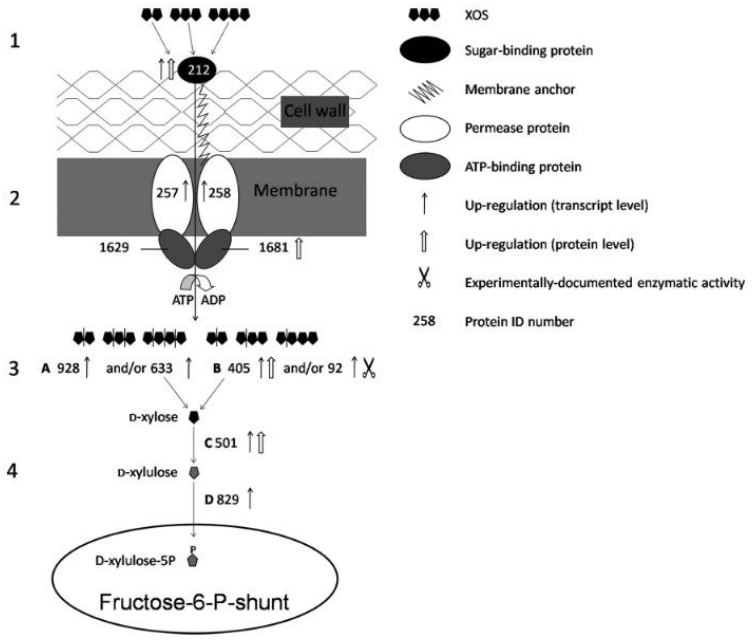

Figure 10. Proposed model for the catabolism of XOS in *B. animalis* subsp. *Lactis* BB-12 comprising the following steps. 1, binding of XOS at the cell surface by a sugar-binding protein. 2, transport of XOS by an ABC transport system. 3, degradation of XOS to D-xylose (by a combined action of an endo-1,4-β-xylanase and aβ-xylosidase). 4, conversion of D-xylose to xylulose-5-P, a key metabolite of the fructose-6-P shunt. A, endo-1,4-β-xylanase; B,β-xylosidase; C, xylose isomerase; D, xylulose kinase. The numbers indicate the respective genes in the genome of BB-12, omittingthe "BIF 0(Gilad et al., 2010).

CHAPTER 2: FEASIBILITY OF DIRECT USE OF HEMICELLULOSE HYDROLYZATE AS A PREBIOTIC COMPOUND IN IN VITRO TESTS

2.1 ABSTRACT

Xylooligosaccharides (XOS) can be extracted in different ways, and this results in structural differences that can influence their prebiotic capacity. This work aimed to evaluate the prebiotic potential of XOS contained in the hemicellulosic hydrolysates obtained from sugarcane bagasse by hydrothermal treatment under different process conditions and within biorefinery concept. For this, anaerobic fermentation was carried out using three probiotic microorganisms (*B. adolecentis, B. infantis* and *B. lactis*). First, the hydrolysates were characterized in relation to their carbohydrates, degree of polymerization, short-chain fatty acid (SCFA), hydroxymethylfurfural and furfural. As a control carbohydrate source for comparison, two commercial prebiotics, XOS and FOS, and glucose were used. The presence of compounds such as HMF and furfural were not inhibitory to the growth of *bifidobacterium*, and the levels of substituent acetyl groups had a moderate impact on the levels of SCFA released. It was possible to notice a significant difference between the growth of probiotic strains on the different carbohydrate's sources, with some hydrolysates promoting the growth and release of SCFA at levels close or superior to commercial XOS. The size of the XOS chain had an influence on its ability to be used as a carbon source, with XOS with DP superior to six showing lower performance than with DP two to six. This chapter may point out that process conditions that result in higher XOS extraction and selectivity of XOS to xylose do not necessarily present XOS with better prebiotic properties, being the XOS structure more important.

2.2 INTRODUCTION

Enzymatic processes preceded by alkaline extraction are the most applied methods for XOS production (Carvalho et al., 2013). However, an alternative route is the production by hydrothermal processes in an acid medium, which are potentially more economical due they do not use enzymes and obtain the product of interest in a smaller number of steps (Otieno and Ahring, 2012).

Among the disadvantages of using a hydrothermal process to produce XOS is the formation of sugar degradation products such as hydroxy-methylfururaland furfural from the degradation of glucose and xylose, respectively (Akpinar et al., 2010). Commonly, hydroxy-methylfurfural and furfural compounds are known as inhibitors, probably because their

presence is inhibitory, at certain concentrations, for aerobic fermentations (Klinke et al., 2004). However, there has been no studies that has inferred this inhibitory property to intestinal microbiota. Thus, this is a disadvantage not yet confirmed. In fact, there is a work that analyzed foods that were heat treated, thus generating compounds such as furfural and hydroxy-methylfurfural through Mailard reactions, and, after statistical analysis, the authors found a positive correlation between the presence of these degradation compounds and the development of some bacteria that exert positive functions in the human microbiota (Pérez-Burillo et al., 2018). In this respect, it may be that these degradation compounds, within ranges to be determined, present in hemicellulose hydrolysates obtained by hydrothermal treatment, do not cause an adverse effect to human health, and may be part of prebiotic compounds.

Another potential advantage of using the hydrothermal process is the possibility of obtaining XOS with distinct structures, for example with varying degrees of polymerization (DP). This is relevant because diversity of structures, such as DP and acetyl groups, can provide different prebiotic effects (Mäkeläinen et al., 2010b). The XOS extraction process conditions can also be manipulated to obtain a higher yield of XOS (Carvalho et al., 2018; Marcondes et al., 2020; Schell et al., 2003)

For a compound to be considered prebiotic, it is necessary to verify if this compound was selective for part of the target microbiota and that its administration results in an improvement in health, and this verification is only done with *in vivo* tests (Rowland et al., 2018). However, preliminary tests can be performed to verify potential prebiotics among the compounds, such as, for example, the ability to serve as a selective substrate for certain genera of bacteria that are already consolidated as probiotics (eg *lactobacillus* and *bifidobacterium*). In addition, the characterization of a compound to be evaluated for its prebiotic property needs to be carried out, such as the origin of the material, production method, chemical and structural characteristics, purity, concentration and the amount to be used per application (Pineiro et al., 2008).

Bifidobacterium, together with *Lactobacillus*, are the genera most recognized as probiotics and for this reason they are the most used for in vitro tests to analyze prebiotic potential (Moura et al., 2007). Using the specific metabolic pathway for *Bifidobacterium*, the bifido-shunt, this microorganism has SCFA as main metabolic products, such as acetic acid and lactic acid. Although the dynamics of interaction between *Bifidobacterium* and other bacteria in the microbiota and the host itself is not perfectly clear, several studies have indicated that the development of *Bifidobacterium* cause inhibitory effects to some potentially pathogenic

bacteria due to the production of SCFA, antibacterial peptides, inhibitors of quorum-sensing or by stimulating the immune system (Hidalgo-Cantabrana et al., 2017).

Considering the possible advantages of extracting XOS from SCB by hydrothermal processes mentioned above, a study was carried out by applying a designer of experiment with the process conditions (time, temperature, and acid concentration) of XOS extraction on a bench scale in a pressure vessel reactor, thus obtaining several hemicellulosic hydrolysates with different chemical compositions and structural characteristics of XOS (Marcondes, 2018). Thus, this chapter evaluates the prebiotic potential of these hydrolysates through anaerobic fermentation with isolated strains of *Bifidobacterium*.

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

The SCB hemicellulose hydrolysates used in this work were obtained in a previous work (Marcondes et al., 2020). Briefly, hydrothermal treatments under different process conditions following a statistical design of experiments were applied to maximize the extraction of XOS by varying temperature (150-190 ºC), time (5-45 min) and concentration of sulfuric acid (0-0.1 % w /w). The treatments were carried out in a stainless-steel pressured reaction vessel (Parr - 2L).

The isolated strains of *B. lactis*, *infantis* and *adolecentis* were obtained from a commercial source (THT Belgian company). Glucose (Sigma-Aldrich), commercial FOS (NEWNUTRITION) and commercial XOS (PrecticX 95P) were used as controls.

2.3.2 QUANTIFICATION OF CARBOHYDRATES AND SHORT CHAIN FATTY ACIDS

The characterization of the hemicellulosic hydrolysates was performed in previous studies (Marcondes, 2018) and is described in Table 1. The previous hydrolysates were named PHX, where X is the number of the respective process condition described in the previous work, and the fermentation medium using the respective hydrolysates was named HX.

The culture media, before and after fermentation, was carried out according to the protocol of NREL (Sluiter et al., 2008) in relation to carbohydrates (total monomeric and oligomeric), SCFA, such as acetic to lactic acid, and pentose carbohydrate degradation products (furfural) using high performance liquid chromatography (HPLC). Briefly, monomeric carbohydrates and SCFA were quantified using an HPX-87H separation column (Bio-rad) at 45°C, eluted with 5 mmol/L sulfuric acid at 0.6 mL/min, with the refractive index detector. To quantify total XOS (X_T), an acid hydrolysis step (4% w/w H_2SO_4) was carried out for 1 h at 121 ºC to hydrolyze all oligomers to monomers, which were as mentioned above. The difference between the monomers obtained after hydrolysis in acidic medium and that quantified before acid hydrolysis was referred to as the amount of total oligomers. A correction factor of 0.88 was used to account for the hydration of xylose obtained in the hydrolysis of the oligomers. This technique also allowed to quantify how many acetyl groups were released in acid hydrolysis, with a correction factor of 0.983 (Sluiter et al., 2008). For the determination of furfural, a Resolve 5µm Spherical C18 column (3.9 x 300 mm) at 25 °C was used, with a mobile phase of aceto-nitrile solution and water (1:8) with 1% acetic acid (v/v), using the SPD-10A UV-VIS ultraviolet detector (Esteve and Carvalho, 2011).

The characterization of the XOS also included the determination of the DP, which was analyzed as described in (Vacilotto et al., 2022). Briefly, a high-performance anion exchange chromatography (HPAEC) system Dionex ICS-5000 (Thermo Scientific, Waltham, USA), coupled with an ion exchange column CarboPAC1 (250 mm × 2 mm) (Thermo Scientific, Waltham, USA) was set up with flow rate of 0.3 mL/min at 30 °C, injection volume equal to 1 µL, and 100 mM NaOH (buffer A) and 500 mM sodium acetate with 100 mM NaOH (buffer B) as buffers for the elution. The available standards for calculating the DP were xylobiose, xylotriose, xylotetraose, xylopentonse and xylohexose, represented by Xyl2, Xyl3, Xyl4, Xyl5 and Xyl6, respectively. The sum of fractions Xyl2 to Xyl6 and the XOS with DP higher than six are named $XOS_{(2-6)}$ and $XOS_{>6}$, respectively.

2.3.3 IN VITRO ASSAYS

To assess the suitability of XOS present in the hydrolysates as a prebiotic compound, fermentation was performed with probiotic microorganisms, specifically *Bifidobacterium lactis*, *infantis* and *adolescents*. Fermentation was carried out at 37 ºC, similar to that of the human body, and the incubation time was 3 days in an anaerobic condition. To ensure anaerobic

condition of the process, fermentation took place in tubes that provided a 5 cm column of liquid, with sterile culture medium. Although the sterilization process (121 ºC for 15 min) also acts as a process for removing oxygen, the tubes cannot be shaken after this step. Therefore, rezasurin (0.01% w/v) was used as an anaerobic indicator and all culture media did not show the reddish color that is characteristic of medium with oxygen in the presence of the indicator, thus establishing that the proposed procedure was suitable for anaerobic environments.

An adaptation of *Bifidobacterium* medium (DSMZ 58) was used to grow *Bifidobacterium*, with the composition per liter of: 50 g of casein peptone; 25 g of yeast extract; 25 g of meat extract; 10 g of K_2HPO_4; 1 g $MgSO_4$ x 7 H_2O; 0.25 g $MnSO_4$ x H_2O; 5 g of NaCl, 2.5 g of cysteine-HCl x H_2O and 40 ml of resazurin (0.025% w/v). The hydrolysates were used as carbon sources, standardizing the X_T concentration at 3 g/L. The pH of the media was adjusted to 7.2 with 0.5 M NaOH or 1M HCl solution, before the autoclave sterilization process at 121 ºC for 15 min. Glucose, XOS and commercial FOS were used as comparative growth carbohydrates for the probiotic bacteria (3 g/L), with a concentration fixed at 3 g/L for the X_T.

To assess the prebiotic activity, three parameters were used: cell growth, measured by optical density at a wavelength of 650 nm (OD_{650}) (Palframan et al., 2003); variation in the pH of the medium and the formation of SCFA.

2.3.4 XYLANASE ACTIVITY

To determine whether the bacteria secreted enzymes to help metabolize XOS, the activity of xylanases and β-xylosidase in the culture medium was verified. For this, the culture medium after the fermentation process was first centrifuged at 400 g for 10 min to precipitate the bacterial cells. The liquid fractions were collected and assayed for the enzymatic activities.

Endoxylanase activity was detected according to a procedure adapted from (Tanaka et al., 1981). Standard birch xylan substrate solution (Sigma, 1 % w/w in 0.05 M citrate-phosphate buffer pH 6.0 solution) in a 1:1 ratio with the liquid sample (100 µL of each) was added in a conical tube (volume 2 mL), allowing to react for 12 h at 37 °C and 100 rpm. Blank solutions for each carbon source were performed simultaneously with the samples without the presence of substrate (xylan). Another blank solution was prepared only with the presence of substrate and buffer. After the reaction period, 300 µL of DNS reagent solution (3,5-dinitrosalicylic acid), prepared according to (Miller, 1959) were added into the medium to stop the reaction. Xylose

solutions were used to prepare the standard curve and were mixed with DNS in the same proportion as the samples. The samples and standards were boiled in a water bath for 5 min. 200 µL of the solutions were added to a 96-well flat-bottom plate for reading in a spectrophotometer (Epoch2 – Biotech) at 540 nm.

For the β-xylosidase activity, the method was adapted from the procedure of (Tan et al., 1998), where the enzyme acts on the p-nitrophenyl-β-xylopyranoside (pNPX) substrate releasing *p*-nitrophenol (pNP), which is analyzed by spectrometry. For this, 0.5 µL of pNPX (Sgima, 0.1 % w/v in 0.05 M citrate-phosphate buffer pH 6.0) and 0.5 µL of the sample were mixed in a 96-well plate and incubated for 12 h at 37 °C under stirring at 100 rpm. Substrate blank for each carbon source was performed by replacing pNPX with buffer. Sample blanks were performed by replacing the sample volume for buffer and pNP was used to standard curve. After the incubation period, the reaction was stopped by adding 200 µL of sodium bicarbonate (10 % w/w in distilled water) and the absorbance was read at 410 nm.

2.4 RESULTS AND DISCUSSION

2.4.1 CHARACTERIZATION OF HYDROLYSATES

The study that generated the hydrolysates used in this research was carried out in order to study the process conditions aiming extract XOS with at high yield (Marcondes, 2018). A statistical design of experiment was used, which varied temperature, time, and sulfuric acid concentration in a pressure vessel reactor (PAR – 2 L). As different process conditions can result in XOS with different structures (Carvalho et al., 2018), several hemicellulosic hydrolysates were generated with different compositions between oligomers and monomers (Table 1), in addition to obtaining XOS with different structures (i.e, DP).

The low concentration of glucose in relation to the products of hemicellulose hydrolysis (xylose, arabinose and acetyl groups) evidences the high selectivity of the process for the extraction of hemicellulose, which was in accordance with the objective of the work to obtain processes that were within the concept of fractionation of biorefinery (Marcondes, 2018). This was possible due to the choice of working ranges, which sought not to be highly severe in order not to reduce the selectivity of the process and not to high obtain xylose as the main product. The products of xylan extraction and hydrolysis are predominant, with the xylose released in dilute acid hydrolysis (post hydrolysis) representing the concentration of XOS in the

hydrolysates. The substituent groups of XOS are understood as arabinose and acetic acid released in the post-hydrolysis process, with arabinose coming from arabinosyl groups and acetic acid from acetyl groups (Marcondes, 2018).

All hydrolysates that showed a minimum concentration of 5 g/L of X_T (PH3, PH4, PH5, PH7, PH8, PH10, PH11, PH13, PH16, PH18 and PH19 – Table 1), including the optimized condition for XOS extraction (OT), were explored in this chapter aiming to study the influence of these structural differences on the prebiotic capacity of XOS through fermentation of isolated strains of *bifidobacterium*.

Table 1. Chemical characterization of hemicellulose hydrolysates; Post hydrolysis*: process of breaking carbohydrate chains with release of monomers and substituent groups; Hydr. #: Code of each hydrolysate obtained in the work Marcondes et. Al. 2020.

	Components [g/L]							
	before post hydrolysis *				post hydrolysis *			
Hydr. #s	**Glucose**	**Xylose**	**Arabinose**	**Acetic acid**	**Glucose**	**Xylose**	**Arabinose**	**Acetic acid**
PH1	0.12	3.84	0.23	1.59	0.72	2.02	0.19	0.74
PH2	0.02	0.39	0.46	0.42	0.74	2.03	0.40	0.06
PH3	0.02	2.09	0.60	1.16	1.21	13.16	0.56	1.57
PH4	0.02	0.57	0.92	0.49	0.60	5.83	0.52	0.70
PH5	0.02	0.59	0.84	0.76	0.87	10.69	0.79	1.06
PH6	1.93	5.62	0.58	3.35	0.92	0.38	0.18	0.22
PH7	0.52	8.16	1.69	1.49	0.93	8.59	0.12	1.29
PH8	0.19	8.33	1.66	1.26	1.24	9.72	0.13	1.59
PH9	1.26	13.57	1.57	2.60	0.77	0.32	0.00	0.49
PH10	0.21	7.84	1.62	1.44	1.21	10.07	0.20	1.42
PH11	0.40	10.44	1.69	1.57	0.87	6.55	0.21	0.97
PH12	0.25	5.50	1.53	1.10	0.91	8.87	0.38	1.01
PH13	0.15	6.33	1.49	1.01	1.15	10.49	0.28	1.50
PH14	1.24	14.95	1.66	2.51	0.57	1.49	0.00	0.54
PH15	0.13	1.89	1.04	0.54	0.48	3.75	0.43	0.40
PH16	0.16	6.69	1.51	1.01	1.07	9.91	0.25	1.55
PH17	1.63	3.09	0.21	4.24	1.06	0.12	0.19	0.11
PH18	0.66	8.92	1.30	1.95	0.61	6.05	0.00	0.81
PH19	0.12	3.78	1.41	0.63	0.98	11.72	0.39	1.55
POT	0.02	0.21	0.45	0.28	0.69	5.21	0.23	0.54

2.4.2 CHARACTERIZATION OF FERMENTATION MEDIUM

The characterization of the hydrolysates in the fermentation process(HX) was performed after the sterilization step, once the conditions applied of 121 ºC during 15 min can

change chemical structure of the XOS and our objective was to evaluate the structure (DP and DS) of XOS available for *Bifidobacterium* in the fermentation process (Table 2).

Only two HX showed selectivity of XOS for xylose lower than 1 (H11 and H18), i.e., it resulted in the extraction of more xylose than XOS (Table 2). The hydrolysate generated at the optimized condition (OT) was the one that presented the highest selectivity, which was the objective of the experimental design applied in the previous study aimed at XOS extraction from sugarcane bagasse (Marcondes et al., 2020). The commercial XOS had the highest selectivity, as it has undergone purification and fractionation processes that result in the removal of monomers. The XOS' DP distribution, within range xylobiose to xylohexaose, of most HX (H3, H7, H8, H10, H12, H19) has a high correlation (R^2>0.9) to commercial XOS' DP distribution, however they also contained xylose, acetyl groups and furfural, even at low concentration, which are not found in the commercial XOS (Table 2). The high correlation between the proportions of $XOS_{(2-6)}$ compared to commercial XOS has a moderate inverse correlation with the selectivity of each fraction (R^2=-0.82), meaning that with higher selectivity generates lower similarity with the commercial XOS.

2.4.3 *BIFIDOBACTERIUM* GROWTH

Optical density is one of the most common means for determining bacterial growth in liquid media due to its rapid performance. However, this method measures the increase in the total turbidity of the medium, encompassing active and dead cells and other compounds present in the medium, such as products that may have been generated in the fermentation process. Based on this, it is interesting to analyze the increase in optical density together with other parameters, according to the biochemical process being studied. In the case of fermentation with *bifidobacterium*, whose main products excreted in the medium are acetic and lactic acid, parameters such as the decrease of the medium pH and concentration of substrate and products at the end of the process can be considered.

On non-XOS carbon sources, there was a greater growth of *B. adolecentis* and *B. infantis* when compared to *B. lactis* (Figure 11). In general, *B. infantis* was the *Bifidobacterium* that grew best on different carbon sources while *B. lactis* was the worst. The XOS carbon source that resulted in lower growth measured by OD_{650} was the same that presents low amount of $XOS_{(2-6)}$ (H4, H5 and OT), but the level of correlation between these two variables was not high (R^2<0.491). In fact, no structural characteristic had a high correlation with the optical density

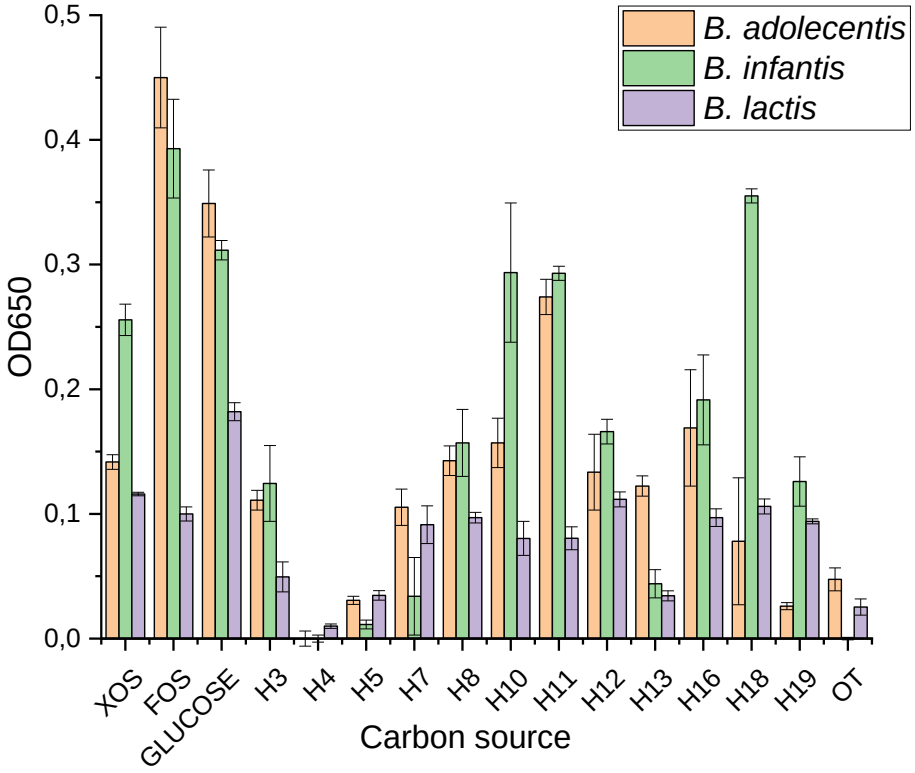

Figure 11. Growth of *B. adolecentis*, *infantis* and *lactis* by different carbon sources, expressed in Optical Density at 650 mm wavelength (OD_{650}).

variable (Table 3), indicating that components other than the chemical composition of the hemicellulosic fraction of the hydrolysate could be affecting the development of *bifidobacterium*.

B. adolecentis and *infantis* showed similar decrease in pH levels in most carbon sources, while *B. lactis* showed lower decrease in pH among *bifidobacterium* in practically all carbon sources (Figure 12), a result also found by (Mäkeläinen et al., 2010b). On the other hand, (Mäkeläinen et al., 2010b) reported that XOS was more effective for the growth of *B. lactis*, a fact that was not confirmed in this work. Even though there was not a high correlation between the structural characteristics of the XOS with the decrease in pH, it is possible to notice that the lower values of $XOS_{(DP\ 2\text{-}6)}$ resulted in a smaller decrease in pH.

The decrease in pH at the end of the fermentation process is an indirect indication of the metabolism of the microorganism that has acids as its product and is another way of analyzing microbial activity. It can be noted that the decrease in pH was similar among the hydrolysates (Figure 12), except for H4, H5 and OT, that did not decrease so much. This result was expected as these hydrolysates showed lower values of OD_{650}, and consequently less metabolic activity

Table 2. Characterization of fermentation medium with different hydrolysates at the beginning of the process.

Xylan's Components (% w/w)		XOS*	fermentation medium with different hydrolysates												
			3H	4H	5H	7H	8H	10H	11H	12H	13H	16H	18H	19H	OT
ratio XOS/xylose		100.01	4.82	8.36	11.50	1.71	2.11	1.82	0.79	2.05	1.32	1.70	0.85	3.96	9.31
X_T		99.01	82.83	89.32	92.00	63.11	67.85	64.56	44.25	67.25	56.93	63.01	45.87	79.83	90.30
$X_{(2-6)}$		65.75	80.50	0.00	6.91	37.15	36.63	19.95	32.05	25.32	28.64	37.09	66.27	13.53	4.43
XOS component (% w/w)**	Xyl2	40.24	34.43	67.12	25.45	46.71	41.34	40.35	51.45	35.66	41.73	44.50	59.72	32.50	39.36
	Xyl3	36.21	25.26	18.49	18.45	24.95	24.82	25.02	20.80	23.01	25.61	21.83	22.44	23.15	18.47
	Xyl4	16.94	18.97	11.64	20.25	16.15	17.81	17.85	16.08	18.66	17.82	15.76	14.02	19.80	14.06
	Xyl5	6.47	14.88	2.74	21.63	9.08	11.28	11.70	9.65	14.57	11.00	14.14	3.82	15.70	23.69
	Xyl6	0.15	6.46	0.00	14.21	3.11	4.76	5.08	2.02	8.10	3.84	3.77	0.00	8.85	4.42
Acetyl***			0.12	0.12	0.10	0.15	0.16	0.14	0.15	0.11	0.14	0.16	0.13	0.13	0.10
Furfural***			0.02	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.03	0.00	
XOS consumption		76.00	61.22	13.40	34.47	56.45	57.87	55.48	72.17	72.96	59.55	50.15	60.25	52.25	18.93

* Commercial XOS; ** percentage of each degree of polymerization in the XOS fractions within XOS 2-6 – Xyl2 (xylobiose); Xyl3 (xylotriose); Xyl4 (xylotetraose); Xyl5 (xylopentaose), Xyl6 (xylohexaose); *** Weight of furfural/acetyl relative to xylose units on XOS.).

Table 3. Correlation factors between the structural characteristics of XOS and the metabolism parameters of *Bifidobacterium*. Adol: *Bifidobacterium adolecentis*; Inf.: *Bifidobacterium Infantis*; Lac.: *Bifidobacterium* lactis.

Xylan's Components (% w/w)		DO650			pH			Acetic acid			Lactic acid			Lactic + acetic acid		
		Adol.	Inf.	Lac.	Adol.	Inf.	Lac.	Adol.	Inf.	Lac.	Adol.	Inf.	Lac.	Adol.	Inf.	Lac.
Ratio XOS/xylose		-0.70	-0.68	-0.74	-0.73	-0.79	-0.62	-0.73	-0.87	-0.65	-0.83	-0.83	-0.86	-0.84	-0.86	-0.84
X_T		-0.73	-0.74	-0.62	-0.59	-0.70	-0.42	-0.66	-0.89	-0.45	-0.88	-0.92	-0.78	-0.84	-0.92	-0.71
$XOS_{(2-6)}$		0.36	0.49	0.41	0.56	0.57	0.54	0.36	0.64	0.54	0.44	0.58	0.64	0.43	0.61	0.65
XOS component (% w/w)*	Xyl2	0.04	0.23	-0.06	-0.43	-0.31	-0.54	-0.06	0.15	-0.36	0.20	0.27	0.07	0.08	0.23	-0.10
	Xyl3	0.37	0.30	0.48	0.78	0.74	0.69	0.46	0.63	0.65	0.45	0.52	0.62	0.49	0.57	0.68
	Xyl4	0.12	0.01	0.26	0.66	0.59	0.67	0.25	0.15	0.39	0.05	0.03	0.17	0.16	0.07	0.27
	Xyl5	-0.14	-0.38	-0.20	0.03	-0.08	0.21	-0.18	-0.43	0.17	-0.37	-0.49	-0.31	-0.30	-0.47	-0.13
	Xyl6	-0.24	-0.30	-0.04	0.22	0.13	0.32	-0.03	-0.31	0.06	-0.35	-0.42	-0.26	-0.22	-0.38	-0.14
Acetyl***		0.63	0.42	0.51	0.51	0.54	0.28	0.34	0.74	0.36	0.74	0.66	0.74	0.61	0.70	0.64
Furfural***		0.04	0.60	0.31	0.21	0.30	0.21	0.15	0.51	0.37	0.27	0.53	0.41	0.23	0.53	0.42
XOS consumption		0.72	0.64	0.74	0.90	0.93	0.83	0.83	0.84	0.68	0.78	0.80	0.81	0.87	0.82	0.82

*Percentage of each degree of polymerization in the XOS fractions within $XOS_{(2-6)}$ – Xyl2 (xylobiose); Xyl3 (xylotriose); Xyl4 (xylotetraose); Xyl5 (xylopentaose), Xyl6 (xylohexaose). Color legends: correlation factor ranging from -1 (red) to +1 (green).

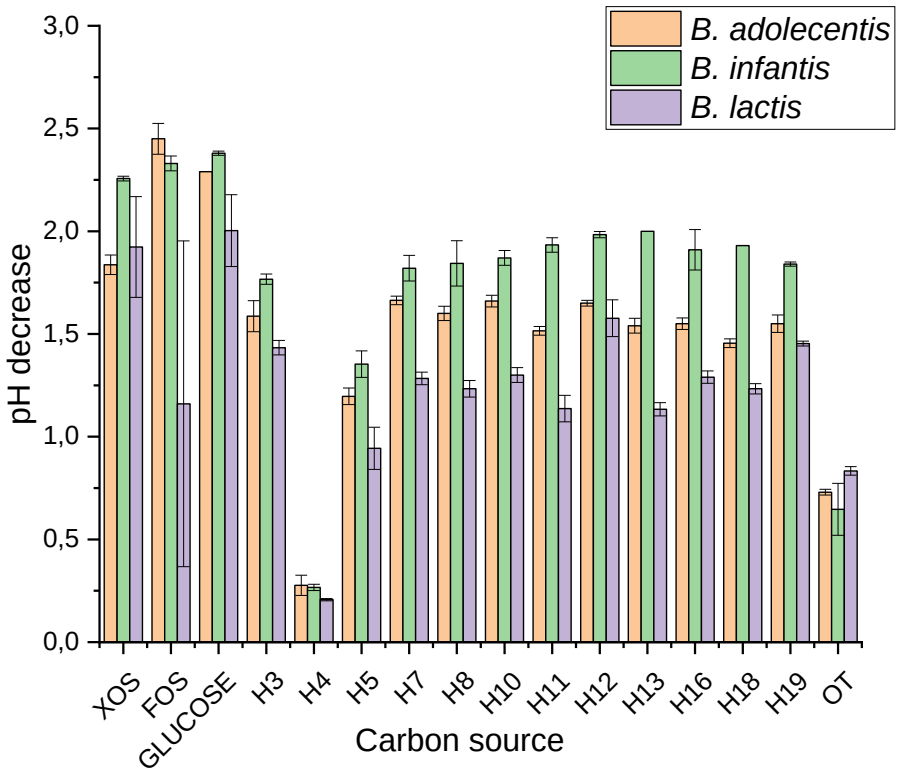

Figure 12. Decrease in pH value at the end of fermentation by different *Bifidobacterium* using different carbon sources from previous work (Marcondes et al., 2020).

As with the OD_{650} data, the decrease in pH was greater for *B. infantis*, *adolecentis* and *lactis*, respectively. The decrease in pH is a result of the accumulation of SCFA that are released as metabolites of *Bifidobacterium*. It is possible to notice that the hydrolysates that had the worst performance in releasing SCFA were the same ones that resulted in less cell growth and a lower decrease in pH of the medium (Figure 13). HX with $XOS_{(2-6)}$ values greater than 10% performed at the same level or higher when compared to commercial XOS or non-XOS carbon source, mainly for lactic acid release by *B. infantis*. Hydrolysates that showed higher selectivity of XOS in relation to xylose showed an inverse correlation with the release of acetic and lactic acid (Table 3), which may indicate that the high concentration of SCFA may come from the higher concentration of monomer (xylose) in the culture medium.

There was a good correlation ($R^2 < 0.7$) between the presence of acetyl groups and the release of lactic acid, which may raise the hypothesis that the release of acetic acid in the medium from the XOS molecules could shifted the balance of *Bifidobacterium* metabolism to the formation

of lactic acid. However, it is necessary a more in-depth study of the metabolic pathways and their possible deviations caused by external factors.

XOS consumption occurred in all carbon sources that contained XOS (Figure 14). Although in different amounts, in percentage, the consumption of XOS was similar among commercial XOS and the H11 and H12 hydrolysates. Accompanying the lower growth, the H4, H5 and OT resulted in lower consumption of X_T. The other hydrolysates showed similar consumption ranges, close to 55%. The low consumption of X_T was not related to the presence of acetyl groups (R^2 = 0.47) however it has a positive correlation with all parameters that represent *bifidobacterium* metabolism as pH decrease (R^2 > 0.83), growth by OD_{650} (R^2 = 0.64) and release of SCFA (R^2 = 0.68) (Table 3). When compared with X_T and XOS/xylose selectivity, the consumption of XOS presented an inverse correlation (R^2 = -0.74 and -0.82, respectively), reinforcing that the treatments, within the

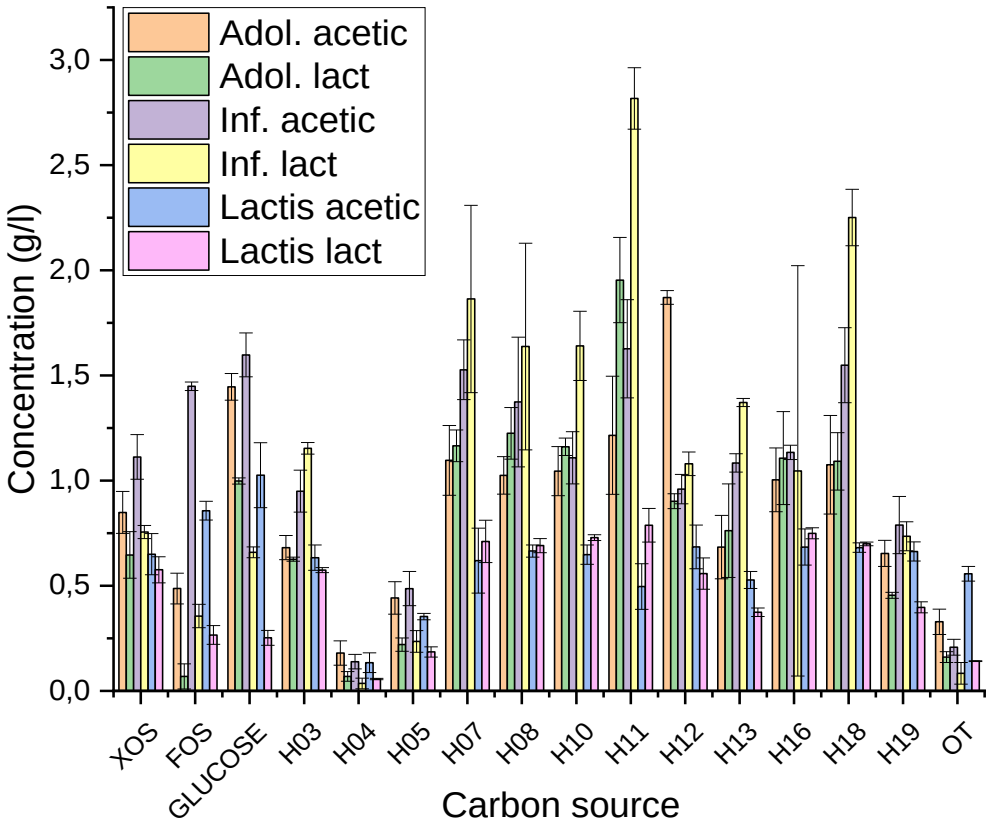

Figure 13. Release of SCFA, acetic and lactic acid in the culture medium after anaerobic fermentation by bifiddobacteria Adol: *B adolecentisl* Inf.*: B. Infantis*; Lactis: *B. lactis*. Carbon source from previous works (Marcondes et al., 2020).

conditions studied, with the highest selectivity and yield did not result in an XOS hydrolysate suitable to be consumed by the *bifidobacterium* studied.

Among the monomers, glucose was consumed from all carbon sources. Xylose and arabinose had a low consumption rate, and, in some cases, these monomers were released into the medium (Table 4). This release may have occurred due to the hydrolysis of XOS before being metabolized by the bacteria, releasing monomers (xylose) and side groups (arabinose and acetic acid). To verify if *Bifidobacterium* would have secreted enzymes to break down XOS and thus metabolize the depolymerization products, the presence of the xylan degrading activities endoxylanase activity and β-xylosidase were performed (Figure 15).

In all carbon sources that contain XOS or xylose, endoxylanase activity, that act on xylan chains with the objective of hydrolyzing them to smaller chains, was detected. B-xylosidase activity, which is related to hydrolysis of xylobiose and xylotriose releasing xylose monomers, was detected in only five carbon sources and at very low levels (Figure 15). Considering that not all hydrolysates showed β-xylosidase activity but showed growth and XOS consumption (Figure 14),

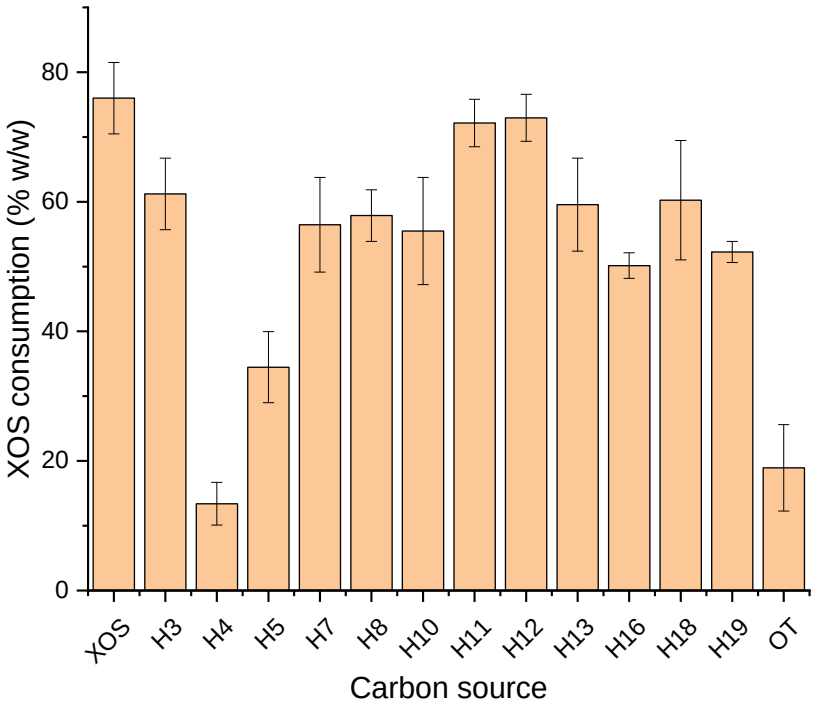

Figure 14. Total XOS consumption during fermentation with *B. Adolecentis*

it is reasonable to say that *B. adolecentis* can use more than one mechanism for XOS consumption in addition to hydrolyze it outside the cell into a monomer for its consumption. (Yang et al., 2019) studied the behavior of *B. adolecentis* in the presence of XOS and xylose and observed that there was a significant increase in the expression of several genes related to the ABC transport of xylo-oligomers in the presence of XOS, evidencing that the bacterium adapts to the use of the oligomer without first degrading it into monomer in the external environment, performing this function only inside the cell. This behavior may explain the fact that even in the absence of β-xylosidase activity, XOS was consumed by *B. adolecentis*. However, this mechanism may not be efficient for XOS with very high DP since hemicellulosic hydrolysates with high fractions higher than xylohexaose showed lower growth and release of SCFA (Figure 13).

Table 4. Balance of monomeric carbohydrates after fermentation with *B. adolecentis* in different carbon sources. Balance considered the carbohydrate concentration before fermentation subtracted from the carbohydrate at the end of fermentation.

Carbon source	**Monomeric carbohydrates (g/L)**			
	Xylose	**SD**	**Arabinose**	**SD**
XOS	-0,58	0,09	ND	ND
H03	0,08	0,08	0,07	0,02
H04	0,01	0,00	0,06	0,01
H05	-0,21	0,01	0,04	0,01
H07	0,76	0,06	0,12	0,02
H08	0,32	0,13	0,03	0,03
H10	0,41	0,51	-0,05	0,06
H11	1,88	0,03	0,16	0,03
H12	-1,13	0,01	-0,03	0,01
H13	0,34	0,04	0,11	0,03
H16	0,40	0,11	0,06	0,03
H18	1,66	0,11	0,15	0,02
H19	-0,14	0,03	0,01	0,01
OT	-0,24	0,01	0,01	0,00

ND: not detected; SD: standard deviation.; Negative values mean that the monomers were released into the fermented medium. Carbon source from previous works (Marcondes et al., 2020).

Regarding the presence of degradation compounds, HMF and furfural, there was no correlation between the presence of these compounds and the growth of *Bifidobacterium*, both by OD_{650} nm and decreases in pH. Interesting, the furfural compound, most present in the hemicellulosic hydrolysate, does not have the median lethal dose (LD50, or LD50) values, which

is the lethal dose to eliminate 50% of the test population, known to humans. However, for rats the value is 122 mg/Kg, 950 mg/Kg for puppies and 400 mg/Kg for mice (Database, 2019). In addition,

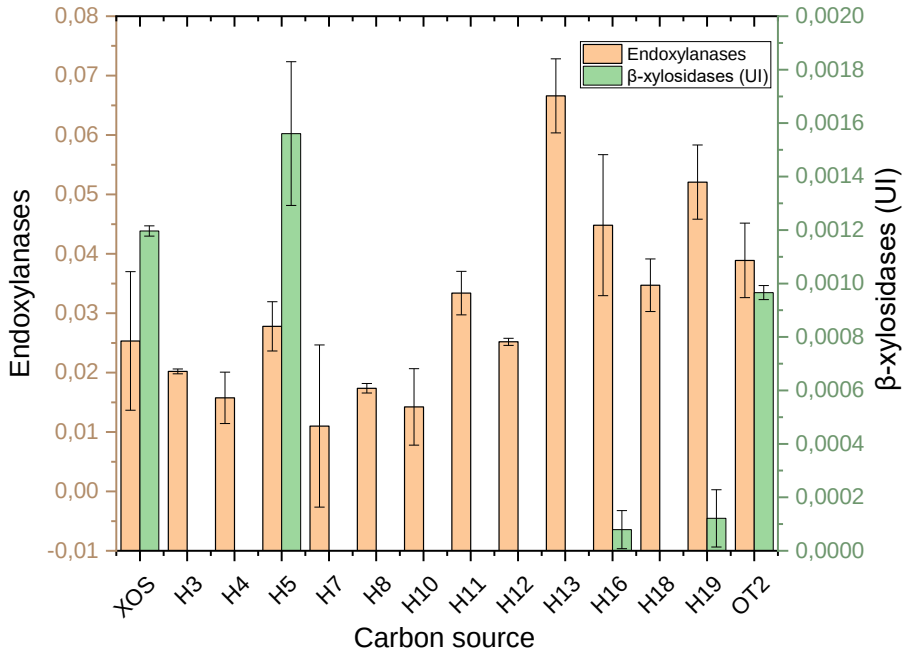

Figure 15. Enzymatic activities of endoxylanase and β-xylosidase in the culture medium after the fermentation process by *B. adolecentis*

furfural is normally found in heat-processed foods and consumed daily in the human diet. Thus, further studies on the influence of these degradation compounds need to be developed to clarify their real impact on human health, and so far, it is not evidence that their presence is a disadvantage for the use of hydrothermal treatment to produce XOS.

2.5 CONCLUSION

It is possible to state that the hemicellulosic hydrolysate, obtained by hydrothermal treatment of sugarcane bagasse and with DP distribution similar to XOS commercial, can be used as a carbohydrate source for the development of *B. adolecentis, B. infantis* and *B. lactis*. The main products of carbohydrate fermentation by *bifidobacterium* were acetic and latic acids, showing high a correlation with XOS consumption. Apparently, *Bifidobacterium* use another method than the excretion of xylan-depolymerizing enzymes to metabolize XOS. The presence of the carbohydrate-degrading compounds furfural and HMF did not affect the growth of

Bifidobacterium, as the acetyl groups attached to XOS. These findings indicate that XOS obtained by hydrothermal treatment of sugarcane bagasse has the potential to be considered prebiotic compounds, since they can be used as a substrate by *Bifidobacterium*. However, it was shown that XOS with a long chain (higher than xylohexaose) resulted in lower metabolic activity of the studied *Bifidobacterium*.

CHAPTER 3: ANALYSIS OF THE PREBIOTIC POTENTIAL OF XOS EXTRACTED FROM SUGARCANE BAGASSE ON A PILOT SCALE WITHIN A BIOREFINERY CONCEPT

3.1 ABSTRACT

This chapter aims to explore the prebiotic potential of XOS extracted on a pilot scale, which is part of the published article entitled "*High yield biorefinery products from sugarcane bagasse: Prebiotic xylooligosaccharides, cellulosic ethanol, cellulose nanofibrils and lignin nanoparticles*". Based on previous studies, it was possible to conclude that XOS extraction conditions with high selectivity (xylooligosaccharides > xylose) resulted in XOS with lower prebiotic activity, possibly due to the high concentration of XOS with size greater than six xylose units. This led us to explore process conditions for extracting XOS with lower selectivity but with higher concentration of $XOS_{(2-6)}$, a process condition also suitable for increasing the efficiency of ethanol production. Therefore, in this chapter XOS was extracted within a pilot scale biorefinery concept with medium selectivity but high concentration of $XOS_{(2-6)}$. This XOS was evaluated for its prebiotic potential using isolated strains of *Bifidobacterium*. The results showed that the XOS extracted in this way has the potential to be used as a prebiotic since it showed similar efficiency to commercial XOS as a source of substrate for the *Bifidobacterium* studied.

3.2 INTRODUCTION

This chapter is part of the article "High yield biorefinery products from sugarcane bagasse: Prebiotic xylooligosaccharides, cellulosic ethanol, cellulose nanofibrils and lignin nanoparticles" (Bioresource Technology – Volume 342, December 2021, 125970).

Over the last two decades, intensive efforts have been dedicated worldwide to the development of technologies for the biotechnological conversion of lignocellulosic feedstock into cellulosic ethanol as a biofuel. However, although the purchase price of cellulosic feedstock is generally competitive with petroleum on an energy basis, the cost of conversion using present's technology is high. The main economic obstacle to cost-competitive cellulosic biofuel production is the cost of conversion, rather than the cost of feedstock (Lynd et al., 2017).

The key factor responsible for the high cost of processing cellulosic biomass using current technology is the recalcitrance of lignocellulose, that is, the difficulty of its conversion to reactive intermediates (fermentable sugars). Such recalcitrance barrier is manifested in costs associated with the two unit operations aimed at rendering cellulosic biomass fermentable: thermochemical pretreatment and enzymatic hydrolysis (Lynd et al., 2017). Therefore, production of cellulosic ethanol as a single-product is still not cost-competitive in large scale (Xu et al., 2019) and commercial projects are limited to the remaining first few plants at different stages of development and commercialization (Rosales-Calderon and Arantes, 2019). Thus, the production of co-products alongside cellulosic ethanol, the so called biorefinery concept, has been recognized as the most promising strategy that can benefit not only process economics but environmental performance as well (Mussatto and Bikaki, 2016; Rosales-Calderon and Arantes, 2019).

A biorefinery integrates different technologies to selectively fractionate lignocellulose biomass in its major constituents (cellulose, hemicellulose, and lignin) and to convert them into a spectrum of bio-based products (chemicals, food and feed ingredients, materials) and bioenergy (biofuels) (Demirbas, 2010). In this context, the selective fractionation of hemicellulose and lignin in a cellulosic ethanol-driven biorefinery also offers various advantages for production of cellulosic ethanol. For example, residual hemicellulose associated with the cellulose has been widely demonstrated to have a significant negative effect on the enzymatic hydrolysis as it decreases the access of enzymes to cellulose and its solubilization during hydrolysis can require various hemicellulose-degrading enzymes, which are highly dependent on the lignocellulose type, pretreatment technology and its severity (Hu et al., 2013; Öhgren et al., 2007). In addition, pentose sugars (mainly xylose) released from residual hemicelluloses during enzymatic hydrolysis are not fermentable with typical industrial ethanol producing *Saccharomyces cerevisiae*, thus requiring an efficient recombinant strain engineered with pentose metabolism to produce ethanol (Lynd et al., 2017). Therefore, efficient fractionation of hemicellulose prior to cellulose hydrolysis can eliminate the necessity for laborious design and expensive development of custom-made enzyme preparations and overcome the limited cellulose accessibility, consequently enhancing cellulose hydrolysis efficiency.

To fractionate and recover, at high yield, hemicellulose from lignocellulose, hydrolysis under acid condition is required and hydrothermal treatments using only water (liquid hot water)

or steam (steam explosion), also known as autohydrolysis pretreatments, have been considered the most attractive options (Duque et al., 2016; Ramos et al., 2020). First because they are environmentally friendly, relatively inexpensive (no need for chemicals and there are no major corrosion issues due to the mild acidic medium) and have been successfully applied to a wide variety of lignocellulosic biomass (Mosier et al., 2005). Another benefit is that the mild condition widely employed to minimize the formation of sugar degradation products while still solubilizing sufficiently amount of hemicellulose, breaks down the hemicellulose chains generating a hemicellulose-rich hydrolysate high in oligomers of varying degree of polymerization (Carvalho et al., 2018; Marcondes et al., 2020; Mosier et al., 2005). This in fact creates an opportunity to, in a single process step, selectively fractionate hemicellulose directly into a marketable product since oligomers can be used in different areas with significant economic value (i.e., pharmaceutical products, food ingredients, fuels, chemicals, and bioplastics) (Lara-Flores et al., 2018).

In this work, an integrated biorefining strategy was applied to sugarcane bagasse (SCB) to selectively fractionate it into its main constituents and allow production of high value biorefinery products. First, SCB was pretreated by uncatalyzed steam explosion using a continuous pilot-scale reactor to solubilize hemicellulose while generating a XOS-rich hydrolysate, whose in vitro efficacy as prebiotic towards different probiotic bacteria was evaluated without further purification.

3.3 MATERIALS AND METHODS

3.3.1 XOS EXTRACTION

The SCB, kindly supplied by the Brazilian National Biorenewables Laboratory – LNBR (Campinas/SP, Brazil), was processed in a continuous steam explosion pilot unit (ADVANCEbio®) at the pilot plant of the LNBR according to Rocha et al. (2012). Briefly, the reactor was fed simultaneously with bagasse at a rate of 11.7 kg/h and saturated steam at a rate of 25−30 kg/h at 15 bar (approximately 190 °C), with a residence time of 15 min, depressurized and automatically discharged.

To extract the XOS produced during steam explosion, the steam exploded bagasse was added into a tumbling reactor at a 20% w/v for 30 min and 30°C. Then, the mixture was vacuum

filtered over a 25-mesh membrane and the filtrate collected, hereafter referred to as SCB-XOS hydrolysate. To increase the removal of saccharides, distilled water (equivalent to the total weight of the mixture) was used to wash the solid fraction by filtration and the diluted filtrate, hereafter referred to as washing liquor (WL), was collected and used for mass balance purpose. The solid fraction, containing mainly cellulose and lignin, was collected and utilized for lignin fractionation.

3.3.2 CHARACTERIZATION OF THE LIQUID FRACTIONS

Chemical characterization of liquid fractions was carried out according to the following National Renewable Energy Laboratory (NREL, USA) analytical protocol: NREL/TP-510−42623 for the liquids fraction (Sluiter et al., 2008). Acid-soluble lignin was determined at 205 nm considering a absorptivity constant of 105 $L.g^{-1}\,cm^{-1}$ (Dence, 1992). Monomeric sugars, short chain fatty acid (SCFA) and ethanol were quantified by high pressure liquid chromatography (Waters® HPLC) using an HPX-87H (Bio-rad) column at 45 °C, eluted with 5 mmol/L sulfuric acid at 0.6 mL/min and a detector of refractive index at 35 °C. Furfural was determined using an ultraviolet detector at 210 nm wavelength. For the determination of the total oligomeric sugars, a post-hydrolysis step in dilute acid (4% w/w H_2SO_4) was carried out at 121 °C for 1 h to depolymerize all the oligomers to their corresponding monomers, according to protocol NREL/TP-510−42623. The cooled samples were filtered and analyzed by HPLC. The quantification of the oligomeric sugars was made by the difference between the amount of sugars obtained in the post-hydrolysis stage and the sugars obtained directly in the liquid fractions.

To determine the xylooligomers, two coupled columns were used, Bio-Rad HPX-87C and Bio-Rad HPX-42A, with 0.5 mL/min deionized water flow at 65 ºC and a refractive index detector. The standards for the calculation of oligomers were xylobiose, xylotriose, xylotetraose, xylopentose and xylohexose.

3.3.3 IN VITRO PREBIOTIC ASSAY

To assess the suitability of the XOS hydrolysate as a prebiotic compound without any purification step, four probiotic bacteria were used: *Bifidobacterium lactis*, *Bifidobacterium infantile*, *Bifidobacterium adolecentis* and *Bifidobacterium breves* (THT, Gembloux, Belgium). Fermentation was carried out at 37 ºC (temperature similar to that of the human body) for three days under anaerobic condition. The *Bifidobacterium* medium (“German Collection of

Microorganisms and Cell Cultures GmbH: List of Media for Microorganisms," n.d.) used as the base medium consisted of (per liter): 10 g of casein peptone, 5 g yeast extract, 5 g of meat extract, 2 g of K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4$. H_2O, 1 g of NaCl, 0.5 g of cysteine-$HCl.H_2O$ and 8.0 mL of resazurin (0.025% w/v). The final concentration of SCB-XOS in the assays was 5 g/L. Xylose (sigma-aldrich), glucose (sigma-aldrich), commercial XOS (PrecticX 95 P) and commercial fructooligosaccharides (FOS) were used as comparative carbohydrates, also at 5 g/L. The pH of the media was adjusted to 7.2 with 0.5 M NaOH solution before sterilization in autoclave (121 ºC for 15 min). Three parameters were used to assess the prebiotic activity: cell growth, measured by optical density at 650 nm (Mäkeläinen et al., 2010b); final medium pH, an indicator that the bacteria generated short-chain fatty acids; and the production of short-chain fatty acids (acetic and lactic acid).

3.4 RESULTS AND DISCUSSION

Even though the applied hydrothermal treatment has not been optimized for selective extraction of XOS from the SCB, but rather to benefit cellulosic ethanol production (Rocha et al., 2012), it was possible to obtain a XOS-rich hydrolysate with a ratio of oligomers to xylose (selectivity) of 2:1 (Table 5). In general, this SCB-XOS has a composition similar to that of the commercial XOS, however, for SCB-XOS it is worth highlighting the higher concentration of xylobiose and xylotriose and practically no oligomers with size greater than six xylose units (Table 5). Unlike commercial XOS, side groups such as arabinose and acetyl were observed in SCB-XOS, which can result in differentiated prebiotic activities (Aachary and Prapulla, 2011). In addition, the concentration of glucose oligomers and monomers derived from cellulose hydrolysis were very low.

The presence of non-saccharide compounds such as phenolics and sugar degradation products (furfural and hydroxymethylfurfural – HMF) in the SCB-XOS hydrolysate was very low (Table 5), which is likely due to the mild conditions of the steam explosion treatment. Indeed, these are commonly considered as undesired and toxic compounds, constituting one of the major disadvantages of the hydrothermal extraction method to produce XOS as compared to enzymatic treatments (Kumar et al., 2021). However, negative effects related to the presence of these compounds in a XOS hydrolysate for probiotic bacteria has not yet been demonstrated. Instead, it

has recently been shown a positive relationship between the presence of sugar degradation (furfural and HMF) in foods, like bread and raw banana, thermally treated and the development of bacteria that play positive roles in the human microbiota (Pérez-Burillo et al., 2018)

Table 5. Characterization of the xylooligosaccharide-rich hydrolysate extracted from sugarcane bagasse (SCB-XOS), XOS*: commercial XOS and the washing liquor (WL).

	Compounds	**SCB-XOS**	**WL**	**XOS***
		Concentration [g/L]		
Monomers	Glucose	1.05	0.18	ND
	Xylose	8.13	2.61	ND
	Arabinose	0.94	0.22	ND
Total oligomers	Glucose	3.00	1.24	ND
	Xylose	16.19	6.17	ND
Side groups	Acetic acid	3.70	1.60	ND
	Arabinose	0.07	0.14	ND
Xylose oligomers	Xylobiose	6.50	2.59	2.99
	Xylotriose	4.06	1.58	2.39
	Xylotetraose	1.77	0.86	1.85
	Xylopentose	2.12	0.49	1.01
	Xylohexose	0.72	0.41	1.26
Others	Soluble lignin	1.51	1.32	ND
	Furfural	0.031	0.007	ND

To assess the potential to modulate the intestinal microbiota, the SCB-XOS used as a substrate for *in vitro* assays with four different bacteria considered beneficial to human health, *B. lactis*, *B. adolescentis*, *B. infantis* and *B. breves* (Mäkeläinen et al., 2010b). We emphasize that the focus of this study was not to prove the effectiveness of XOS as a prebiotic, as this has already been well established (Aachary and Prapulla, 2011), but rather to assess the prebiotic potential of the produced SCB-XOS (without any purification step – containing xylose, minor quantities of phenolics and sugar degradation compounds as well as XOS with varying degree of polymerization) obtained via steam explosion from SCB in a biorefinery concept on a pilot scale as compared to commercial prebiotic XOS produced by chemical extraction of xylan followed by enzymatic treatments.

When xylose was used as the sole carbon source, xylose consumption varied depending on the *Bifidobacterium* (Figure 16). *B. lactis* practically did not consume xylose, resulting in no bacterial growth and no change in the pH of the medium, while *B. infantis* consumed part of the

xylose and the other *Bifidobacterium* consumed all xylose. For the *Bifidobacterium* that consumed xylose, acetic acid was the predominant metabolite in relation to lactic acid (Figure 16C). The use of xylose as a carbon source for *Bifidobacterium* was carried out because xylose was found in the SCB-XOS. However, it is important to mention that xylose is unlikely to reach the intestinal microbiota, as it is absorbed by the small intestine (Craig and Atkinson, 1988), but if it reaches the gut microbiota, it is possible to verify that it would serve as a carbohydrate for some *Bifidobacterium*.

Glucose, used as a comparative sugar, was the carbohydrate source that best stimulated the growth of the *Bifidobacterium*, even compared with commercial prebiotic, as can be seen for the generation of the greatest cell mass, the highest reduction in final pH, in addition to being practically all consumed (Figure 16). Compared to SCB-XOS and commercial XOS, commercial

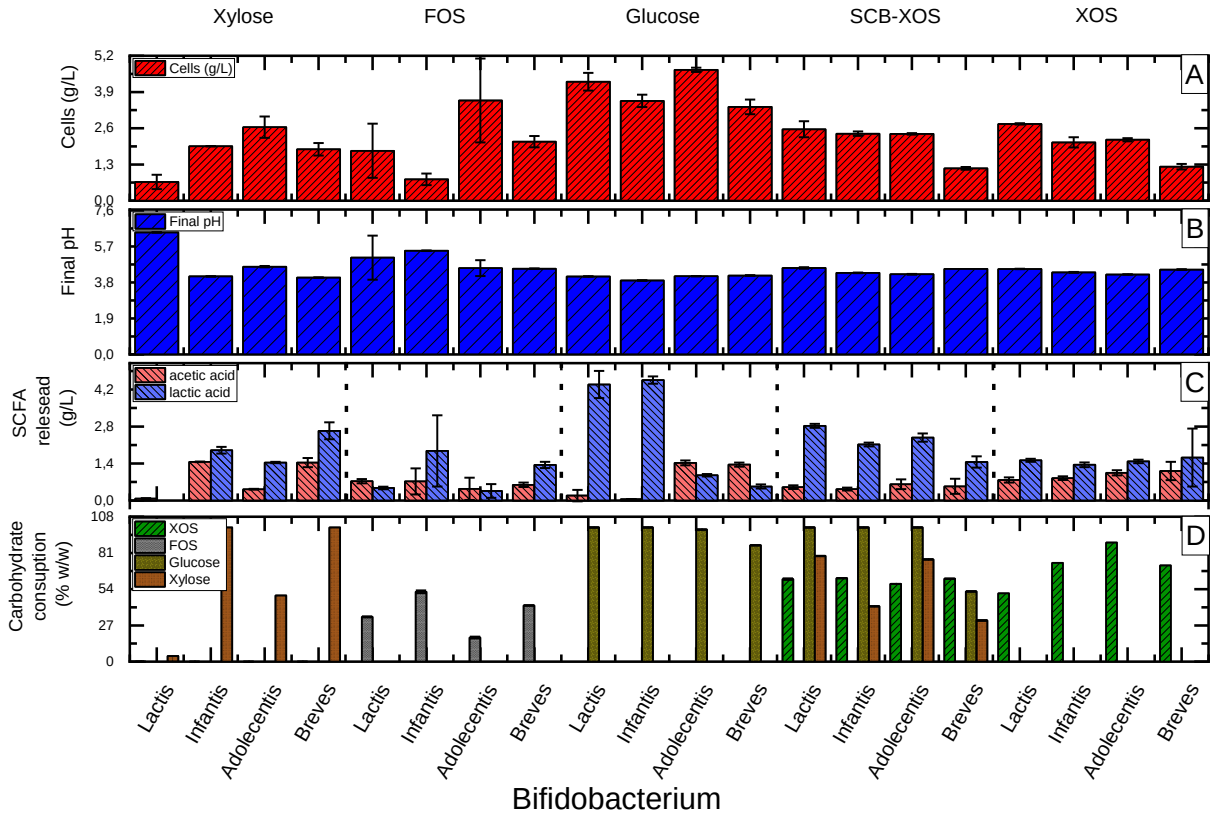

Figure 16. Metabolic parameters and carbohydrate consumption by *Bifidobacterium* over 72 h. A – final cell concentration (g/L) calculated by optical density at 600 nm; B – pH after prebiotic fermentation; C – Short chain fatty acid released during prebiotic fermentation; D – consumption of carbohydrate after prebiotic fermentation.

FOS, used as a comparative prebiotic, promoted similar growth of *B. lactis* and *B. breves*, but a higher growth of *B. adolescentis* and a lower growth of *B. infantis*.

SCB-XOS showed similar bacterial growth development and pH reduction to the commercial XOS, which was used as a prebiotic for comparison (Figure 16A-B). The amount of lactic acid secreted in the medium was higher with SCB-XOS for three of the *Bifidobacterium* (*B. lactis*, *B. infantis* and *B. adolecentis*), while with commercial XOS the production of acetic acid was more pronounced for all *Bifidobacterium* (Figure 16C). For three of the *Bifidobacterium* studied (*B.infantis*, *B. adolecentis* and *B. breves*), XOS consumption was slightly higher in commercial XOS than in SCB-XOS, while for *B. lactis*, XOS consumption was similar (Figure 16D).

The results from the *in vitro* prebiotic assays with the four *Bifidobacterium* evidence that the metabolism data using as-produced SCB-XOS and commercial prebiotics (XOS and FOS) were very similar, even though the SCB-XOS contained other compounds (monomers, sugar degradation compounds and phenolics) in addition to xylooligomers with varying degree of polymerization. The production of lactic and acetic acids indicates that XOS from SCB has the potential to benefit human health since these acids released by *Bifidobacterium* are considered responsible for improving the functioning and regulation of the human microbiota (Aachary and Prapulla, 2011).

3.5 CONCLUSION

The XOS extracted on a pilot scale by the steam explosion process showed a prebiotic potential similar to the commercial XOS when compared to the suitability of being a carbon source for the *Bifidobacterium* studied. This work reinforces what was noted in the previous chapter, in which XOS extracted at median selectivity showed prebiotic activity equal to or greater than commercial XOS, but now obtained on a pilot scale and within a biorefinery concept.

CHAPTER 4: INFLUENCE OF STRUCTURAL PROPERTIES OF XOS ON ITS POTENTIAL AS A PREBIOTIC COMPOUND IN HUMAN FECAL FERMENTATION.

4.1 RESUME

Moving forward in the studies of the potential properties of XOS extracted from sugarcane bagasse, this chapter is dedicated to analyzing the influence of the XOS structure, the degree of polymerization (DP), on its prebiotic potential through fecal fermentation. For this, XOS with different DP was obtain by a process for fractionation of XOS-SCB using a chromatographic column permeated with Bio-gel P-2, where two new fractions of XOS were obtained, one enriched with shorter chains (XOS-short) and another enriched with longer chains (XOS-long). Commercial XOS, XOS-SCB and the two new XOS fractions were subjected to human fecal fermentation from three donors. Fermentation was carried out in an agitated anaerobic system, at constant temperature of 37 ºC, and samples were taken at 0, 4, 8 and 24 h. The results on the pH profile and ammonia accumulation in the fermentation medium did not indicate an influence of the XOS DP on the metabolism of the bacteria, with the difference among individuals being a more relevant factor. Cytotoxicity analysis showed that XOS-Long and the fermentation product of XOS-SCB have an adverse effect on cell viability, while all XOS from sugarcane bagasse present a protective action against the cell membrane damage, both before fermentation as after 24 hours of fermentation.

4.2 INTRODUCTION

The structural properties of oligosaccharides can be extremely important for their use, since their structure can influence their prebiotic activity (Sanz et al., 2005; van Craeyveld et al., 2008). Sanz et al. studied the influence of linkages on glycoside groups in disaccharides and concluded that 1-2, 1-4 and 1-6 linkages generated higher prebiotic activity, especially Kojibiose and sophorose, and that disaccharides containing mannose showed low prebiotic levels, except for 6α-mannobiose. Craeyveld et al. (2008) studied the effect of the arabinoxylan-oligosaccharides (AXOS) structure on the prebiotic effect in rats, considering the average degree of polymerization (avDP)), evidencing that an avDP value ≤5 stimulates a growth of *Bifidobacterium* while an avDP value > 12 does not stimulate the growth of these same bacteria (van Craeyveld et al., 2008).

Although there is no consensus on which DP range XOS has the best prebiotic properties, some studies indicated the limited size of XOS that can exert a prebiotic activated, as XOS with DP < 12 (van Craeyveld et al., 2008) and XOS < 5 (Mäkeläinen et al., 2010b), and further research on the influence of XOS structure on prebiotic activity should be carried out. This observation was also demonstrate about the limited size to be a carbon source to *Bifidobacterium* in the previous chapter (chapter 2), when XOS with DP greater than six ($XOS_{(>6)}$) resulted in lower growth of isolated strains of *Bifidobacterium* as compared to hemicellulosic hydrolysates with higher concentration of $XOS_{(2-6)}$.

The structural differences of XOS, such as DP, have been shown to significantly influence the growth of isolated bacterial strains (Mäkeläinen et al., 2010b). This may be a relevant factor when one wants to obtain a prebiotic compound with specific actions.

One of the most used techniques for fractionation of oligomers in relation to their size, which reflects the DP, are the gel permeation chromatographic. Although this technique has a limitation of use due to its high cost in large-scale production (Manisseri and Gudipati, 2010), it allows the separation of oligomers into fractions with different DP, depending on the pore size of the bio-gel column used (Palaniappan et al., 2021).

To study the influence of the DP of XOS extracted from SCB on prebiotic activity using human fecal samples, in an attempt to study the prebiotic potential in an environment closer to the human microbiota, in this chapter XOS-SCB was submitted to a fraction process by liquid permeation chromatography technique in Bio-Gel P-2, obtain two major new fractions, one with short-chain XOS (XOS-short) and other with long-chain XOS (XOS-long).

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

The xylooligosaccharides used were from a commercial source (PrecticX 95P) and from sugarcane bagasse (XOS-SCB) as reported in previous work (Pereira et al., 2021) and used in the chapter 3.

4.3.2 PURIFICATION AND FRACTION OF XOS

Preliminary tests for the fractionation of XOS-SCB was carried out with a 25 cm x 1 cm (80 mL) column packed with Bio-gel P-2 at a 1 ml/min flow, with fractions being collected every 2 min. After this validation step, the XOS-SCB hydrolysate was fractionated on a Bio-Gel P-2 column 45 × 1.5 cm (320 mL) eluted with ultra-pure water at a flow rate of 0.5 mL/min. Fractions were collected with the aid of an automatic collector programmed to collect fractions every 10 min. A total sample volume of 5.0 mL was used for each run on the column. After each fractionation cycle, the column was eluted with ultra-pure water until it was fully restored. Each fraction was subjected to characterization to determine the DP of the XOS obtained.

XOS fraction with short-chain (xylobiose and xylotriose) was pooled together as well as XOS fraction with long-chain (xylotetraose until xylohexaose), being named XOS-short and XOS-long, respectively. These new samples were concentrated by a combination of vacuum evaporation system at 70 °C and lyophilization, and storage at -20 ºC until further use.

4.3.3 XOS' DEGREE OF POLIMERIZATION

The characterization of XOS DP was performed by two techniques due to availability of equipment. The first was applied to the characterization of XOS-SCB and its fractionation in a Bio-gel P-2 column. The second technique was used to confirm the efficiency of the fractionation system by gel permeation chromatography.

First technique: To determine the DP of the xylooligomers in the fractions collected after gel permeation chromatography, two coupled columns were used (Bio-Rad HPX-87C and Bio-Rad HPX-42A), with 0.5 mL/min deionized water flow at 65 °C and a refractive index detector. The standards for the calculation of oligomers were xylobiose, xylotriose, xylotetraose, xylopentose, and xylohexaose.

Second technique: The XOS-SCB, XOS-Long and XOS-Short were resuspended in pure water was analyzed as describe in (Vacilotto et al., 2022). Briefly, samples were analyzed using the high-performance anion exchange chromatography (HPAEC) system Dionex ICS-5000, coupled with an ion exchange column CarboPAC1 (250 mm × 2 mm) (Thermo Scientific, Waltham, USA). Running conditions were as follow: flow rate of 0.3 mL/min at 30 °C, injection volume equal to 1 µL, 100 mM NaOH (buffer A) and 500 mM sodium acetate with 100 mM NaOH

(buffer B) as buffers used in the elution. The standards used was xylose xylobiose, xylotriose, xylotetraose, xylopentose, and xylohexaose.

4.3.4 FECAL FERMENTATION

4.3.4.1 FECAL SAMPLES PREPARATION

Three independent biological triplicates were performed, using fecal samples from three healthy donors. The samples were collected with the approval of the Swedish Ethical Review Authority (Dnr 2022-01696-01), and all participants signed an informed consent prior to the initiation of the experiment. The participants had not used antibiotics or fiber supplementation in the previous six months of the sample collection. Fresh fecal samples were collected and kept in an anaerobic environment with the aid of an anaerobic punch (Oxoid AnaeroGen W-zip compact gas generator system, #12201972 - AN0010W) until homogenized in a sterile 50 mM PBS buffer in a ratio of 200 g/L. The slurry was prepared just prior to inoculation in the bioreactors.

4.3.4.2 FERMENTATION

The basal medium ingredients in pure water per liter were 2 g of Peptone water, 2 g NaCl, 0.04 of K_2HPO_4, 0.04 g of KH_2PO_4, 0.01 g of $MgSO_4.7H_2O$, 0.04 g of $CaCl_2.6H_2O$, 2 g of $NaHCO_3$, 2 mL of Tween 80, 0.05 g of Haemin (Sigma, #51280), 10 µL of Vitamin K1 (Sigma, #V3501), 1 g of Cysteine.HCl (Sigma, #C7477) and 0.5 g of Bile salts (Sigma, #48305). The correspondent substrates (1 % w/w) and 100 mL of the basal medium, previously bubbled with sterile nitrogen, were mixed in the anaerobic chamber (37ºC; 10% H_2, 80% N_2, 10% CO_2; Ruskinn, UK) and inoculated with 10 % v/v of fecal slurry, reaching a final fecal concentration of 2 % w/v. Fecal fermentation was carried out in a Gas Endeavor system using 500 mL bottles during 24 h, with stirring at 100 rpm. Aliquots of the samples were collected at 0, 4, 8 and 24 h, centrifuged and both supernatant and pellet were storage at -80 ºC until further use.

4.3.5 METABOLITES ANALYSIS

Ammonia was analyzed using a “Ammonia assay Kit” from Megazyme (Product code: K-AMIAR) strictly following the manufacturer’s protocol (Megazyme, 2020)

4.3.6 CYTOTOXICITY

4.3.6.1 CELLS CULTIVATION

Caco-2 cells were kindly provided by Dr Ignacio Rangel and were cultured according ATCC guidelines. Briefly, cells were cultured in DMEM containing penicillin and streptomycin with 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. Cells were passed to new culture plates by using trypsin/EDTA when they reached 70–90% of confluence. Before treatments, cells were added to the culture plates at the desired concentration and left overnight on the incubator. Supernatants from the fermentation were filtered (0.22 µm), sPBS was used as negative control and Triton-X (0.2% - final concentration) as positive control.

4.3.6.2 IN VITRO TOXICOLOGY ASSAY BASED IN RESAZURIN

“*In Vitro Toxicology Assay Kit, Resazurin based*” (Sigma, n.d.) was used to determine the toxicology assay for Caco-2. Briefly, cells were plated on a 96-well cell culture plates at a density of 1×10^4 cells/well (180 µL) overnight. Twenty uL of tsPBS (0.5% Triton X-100 and 0.1% saponin in PBS – Control) or 20 µl of the filtered supernatants samples from the fermentation was added in the cells which were incubated for 24 h. A blank containing complete medium without cells was also used. After incubation, 20 uL of resazurin dye solution was added into each well. Plates were incubated for more 3 h and plates were measured by fluorescence at a wavelength of 590 nm using an excitation wavelength of 560 nm. Cells viability at each incubation time was expressed in relation to the untreated cells (negative control).

The relative percentage of viable cells (VC) for each sample in relation to the positive control was used according to equation 1, with the positive control being defined as 100%.

$$VC = \frac{Flu_{sample}}{Flu_{nc}} x100 \quad (1)$$

Where VC is the viable cells in each sample, Flu_{sample} is the fluorescence of the sample and Flu_{nc} is the fluorescence of negative control.

4.3.6.3 LDH ASSAY

Cells were plated overnight as described for resazurin assay, including the same treatments and controls, and LDH was measured by using the "lactate dehydrogenase assay kit – Sigma" following the manufacturer's instructions (Sigma-Aldrich, n.d.). Briefly, after incubation, supernatants (20 µL) were transferred to 96-well cell culture plates, mixed with LDH Assay Buffer and Master Mix Reaction. A LDH standard curve was used, as well as the positive control provided in the assay kit. The plates were mix using a horizontal shaker, incubated at 37 ºC and the absorbance was read at 450 nm. The final measurement is when the most active sample is near or exceeds the end of the linear range of the standard curve. A factor (F) was used to compare the samples to negative control as show in equation 2.

$$F = \frac{AD_{sample}}{AD_{pc}} \quad (2)$$

AD_{sample} is the difference between absorbance at T_{final} and T0 (blank) of the samples and AD_{pc} is the difference between absorbance at T_{final} and T_0 (blank) of the positive control.

4.4 RESULTS AND DISCUSS

4.4.1 PURIFICATION STEP

The fractionation technique by gel permeation chromatography exploits the difference among the sizes of the molecules of the components to be fractionated. In this technique, there is a stationary phase, which is composed of a porous gel, and a mobile phase that is the eluent. The principle of the technique is based on the ability of smaller molecules to permeate the stationary phase within its pores, resulting in a longer retention time in the column, while larger molecules

have a shorter retention time because they are not able to enter the pores. This difference in retention time is then used to fractionate the materials (Takeuchi et al., 1983). According to the manufacturer, Bio-gel P2 has a working range for the separation of molecules with sizes of 100-1900 MW (Bio-Rad, n.d.) which would be suitable for the separation of XOS with sizes up to six xylose units (xylohexaose, MW = 810) and even xylose monomer (MW=100).

To verify the suitability of Bio-gel P2 for the separation of XOS contained in XOS-SCB, a validation test was carried out in a small column (25 cm x 1 cm) and each fraction was collected and characterized in terms of monomers and oligomers (Figure 17). As expected, the xylooligosaccharides were eluted earlier than the xylose since they have higher molar mass than xylose. Up to 45 min, only the xylooligomers were eluted, indicating high purity in relation to the presence of monomers. However, from this time onwards the elution of oligomers decreased, and the elution of monomers increased. As a validation experiment, these results showed that it was possible to fractionate XOS using Bio-gel P2. Then, a larger volume column (45 cm x 1.5 cm, 320 mL)) was packed using this gel.

In a column with a volume four times greater than the one used in the preliminary tests, the fractionation was performed at the same the flow rate but increasing the amount of sample to be fractionated. It was possible to collect samples with a high degree of purity for xylose, xylobiose, a mixture of xylobiose with xylotriose and mixtures of XOS with sizes greater than xylotriose (Figure 18). It was observed that the difference between the molar mass of the compounds to be

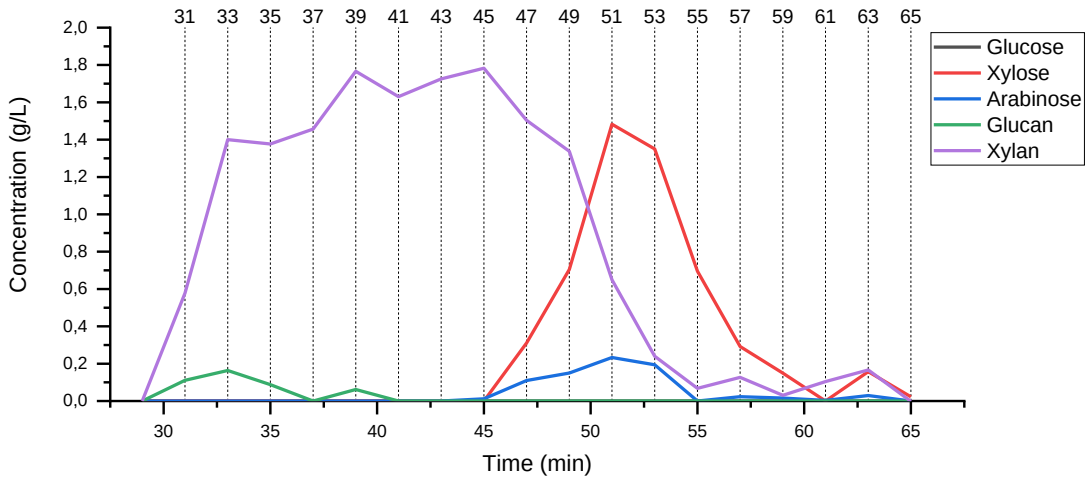

Figure 17. Fractionation profile of hemicellulosic hydrolyzate by liquid chromatography on a Bio-gel P2 column under the following conditions: Flow 1mL/min, sample volume 2 mL, collection every 2 min. Column 80 ml.

fractionated had a great impact on the purity of each fraction obtained, and the separation of compounds with a lower molar mass presented greater purity than those with a higher molar mass. As a comparison, separating xylose (MW=150 g/mol) from xylobiose (MW=282.25 g/mol) was much more efficient since the difference between the molar mass of the two compounds is 88% in relation to the molar mass of the smallest compound (xylose). For xylotriose (MW=414.4 g/mol) and xylobiose, the difference between the masses in relation to xylobiose is 50%, which already reduces the separation efficiency between the compounds. Thus, the lower this percentage in difference, the more difficult the separation is, as can be seen in the separation between xylohexaose (MW= 810.7 g/mol) and xylopentose (MW=678.6 g/mol), with a difference of 19.5% and has a lower fractionation efficiency. Other peaks for compounds with a molar mass greater than the molar mass of xylohexaose were also observed (Figure 18). However, there were no standards for the quantification of these compounds.

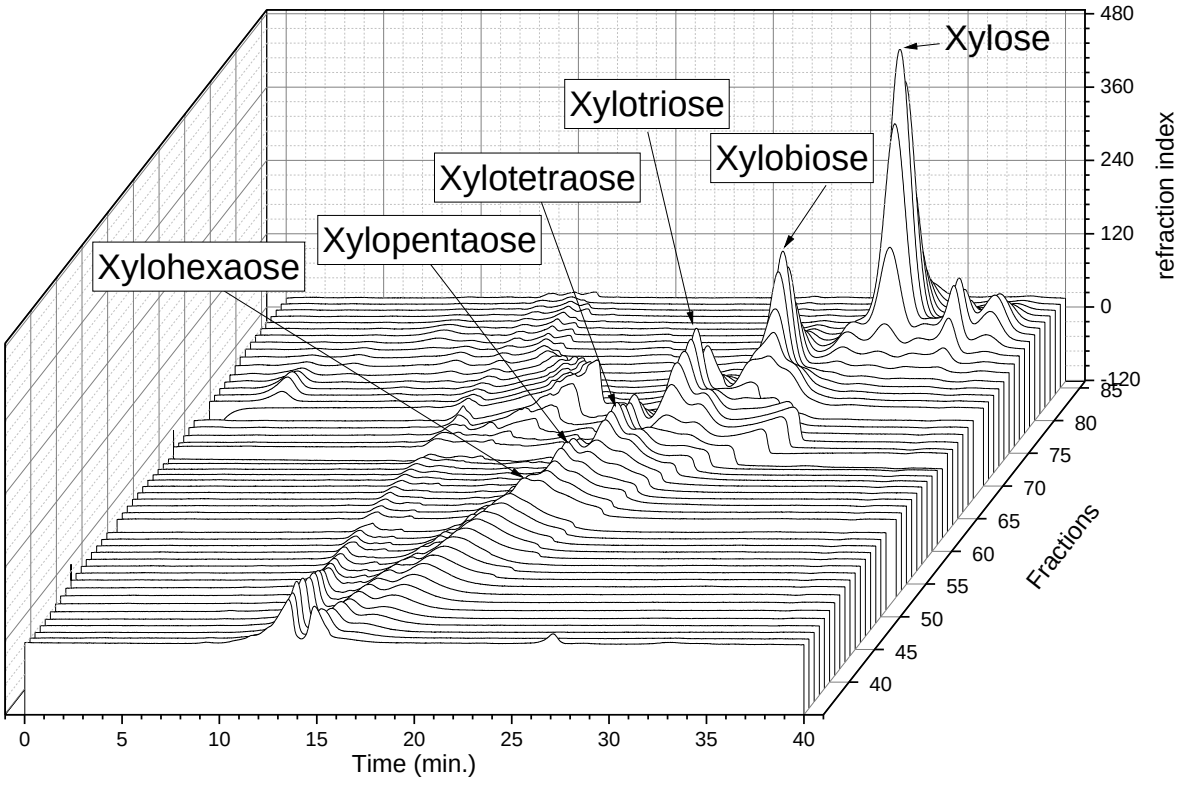

Figure 18. Chromatograms showing the elution profile for the fractionation of XOS-SCB by a Bio-gel P-2 chromatographic column. Characterization by high performance chromatography using two coupled columns (Bio-Rad HPX-87C and Bio-Rad HPX-42A).

The chromatograms for each collected fraction also show an area attached to the main peak of the compounds forming a kind of "shoulder peak". This may be due to the presence of substituent groups in the main chain of the XOS, such as acetyl group, as their presence increase the molar weigh. However, the presence of such groups was not performed.

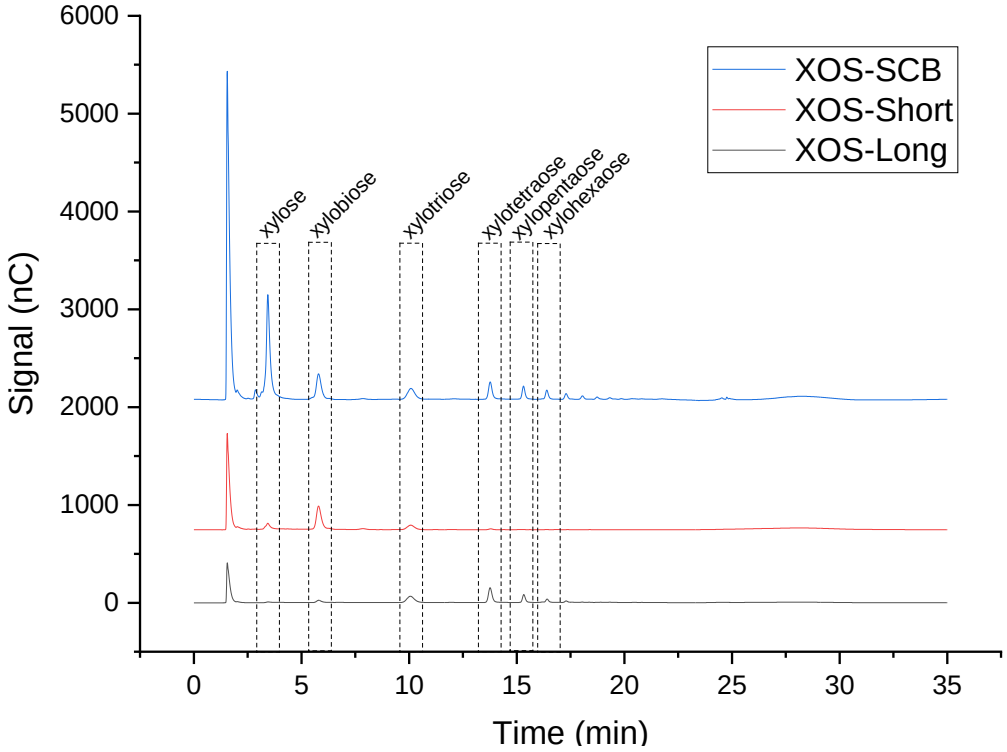

Figure 19. Chromatogram of the XOS-SCB in natura and after the fractionation process, such as fractions with XOS short-chain (XOS-Short) and fractions with XOS long-chain (XOS-long). Characterization by high-performance anion exchange chromatography (HPAEC) system Dionex ICS-5000, coupled with an ion exchange column CarboPAC1.

By grouping the eluted fractions with the most similar XOS DP, it was possible to separate the XOS-SCB into two main fractions, the first with short-chain XOS (XOS-short) and the second with long-chain XOS (XOS-long). The composition of these two new fractions was further confirmed (Figure 19).

The eluted fractions containing short and long XOS showed much higher purity than XOS-SCB relative to XOS than xylose. This is because the fraction containing monomers was easily discarded and only fractions containing XOS was grouped (Table 6). XOS-short fraction was mostly composed of xylobiose and xylotriose while the XOS-long fraction was poor in xylobiose and had more XOS with DP up to 6 xylose units. This higher purity allows to better study the

influence of the XOS DP as a prebiotic, since there is no interference of monomers in the probiotic fermentation.

Together, these results show the potential of XOS fractionation using Bio-gel P-2 as a chromatographic column. Although it is known that the fractionation process could be improved, this would require a detailed study of the experimental conditions (i.e., flow rate, temperature, fraction collect time and volume of samples).

Other compounds present in XOS-SCB, such as degradation compounds (i.e., furfural and HMF) or lignin (polyphenols) were not quantified in this work but may still be present in these XOS fractions.

Table 6. Degree of polymerization of XOS extracted from SCB before and after fractionation process by gel permeation chromatography, expressed as % w/w. Xylobiose, Xylotriose, Xylotetraose, Xylopentaose and Xylohexaose are expressed in relation to Total XOS.

Xylan's components	**XOS**			
	XOS commercial	XOS-SCB	XOS-Long	XOS-Short
Xylose	0,05	39,10	0,02	6,74
Total XOS	99,05	60,90	99,98	93,26
Xylobiose	31,47	30,37	9,08	72,63
Xylotriose	25,16	24,03	32,61	24,94
Xylotretraose	19,47	20,58	32,28	2,42
Xylopentose	10,63	16,14	18,02	0,00
Xylohexaose	13,26	8,88	8,01	0,00

4.4.2 FECAL FERMENTATION

Fecal fermentation of commercial XOS, *in vivo* (Chung et al., 2007) and in vitro (Chen et al., 2016), results in a pH decrease due to the production of SCFA. This has been proposed as a mechanism of action for the effect of *bifidobacterium* on the intestinal bacteria (Chung et al., 2007). The decrease in pH was also obtained in this work, regardless of the donor of the fecal sample (Figure 20). According to statistical analysis of Tukey's test, difference between commercial XOS and the different XOS fractions was observed, evidencing that the DP of XOS did not impact the final pH value of fecal fermentation. The greatest impact on the pH profile during fermentation was related to the different donors, which can be explained by the difference in the composition of the microbiota of each individual, with a population of different bacteria.

Ammonia is a metabolite released mainly by the fermentation of proteins and their derivatives in the fecal microbiota and the process of deamination (Diether and Willing, 2019; Tomé, 2021). From Figure 21, it is possible to notice that the highest values of ammonia accumulation occur in the control, a culture medium that does not contain any carbohydrate. This is due the fermentation of the protein itself that makes up the culture medium. In culture media containing XOS, the accumulation of ammonia is lower because there is a greater microbial growth due to the use of XOS as a carbon source, evidenced by the decrease in pH. This results in a greater

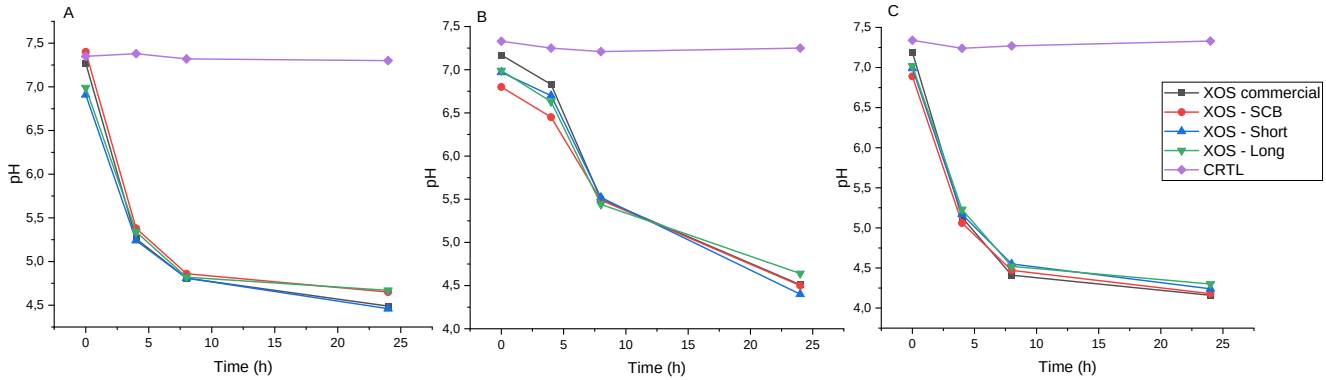

Figure 20. pH profile during fecal fermentation with different donors (A, B and C)

need for nitrogen sources for the growth of bacteria, being the ammonia an important source of nitrogen for microorganisms (Tomé, 2021). As the pH values, the release of ammonia in the medium does not present a significant difference between the different carbon sources but it is more influenced by the individuality of the donors.

A greater diversity in the DP of XOS may provide better growth of *Bifidobacterium* in relation to the XOS with a lower variety of DP, which indicates that a mixture of XOS with different

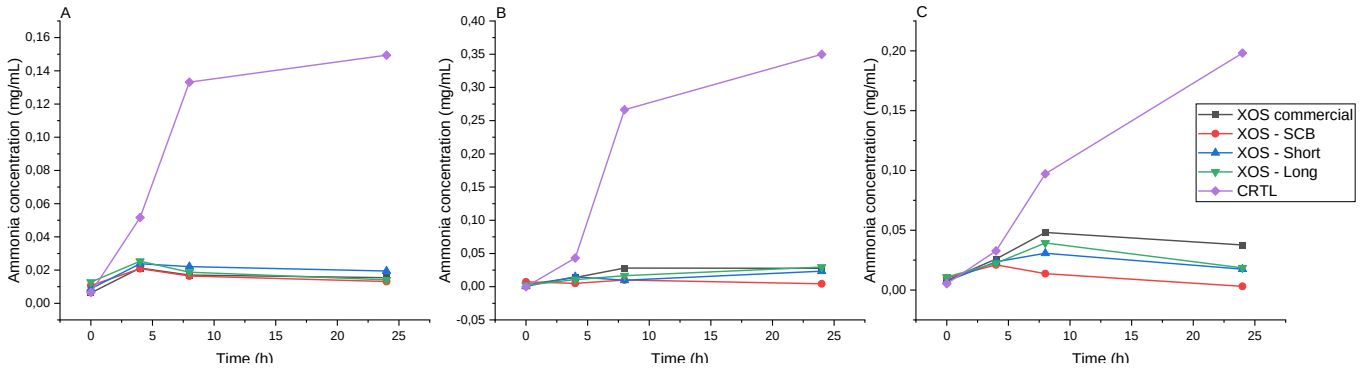

Figure 21. Ammonia concentration profile during fecal fermentation with different donors (A, B and C).

DP can be more effective than a mixture of defined DP (Mäkeläinen et al., 2010a). Other work indicates that XOS with DP_{2-3} have better prebiotic properties (Moura et al., 2007). However, our study showed that there was no significant difference in prebiotic property between short-chain XOS (Xlobiose-xylotriose) and Xos-Long (xylotriose-xylohexaose).

4.4.3 TOXICITY

The accumulation of metabolites in the distal colon, as well as variations in pH, can cause toxic effects on the cells of the intestinal wall, which are some of the conditions that can trigger disturbances in human health (Fung et al., 2013). Caco-2 is an entero-epithelial cell that has the particularity of being self-differentiate in enterocyte-like cells (e.g., polarization, tight junctions and protein expression) and mimic the intestinal epithelial barrier in *in vitro* cultures after 21 day-cultivation, making this cell a good choice for simulating an in vivo environment (Castiaux et al., 2016). The toxicity test using resazurin, also known as the Alamar Blue test, monitors the reducing environment of the living cell. It is based on the property of the blue-colored resazurin being nonfluorescent, whereas in its reduced form, called resorufin, it is pink and highly red fluorescent. With fluorometric methods, it is possible to estimate the number of viables cells by measuring the reduction of resazurin into resorufin (Walzl et al., 2014). The main agent that can reduce resazurin is a mitochondrial NADPH reductase, however other enzymes such as diaphorases and flavin reductase found in the cytoplasm and mitochondria can also play a reducing role in the reagent. Therefore, there is no specificity of resazurin to detect the interruption of electron transport and mitochondrial dysfunction, and it may also detect other impairments of cellular metabolism (Rampersad, 2012). This chosen assay has a good benefit/time, it is not toxic or radioactive, it is safe for the user and the environment, it has high sensitivity and linearity, it does not involve cell lysis, it is ideal for use in post-measurement functional assays, and it is flexible as it can be used with different cell models. However, several studies have adopted the use of more than one toxicity assessment technique to obtain a better understanding of the toxicity effects of the compounds (Rampersad, 2012).

The other method used was the indirect detection of cell membrane damage by the LDH test. LDH is an enzyme present in the cytosol of intact cells, but it is released to the outside of the cell when it suffers plasma membrane damage. In this context, it is possible to relate the amount

of LDH released in the medium to the severity of cell damage that a culture medium can cause to the membrane of Caco-2 cells (Shima et al., 1999).

The XOS fractions before fermentation did not show significant differences in relation to cell viability, except for XOS-long that had a negative impact (Figure 22 A). When there was fermentation, only the experiment with XOS-SCB (X2) had a slight negative impact on cell viability, while the other sources of XOS from sugarcane bagasse had a slightly positive impact. In the LDH assay (Figure X), all hydrolysates containing XOS from sugarcane bagasse, before and after fermentation, had a positive impact on the test. That is, they had lower relative amounts of LDH released in the medium (Figure 22 B). Together, the results of both tests indicate that XOS from sugarcane bagasse, either by its DP or by the presence of other compounds in the hydrolysate, has a cell membrane protection action. However, the cell viability assay points to a lower cell viability when it comes from XOS-Long or the product of fermentation using XOS-SCB, which may represent that the decrease in this cell viability is due to factors other than cell membrane damage.

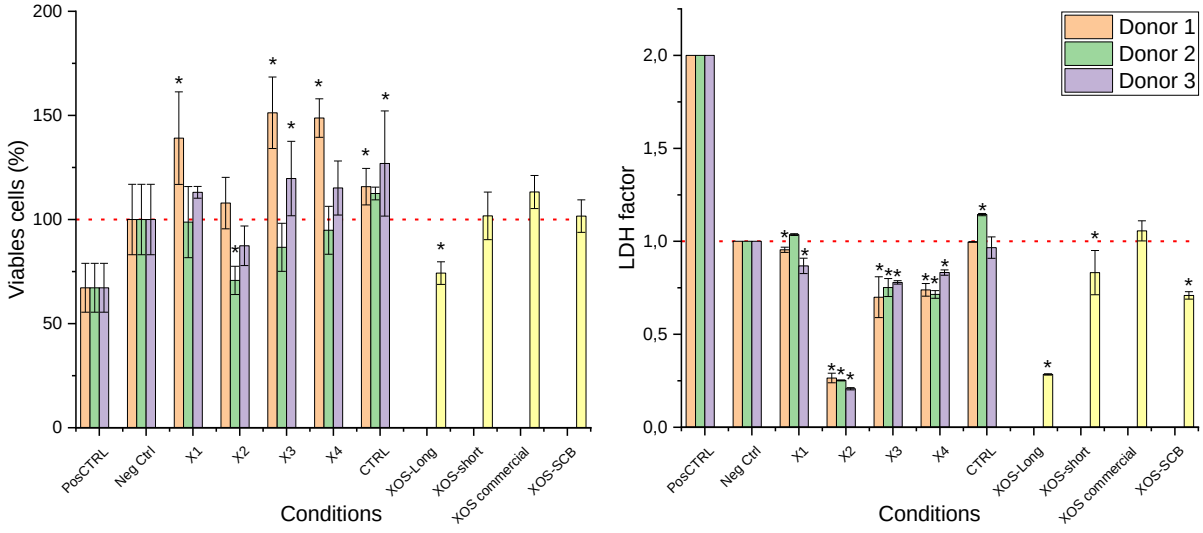

Figure 22. Cytotoxicity assays - A: Analyze viable cells by resazurin test; B – Analysis of cell membrane damage by quantification of LDH (lactate dehydrogenase) released in the medium. PosCTRL: Positive control with agent tsPBS (0.5% Triton X-100 and 0.1% saponin in PBS); NegCTRl: negative control containing only basal medium.

4.5 CONCLUSION

Gel permeation chromatography was efficient in the separation of xylan derivatives, mainly for the separation of monomeric and oligomeric compounds. Consequently, it allowed to obtain two distinct groups of XOS, one with a short-chain (XOS-short) and one with long-chain (XOS-long). The use of XOS-SCB and these different fractions in human fecal fermentation showed that in the cellular metabolism there was no difference in the fermentation profile with the responses studied (pH and ammonia), but that XOS fractions from sugarcane bagasse have a potential to protect against damage in the cell membrane.

CHAPTER 5: POTENTIAL OF XOS TO ATTENUATE THE ADVERSE EFFECTS OF FECAL FERMENTATION OF PROTEINS

5.1 ABSTRACT

XOS-SCB, both fractionated and obtained directly from the extraction process of sugarcane bagasse, proved to be as efficient as commercial XOS in terms of pH profile and ammonia concentration in the medium when administered as a carbon source in human fecal fermentation, as shown in previous chapter. In the literature there are also several works that show the benefit of its use in human health due to its high selectivity for *Bifidobacterium* and the generation of SCFA. However, their benefits can go further as XOS could also have the potential to act to attenuate the adverse effects of high concentration of proteins derivates in the cecal environment, what can prevention some diseases such as colorectal cancer (CRC). This work sought to evaluate the ability of XOS, both commercial and XOS-SCB, to attenuate the adverse effects of high protein derivates concentration in the cecal medium by fecal fermentation, such as the accumulation of ammonia and the high pH in the medium. Therefore, high concentration of proteins, from animal and plant sources, were pre-digested following the INFOGEST protocol and batch fecal fermentations were performed with different XOS supplements, both from commercial sources and XOS-SCB, and microbial functionality was determined by measuring fermentation characteristics such as ammonia concentration, pH variation and cytotoxicity analysis with Caco-2 cells. Supplementation of both XOS was effective to decrease the concentration of ammonia and the final pH of the fecal fermentation when compared with the fermentation of protein derivatives, with emphasis on the XOS-SCB which presented values closer to the fecal fermentation of media without addition of protein. Cytotoxic analyzes showed that the metabolites generated after fermentation showed a decrease in cell viability only for fermentations containing XOS-SCB, but on the other hand, cell membrane integrity was higher in the presence of the same XOS-SCB. This work shows that there is great potential in the use of XOS, both from a commercial source as XOS-SCB, to attenuate the effect of high-protein diets that can cause CRC precursor cells.

5.2 INTRODUCTION

Until this chapter, all studies have focused on the most classic prebiotic property of oligosaccharides, which is the property of being selective for *Bifidobacterium* and generating

higher levels of SCFA that results in lower pH values. In fact, most studies in the literature on the prebiotic properties of XOS have focused on the use of XOS to improve the levels of *Bifidobacterium* and short-chain fatty acids (SCFA) production, both *in vitro* and *in vivo*, and little has been explored its use to attenuate the harmful effect of protein fecal fermentation of proteins derivates (Han et al., 2020; Kajihara et al., 2000; Li et al., 2022), which can add another great benefit to the use of XOS in health.

Proteins are an essential component of the human diet as they are crucial for the synthesis of body tissues proteins, regulatory proteins, and different amino acid-derived metabolites. The digestion of protein by the gastrointestinal system aims to allow the absorption of protein components by the body and, under normal conditions, approximately 95% of ingested protein are absorbed and transferred into the bloodstream (Tomé, 2021). However, some factor as the quality and quantity of the ingested protein or problems during the digestion process can result in a lower absorption of these proteins and their derivatives, generating a high availability of proteins in the distal colon, in addition to the endogenous proteins themselves (Dave et al., 2016).

Due the slow transit time and limited host absorption of the protein when it reaches the distal colon, there are a facility to intense microbial proteolysis and accumulation of metabolic end products (Diether and Willing, 2019), and the bacterial activity in distal colon not only produces metabolites beneficial to intestinal tract but also produces substances harmful to human health (Zhang et al., 2020). Ammonia is one of the metabolites that accumulate when there is a large availability of proteins and their derivatives in the distal colon, that is a result from the cumulative effects of enterocyte metabolism, microbial deamination, and microbial protein synthesis (Diether and Willing, 2019). Long exposure time of the luminal environment to components such as ammonia can cause health damage. Normal colonocytes from luminal environment, when exposed for a long time to cytotoxic agents such as ammonia, can result in a cellular response to adapt and result in colorectal cancer (CRC) cells (Fung et al., 2013). Another factor that can increase the risk of CRC is the high pH of the environment, with most cases of CRC occurring in the distal colon, the region of the intestine that has the highest pH values. Environments with a more acidic pH, below 5.5, favor the development of bacteria that result in health improvements, such as butyrate producing *Roseburia* species and inducing apoptosis of cancerous cells (Duncan et al., 2021; Fung et al., 2013).

It is observed that diets high in protein and low in carbohydrate can result in cellular damage, increasing the risk of CRC. However, this damage can be reversed feeding fermentable fiber like resistant starch or arabinoxylan (Fung et al., 2013; Lutz and Schlatter, 1992).

In this context, considering the benefits that non-digestible carbohydrates can bring to human health, this study aimed to evaluate the potential of using xylooligosaccharides, from different source (commercial and from sugarcane bagasse), to attenuate the harmful effects of protein fermentation in the distal colon.

5.3 METHODS

5.3.1 MATERIALS

Two commercial protein sources were used. The first was from a vegetable source (Pea Protein 88%, Piscane C9- Cosucra) and the second from an animal source (Casein 77.5%, MYPROTEIN). The commercial, purified XOS was the XOS 95% (PrecticX 95P). XOS produced at a pilot-scale continuous reactor for hydrothermal processing from sugarcane bagasse (XOS-SCB) in a previous work (Pereira et al., 2021) was also utilized. The characterization results of this XOS were made in the previous chapter (4) and is summarized at Table 7.

Table 7. Degree of polymerization of XOS from commercial source PrecticX and from sugarcane bagasse XOS-SCB). Xyl1: xylose; Xyl2: Xylobiose; Xyl3: Xylotriose, Xyl4: Xylotetraose; Xyl5: Xylopentaose and Xyl6: Xylohexaose.

Fraction	**Xylan components (% w/w)**						
	Xyl1	Xyl2	Xyl3	Xyl4	Xyl5	Xyl6	Total XOS
XOS commercial	-	29,9	23,9	18,5	10,1	12,6	95,00
XOS-SCB	39,08	18,50	14,64	12,54	9,83	5,41	60,92

5.3.2 PROTEIN DIGESTION

To simulate the human digestive system (oral, gastric and intestine phase), the protocol adapted from INFOGEST (Brodkorb et al., 2019) was used. The procedure for the digestive system was performed in a shaker with a temperature of 37 °C at agitation of 100 rpm and the solution to

simulate each digestive phase (simulated Salivary fluid – SSF; gastric fluid – SGF; Intestinal fluid - SIF) was done exactly as the INFOGEST protocol. The enzymes and bile activities assays are given in the supplementary material. To simulate the oral phase, 13.2 g of pure protein (15.0 g of Pea Protein and 17.0 g of Casein) was added into a 250 mL bottle and diluted with 24.0 mL de SSF, 150 µL of $CaCl_2$ and 20.0 mL of H_20 (final volume of 60 mL) and left to mix for 2 min. To simulate the gastric phase, 48.0 mL of SGF was added in the mixture from oral phase and the pH was adjusted to 3 using 6M HCl. Then, 30 µL of $CaCl_2$, 3 mL of Rabbit gastric extract (7200 U Lipase and 240000 U Pepsin - Lipolytech, cat. no. RGE 25-100MG) and H_20 were added to reach a final volume of 120 mL. The mixture was left to mix for 2 h. To simulate the intestinal phase, 51.0 mL of SIF and 15 mL of 2.4 mmol Ox bile solution (Sigma - Aldrich, cat. No. 70168) were added at the mixture from gastric phase and let to mixed for 30 min to achieve complete bile solubilization. 240 µL of $CaCl_2$ was added and the pH was adjusted to 7 with 6N NaOH. Thirty mL of pancreatin (24000 U Trypsin - Sigma-Aldrich, cat. no. P7545) was added to the mixtures and the final volume was adjusted to 240 mL with H_2O. After 2 hours of agitation, the samples were freeze dried and kept at -20°C until further use.

5.3.3 FECAL FERMENTATION

5.3.3.1 FECAL SAMPLES PREPARATION

Three independent biological triplicates were performed, using fecal samples from three healthy donors. The samples were collected with the approval of the Swedish Ethical Review Authority (Dnr 2022-01696-01), and all participants signed an informed consent prior to the initiation of the experiment. The participants had not used antibiotics or fiber supplementation in the previous six months of the sample collection. Fresh fecal samples were collected and kept in an anaerobic environment with the aid of an anaerobic punch (Oxoid AnaeroGen W-zip compact gas generator system, #12201972 - AN0010W) until homogenized in a sterile 50 mM PBS buffer in a ratio of 200 g/L. The slurry was prepared just prior to inoculation in the bioreactors.

5.3.3.2 FECAL FERMENTATION

The basal medium ingredients in pure water per liter were 2 g of Peptone water, 2 g NaCl, 0.04 of K_2HPO_4, 0.04 g of KH_2PO_4, 0.01 g of $MgSO_4.7H_2O$, 0.04 g of $CaCl_2.6H_2O$, 2 g of $NaHCO_3$, 2 mL of Tween 80, 0.05 g of Haemin (Sigma, #51280), 10 µL of Vitamin K1 (Sigma, #V3501), 1 g of Cysteine.HCl (Sigma, #C7477) and 0.5 g of Bile salts (Sigma, #48305). The basal medium, previously bubbled with sterile nitrogen, and the substrates, added as the Table 8, were mixed in the anaerobic chamber (37ºC; 10% H_2, 80% N_2, 10% CO_2; Ruskinn, UK) and inoculated with 10 % v/v of fecal slurry, reaching a final fecal concentration of 2 % w/v. Fecal fermentation was carried out in a Gas Endeavor system using 500 mL bottles during 24 h, with stirring at 100 rpm. Aliquots of the samples were collected at 0, 4, 8 and 24 h, centrifuged and both supernatant and pellet were storage at -80 ºC until further use.

Table 8. Designer of experiment showing the fraction of each component in each fermentation

Code / **Substrate**	**Addition of each component to the basal culture medium (% w/w)**								
	CTRL	P1	P2	X1	X2	P1X1	P1X2	P2X1	P2X2
Casein Protein	0	1	0	0	0	1	1	0	0
Pea protein	0	0	1	0	0	0	0	1	1
XOS commercial	0	0	0	1	0	1	0	1	0
XOS-SCB	0	0	0	0	1	0	1	0	1

5.3.4 METABOLITES ANALYSIS

Ammonia was analyzed using a “Ammonia assay Kit” from Megazyme (Product code: K-AMIAR) strictly following the manufacturer's protocol (Megazyme, 2020).

5.3.5 CYTOTOXICITY

5.3.5.1 CELL CULTURE

Caco-2 cells were kindly provided by Dr Ignacio Rangel and were cultured according ATCC guidelines. Briefly, cells were cultured in DMEM containing penicillin and streptomycin with 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. Cells were passed to new culture

plates by using trypsin/EDTA when they reached 70–90% of confluence. Before treatments, cells were added to the culture plates at the desired concentration and left overnight on the incubator. Supernatants from the fermentation were filtered (0.22 µm), sPBS was used as negative control and Triton-X (0.2% - final concentration) as positive control.

5.3.5.2 CELLS CULTIVATION

Living cells maintain a reducing environment within their cytoplasm and mitochondria, in which resazurin (blue and non-fluorescent) is reduced by dehydrogenase enzymes to form the red fluorescent dye resorufin (Rampersad, 2012). "*In Vitro Toxicology Assay Kit, Resazurin based*" (Sigma, n.d.) was used to determine the toxicology assay for Caco-2. Briefly, cells were plated on a 96-well cell culture plates at a density of 1 × 104 cells/well (180 µL) overnight. Twenty uL of t sPBS (control) or 20 µl of the filtered supernatants samples from the fermentation was added in the cells which were incubated for 24 h. A blank containing complete medium without cells was also used. After incubation, 20 uL of resazurin dye solution was added into each well. Plates were incubated for more 3 h and plates were measured by fluorescence at a wavelength of 590 nm using an excitation wavelength of 560 nm. Cells viability at each incubation time was expressed in relation to the untreated cells (negative control).

The relative percentage of viable cells (VC) for each sample in relation to the positive control was used according to equation 3, with the positive control being defined as 100%.

$$VC = \frac{Flu_{sample}}{Flu_{nc}} x100 \quad (3)$$

Where VC is the viable cells in each sample, Flu_{sample} is the fluorescence of the sample and Flu_{nc} is the fluorescence of negative control.

5.3.5.3 LDH ASSAY

LDH is released in the cell culture medium in proportion to the cell membrane damage and this released LDH is measured by an indirect method. LDH catalyzes the conversion of pyruvate to lactate and consequently reduces NAD^+ to NADH, so diaphorase uses NADH to reduce the

tetrazolium salt to a red formazan product, which can be quantified by spectrometry at 450 nm (Forest et al., 2015).

Cells were plated overnight as described for resazurin assay, including the same treatments and controls, and LDH was measured by using the "lactate dehydrogenase assay kit – Sigma" following the manufacturer's instructions (Sigma-Aldrich, n.d.). Briefly, after incubation, supernatants (20 µL) were transferred to 96-well cell culture plates, mixed with LDH Assay Buffer and Master Mix Reaction. A LDH standard curve was used, as well as the positive control provided in the assay kit. The plates were mix using a horizontal shaker, incubated at 37 ^{o}C and the absorbance was read at 450 nm. The final measurement is when the most active sample is near or exceeds the end of the linear range of the standard curve. A factor (F) was used to compare the samples to negative control as show in equation 4.

$$F = \frac{AD_{sample}}{AD_{pc}} \quad (4)$$

AD_{sample} is the difference between absorbance at T_{final} and T0 (blank) of the samples and AD_{pc} is the difference between absorbance at T_{final} and T_0 (blank) of the positive control.

5.4 RESULTS AND DISCUSS

5.4.1 FECAL FERMENTATION

Regardless of the donors of the fecal samples, fecal fermentation using only protein derivatives, both from animal and vegetable sources, showed an initial pH reduction and an increase in pH throughout the fermentation (Figure 23). In this scenario, there is a limitation of the carbon source, and the first step of catabolism of the amino acids and peptides is their deamination, most of them generating free ammonia and their respective keto acids or saturated fatty acids, which are precursors for the synthesis of SCFA (Tomé, 2021). After the amino acids most susceptible to be precursors of SCFA are metabolized, and a scenario with scarcity of carbohydrates, there is fermentation of the remaining protein residues, resulting in the consumption of SCFA for the metabolism of these bacteria and the release of nitrogenous compounds, such as free ammonia, leading in an increase of the pH value (Duncan et al., 2021; Windey et al., 2012).

In a scenario of low amount of proteins but abundant in carbohydrates (XOS), it is possible to notice that the pH remained low throughout the fermentation (Figure 23). This result can be related to an *in vivo* study where the administration of XOS (5 g/day) resulted in a decrease in fecal pH (Okazaki et al., 1990). It is possible that this may have occured because the release of nitrogenous compounds is lower as needed to be taken up by the cell's metabolism, coming only from the basal medium, and there is an accumulation of SCFA in the medium that reduces the pH. The same low pH value is observed when there is a mixture of protein and carbohydrate, as seen in experiments P1X1, P1X2, P2X2 and P2X3. XOS is a substrate for the microbiota bacteria, which generates enough SCFA to reduce the pH, and probably the free ammonia released during the deamination step or metabolism of peptides and amino acids is used for cell development as a source of nitrogen.

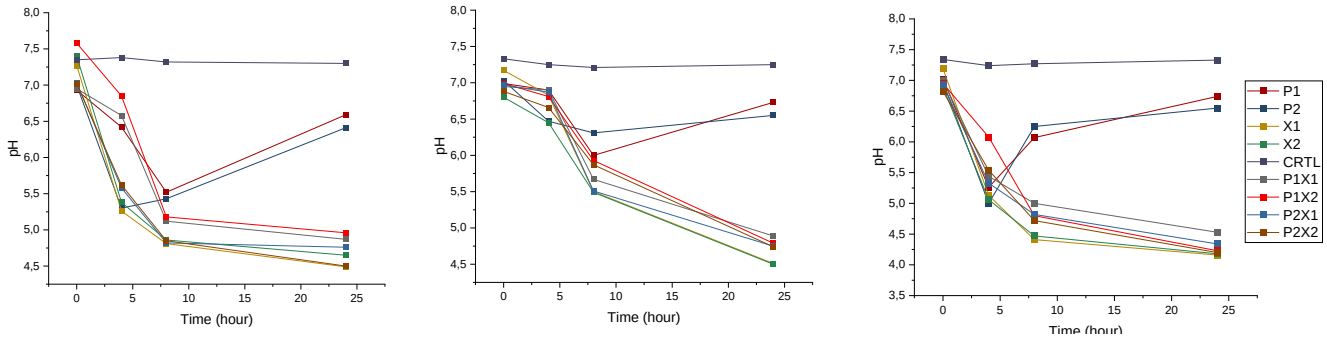

Figure 23. pH variation during fecal fermentation with samples from three different donors

The hypothesis above is in line with the results obtained with the concentration of free ammonia in the medium (Figure 24). When there was just nitrogen source from the basal fermentation medium, experiments containing only XOS result in practically no-free ammonia concentrations, which can be explained by the fact that all nitrogen were used for cell growth. On the other hand, when the culture medium was supplemented only with a protein source, there was a higher accumulation of free ammonia. This can be explained by the high availability of amino acids and a shortage of energy source to use all this substrate for cell growth. When there was a higher balance between protein and carbohydrate sources, with the mix of XOS and proteins, there was a higher balance between free ammonia in the medium, which may represent that part of the free ammonia released in the experiment was used as a source of nitrogen for cell growth, since

there was greater availability of energy sources for the cell due to the presence of XOS as a carbon source.

The use of XOS has already been reported to be beneficial in reducing the level of ammonia in the blood of patients with liver cirrhosis, and, although the mechanism behind this still remains unclear, XOS may exert its effect by inhibiting enteric colonization of ammonia-producing anaerobes such as Bacteroides (Kajihara et al., 2000). *In vivo* studies in broilers have shown that the administration of XOS results in a greater utilization of nitrogen sources and a lower concentration of free ammonia in the fecal samples (Li et al., 2022), while a recent *in vitro* study with swine fecal samples have reported that the use of XOS as a carbon source results in lower pH values and ammonia concentration (Han et al., 2020), results that , although performed within the scope of animals, highlight the potential benefit of the use of XOS to attenuate the high pH value and concentration of free ammonia resulting from the intestinal fermentation of protein residues.

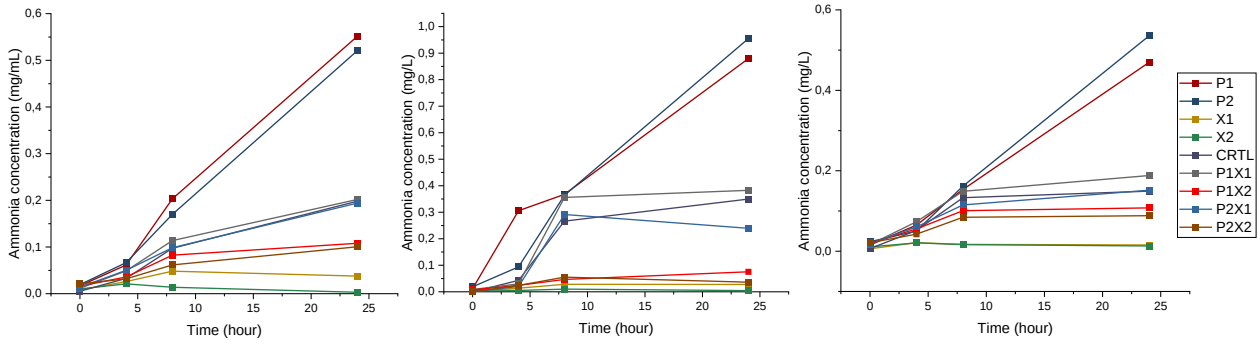

Figure 24. Ammonia concentration variation during fecal fermentation with samples from three different donors

Regarding the studied metabolite and pH profile, it is evident that the addition of XOS (commercial and XOS-SCB) into a culture medium rich in protein derivatives can attenuate the harmful effect of high pH and free ammonia concentration in the medium. However, the XOS-SCB displayed a more accentuated benefit mainly in the levels of free ammonia concentration, with values very close to the XOS without the presence of proteins. Although the concentration of commercial XOS and XOS-SCB was similar, the chemical composition of the mixture containing XOS-SCB is more complex, containing monomers of xylose, furfural from sugar degradation and polyphenols from lignin degradation (Pereira et al., 2021), which may have positively affected the fecal fermentation when analyzing the pH and final free ammonia concentration.

5.4.2 TOXICIT

There was no statistical difference for the cell viability test (resazurin) in any case for the 3 donors when referring to the same substrate, but at least for 1 of the donors there was a statistical difference for each substrate (Figure 25 A) All fermentations with proteins and/or commercial XOS did not present values lower than the negative control, indicating that there was no reduction in cell viability by the metabolites from their fermentations. In fact, it has been reported that cancer cells can develop after long periods of exposure to an environment containing toxic metabolites (Fung et al., 2013) and that this test may not be efficient to note these mutations. The other metabolites from this fermentation also showed no adverse effect on cell viability measured by this method. In contrast, XOS-SCB (X2), used either alone or in mixture with the different proteins, was the only compound that showed a reduction in the cell viability of Caco-2 in at least one of the donors, with its maximum reduction in 30 % cell viability, which is very close to the positive control.

As mentioned, the composition of XOS-SCB is more complex than that of commercial XOS as it has not undergone purification steps and has compounds such as furfural and polyphenols (Pereira et al., 2021). Although it has been shown that furfural results in the accumulation of reactive oxygen species (ROS) in *Saccharomyces cerevisiae* and this can be related to cell damage (Allen et al., 2010), there is no study that has explored this issue in depth with intestinal wall cells.

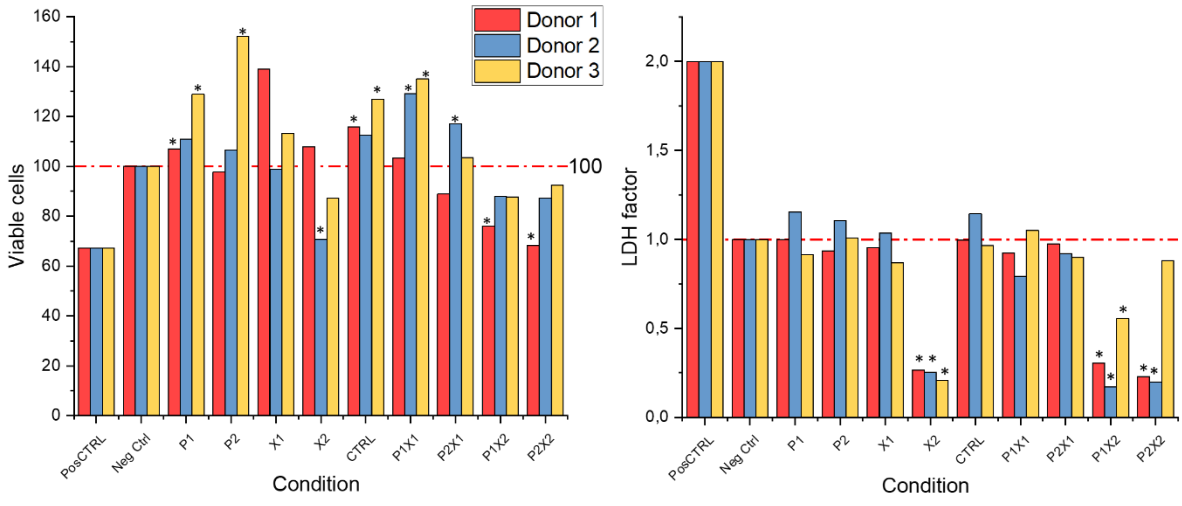

Figure 25. Cytotoxicity tests. A: Analyze viable cells by resazurin test; B – Analysis of cell membrane damage by quantification of LDH (lactate dehydrogenase) released in the medium. PosCTRL: Positive control with agent Triton -X; NegCTRl: negative control containing only basal medium.

In fact, there are studies that have shown that the presence of furfural in the diet can improve the regulation of the intestinal microbiota, favoring the development of beneficial bacteria to health (Pérez-Burillo et al., 2018) and this can have an impact on the improvement of the barrier properties of the intestinal wall. One hypothesis for the reduction in the detection of viable cells may be the presence of polyphenols from lignin degradation, which have an antioxidant propriety (Pandey and Rizvi, 2009) and may reduce resazurin, thus reducing the sensitivity of the method and causing interference in the result.

For the analysis aiming to evaluate cell membrane damage by detecting the LDH released in the medium (Figure 25 B), there was only a statistical difference in the positive control and in the fermentations that used XOS-SCB (X2), both alone and in the mixture, regardless of the donors. Fermentations involving X2 resulted in lower values of LDH compared to other fermentations and controls, thus indicating a possible protection of the cell membrane by the metabolites present in the medium. These results are contrary to those found in the cell viability tests. This may raise two hypotheses such as: 1- The lower cell viability value of the resazurin test is more affected by the presence of antioxidant compounds (polyphenols) than a lower cell viability; 2 - There was actually less cell viability caused by the use of XOS-SCB, but the reason is not associated with cell membrane damage or. In both cytotoxicity tests applied (cells viability or evaluation of cell damage) there is a reaction that involves the reduction of agents mediated by NADH (Forest et al., 2015; O'Brien et al., 2000) and if the culture medium somehow interfered with this reaction, the tests are no longer accurate. Therefore, follow up studies focusing on the influence of the cell-free culture medium with the resazurin and LDH reagent should be carried out to better elucidate this issue. In any case, other techniques must be applied to assess the toxicity that the medium after fermentation presents to Caco-2 cells.

5.5. CONCLUSION

XOS supplementation, from commercial source and XOS-SCB, showed potential to attenuate two of the major consequence of a diet rich in protein and poor in carbohydrate, that is the high value of pH and ammonia concentration in the fecal medium. However, further studies are needed to analyze the cytotoxicity of the metabolites generated after fermentation.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

This set of works that make up the thesis allowed us to explore the prebiotic potential, both with isolated strains of *Bifidobacterium* and in human fecal fermentations, of XOS extracted from SCB by a hydrothermal process within a biorefinery concept.

Bench a pilot scale hemicellulosic hydrolysates were suitable as a carbon source for isolated strains of *Bifidobacterium*, regardless of their chemical composition but containing XOS with DP equal to or less than six xylose units. Fractionation of XOS-SCB by gel permeation was efficient to obtain fractions with high purity of XOS with short chains or long chains, however the fecal fermentation of these different fractions of XOS and XOS-SCB did not show significant differences in relation to the pH of the medium and ammonia accumulation, indicating that this purification may not be necessary for better prebiotic effects.

The exploration of the prebiotic potential of XOS to attenuate the adverse effects of fecal fermentation of high loads of proteins and derivatives proved to be effective in detecting that the addition of XOS resulted in a decrease in pH and ammonia accumulation in the fermentation medium.

In conclusion, it is possible to state that XOS extracted by hydrothermal process under the conditions studied here within the biorefinery concept presents characteristics to be a potential prebiotic compound within the fermentation test applied, since its chemical characteristics encompass a degree of polymerization of up to six xylose units.

Unfortunately, it was not possible to perform the analysis of SCFA and microbial composition of samples from fecal fermentations. However, these samples are stored at -80 ºC for future analysis, which results will allow to deepen the understanding of the use of XOS as a carbon source for this pool of bacteria.

Once the prebiotic potential of the XOS studied herein is evident and the economic potential of the XOS extraction process to be more attractive than other process approaches, the application of these compounds in more advanced tests such as in microbiota simulators or *in vivo* tests are required so that there is the possibility of this XOS reaching the market with a high value product.

REFERENCES

Aachary, A.A., Prapulla, S.G., 2011. Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. Compr Rev Food Sci Food Saf 10, 2–16. https://doi.org/10.1111/j.1541-4337.2010.00135.x

Ahmad, R., 2019. Better health with plant oligosaccharides: trends and future [WWW Document]. The Second International Conference on Food and Agriculture. URL https://core.ac.uk/download/pdf/287231781.pdf (accessed 11.2.22).

Akpinar, O., Erdogan, K., Bakir, U., Yilmaz, L., 2010. Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. LWT - Food Science and Technology 43, 119–125. https://doi.org/10.1016/j.lwt.2009.06.025

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae. Biotechnol Biofuels 3, 2. https://doi.org/10.1186/1754-6834-3-2

Andersen, J.M., Barrangou, R., Hachem, M.A., Lahtinen, S.J., Goh, Y.J., Svensson, B., Klaenhammer, T.R., 2013. Transcriptional analysis of oligosaccharide utilization by *Bifidobacterium* lactisBl-04. BMC Genomics 14, 312. https://doi.org/10.1186/1471-2164-14-312

ben David, Y., Dassa, B., Borovok, I., Lamed, R., Koropatkin, N.M., Martens, E.C., White, B.A., Bernalier-Donadille, A., Duncan, S.H., Flint, H.J., Bayer, E.A., Moraïs, S., 2015. Ruminococcal cellulosome systems from rumen to human. Environ Microbiol 17, 3407–3426. https://doi.org/10.1111/1462-2920.12868

Bio-Rad, n.d. Bio-Gel P-2 Gel #1504118 | Bio-Rad [WWW Document]. 2022. URL https://www.bio-rad.com/pt-br/sku/1504118-bio-gel-p-2-gel?ID=1504118 (accessed 10.20.22).

Brienzo, M., 2010. Extração da hemicelulose do bagaço de cana-de-açúcar para produção de xilo-oligosacarídeos. Thesis, Escola de Engenharia de Lorena, Universidade de São Paulo 137.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.R., Martins, C., Marze, S., McClements, D.J., Ménard, O., Minekus, M., Portmann, R., Santos, C.N., Souchon, I., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Recio, I., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc 14, 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Buckeridge, M.S., de Souza, A.P., 2014. Breaking the "Glycomic Code" of Cell Wall Polysaccharides May Improve Second-Generation Bioenergy Production from Biomass. Bioenergy Res. https://doi.org/10.1007/s12155-014-9460-6

Carlson, S., 2018. GRAS Notice 816, Xylooligosaccharides from sugarcane.

Carvalho, A.F.A., Marcondes, W.F., de Oliva Neto, P., Pastore, G.M., Saddler, J.N., Arantes, V., 2018. The potential of tailoring the conditions of steam explosion to produce xylo-oligosaccharides from sugarcane bagasse. Bioresour Technol 250, 221–229. https://doi.org/10.1016/j.biortech.2017.11.041

Carvalho, A.F.A., Neto, P. de O., da Silva, D.F., Pastore, G.M., 2013. Xylo-oligosaccharides from lignocellulosic materials: Chemical structure, health benefits and production by chemical and enzymatic hydrolysis. Food Research International 51, 75–85. https://doi.org/10.1016/j.foodres.2012.11.021

Castiaux, V., Laloux, L., Schneider, Y.-J., Mahillon, J., 2016. Screening of Cytotoxic B. cereus on Differentiated Caco-2 Cells and in Co-Culture with Mucus-Secreting (HT29-MTX) Cells. Toxins (Basel) 8, 320. https://doi.org/10.3390/toxins8110320

Chen, H., 2014. Biotechnology of Lignocellulose, Biotechnology of Lignocellulose: Theory and Practice. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-007-6898-7

Chen, M.-H., Swanson, K.S., Fahey, G.C., Dien, B.S., Beloshapka, A.N., Bauer, L.L., Rausch, K.D., Tumbleson, M.E., Singh, V., 2016. In Vitro Fermentation of Xylooligosaccharides Produced from Miscanthus × giganteus by Human Fecal Microbiota. J Agric Food Chem 64, 262–267. https://doi.org/10.1021/acs.jafc.5b04618

Cheng, S., Zhu, S., 2009. Lignocellulosic biorefinery [WWW Document]. Bioresources. URL https://eds.p.ebscohost.com/eds/pdfviewer/pdfviewer?vid=1&sid=58d8461b-601f-422b-a9e5-c4c832f316c9%40redis (accessed 11.2.22).

Cherrington, C.A., Hinton', M., Pearson2, G.R.C., 1991. Short-chain organic acids at pH 5.0 kill Escherichia coli and Salmonella spp. without causing membrane perturbation. Journal of Applied Bacteriology 70, 161–165.

Childs, C.E., Röytiö, H., Alhoniemi, E., Fekete, A.A., Forssten, S.D., Hudjec, N., Lim, Y.N., Steger, C.J., Yaqoob, P., Tuohy, K.M., Rastall, R.A., Ouwehand, A.C., Gibson, G.R., 2014. Xylo-oligosaccharides alone or in synbiotic combination with *Bifidobacterium* animalis subsp. lactis induce bifidogenesis and modulate markers of immune function in healthy adults: A double-blind, placebo-controlled, randomised, factorial cross-over study. British Journal of Nutrition 111, 1945–1956. https://doi.org/10.1017/S0007114513004261

Chung, Y.C., Hsu, C.K., Ko, C.Y., Chan, Y.C., 2007. Dietary intake of xylooligosaccharides improves the intestinal microbiota, fecal moisture, and pH value in the elderly. Nutrition Research 27, 756–761. https://doi.org/10.1016/j.nutres.2007.09.014

Craig, R.M., Atkinson, A.J., 1988. d-Xylose testing: A review. Gastroenterology 95, 223–231. https://doi.org/10.1016/0016-5085(88)90318-6

Database, N.L. of M.H., 2019. FURFURAL CASRN: 98-01-1 [WWW Document]. URL https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+542 (accessed 12.8.19).

Dave, L.A., Hayes, M., Montoya, C.A., Rutherfurd, S.M., Moughan, P.J., 2016. Human gut endogenous proteins as a potential source of angiotensin-I-converting enzyme (ACE-I)-, renin inhibitory and antioxidant peptides. Peptides (N.Y.) 76, 30–44. https://doi.org/10.1016/j.peptides.2015.11.003

Demirbas, A., 2010. Biorefinery Technologies for Biomass Upgrading. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 32, 1547–1558. https://doi.org/10.1080/15567030902780394

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J., Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54, 2325–2340. https://doi.org/10.1194/jlr.R036012

Dence, C. W., 1992. The Determination of Lignin, in: Lin, S.Y., Dence, Carlton W. (Eds.), Methods in Lignin Chemistry. Springer Berlin Heidelberg, Berlin, pp. 33–61. https://doi.org/10.1007/978-3-642-74065-7_3

Diether, N.E., Willing, B.P., 2019. Microbial fermentation of dietary protein: An important factor in diet–microbe–host interaction. Microorganisms 7. https://doi.org/10.3390/MICROORGANISMS7010019

Du, J., Li, C., Zhao, Y., Wang, H., 2018. Hemicellulose isolated from waste liquor of viscose fiber mill for preparation of polyacrylamide-hemicellulose hybrid films. Int J Biol Macromol 108, 1255–1260. https://doi.org/10.1016/j.ijbiomac.2017.11.036

Duncan, S.H., Holtrop, G., Lobley, G.E., Calder, A.G., Stewart, C.S., Flint, H.J., 2004. Contribution of acetate to butyrate formation by human faecal bacteria. British Journal of Nutrition 91, 915–923. https://doi.org/10.1079/bjn20041150

Duncan, S.H., Iyer, A., Russell, W.R., 2021. Symposium three: Physiological determinants for protein requirements Impact of protein on the composition and metabolism of the human gut microbiota and health. Proceedings of the Nutrition Society 80, 173–185. https://doi.org/10.1017/S0029665120008022

Duque, A., Manzanares, P., Ballesteros, I., Ballesteros, M., 2016. Steam Explosion as Lignocellulosic Biomass Pretreatment, Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802323-5.00015-3

Esteve, P.J.S., Carvalho, W. de, 2011. Pré-tratamento do bagaço de cana-de-açúcar com H2SO4 diluído em reator piloto aquecido por vapor direto. Dissertation – Escola de Engenharia de Lorena, Universidade de São Paulo.

Felipe, M.G.A., Vitolo, M., Mancilha, I.M., Silva, S.S., 1997. Fermentation of sugar cane bagasse hemicellulosic hydrolysate for xylitol production: Effect of pH. Biomass Bioenergy 13, 11–14. https://doi.org/10.1016/S0961-9534(97)00032-9

Fengel, D., Wegener, G., 1989. Wood: Chemistry, Ultrastructure, Reactions. W. de Gruyter.

Finegold, S.M., Li, Z., Summanen, P.H., Downes, J., Thames, G., Corbett, K., Dowd, S., Krak, M., Heber, D., 2014. Xylooligosaccharide increases bifidobacteria but not lactobacilli in human gut microbiota. Food Funct 5, 436–445. https://doi.org/10.1039/c3fo60348b

Forest, V., Figarol, A., Boudard, D., Cottier, M., Grosseau, P., Pourchez, J., 2015. Adsorption of lactate dehydrogenase enzyme on carbon nanotubes: How to get accurate results for the cytotoxicity of these nanomaterials. Langmuir 31, 3635–3643. https://doi.org/10.1021/acs.langmuir.5b00631

Fung, K.Y.C., Ooi, C.C., Zucker, M.H., Lockett, T., Williams, D.B., Cosgrove, L.J., Topping, D.L., 2013. Colorectal carcinogenesis: A cellular response to sustained risk environment. Int J Mol Sci 14, 13525–13541. https://doi.org/10.3390/ijms140713525

Gao, Z., Yin, J., Zhang, J., Ward, R.E., Martin, R.J., Lefevre, M., Cefalu, W.T., Ye, J., 2009. Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes 58, 1509–1517. https://doi.org/10.2337/db08-1637

Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D., Verbeke, K., Reid, G., 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol 14, 491–502. https://doi.org/10.1038/nrgastro.2017.75

Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125, 1401–12. https://doi.org/10.1093/jn/125.6.1401

Gibson, G.R., Scott, K.P., Rastall, R.A., Tuohy, K.M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E.F., Saulnier, D., Loh, G., Macfarlane, S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkook, I., Walker, C., Buddington, R., 2010.

Dietary prebiotics: current status and new definition. Food Science and Technology Bulletin: Functional Foods 7, 1–19. https://doi.org/10.1616/1476-2137.15880.

Gilad, O., Jacobsen, S., Stuer-lauridsen, B., Pedersen, M.B., Garrigues, C., Svensson, B., 2010. Combined Transcriptome and Proteome Analysis of *Bifidobacterium* animalis subsp . lactis BB-12 Grown on Xylo-Oligosaccharides and a Model of Their Utilization □ † 76, 7285–7291. https://doi.org/10.1128/AEM.00738-10

Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Łukasik, R., 2010. Hemicelluloses for fuel ethanol: A review. Bioresour Technol 101, 4775–4800. https://doi.org/10.1016/j.biortech.2010.01.088

Gobinath, D., Madhu, A.N., Prashant, G., Srinivasan, K., Prapulla, S.G., 2010. Beneficial effect of xylo-oligosaccharides and fructo-oligosaccharides in streptozotocin-induced diabetic rats. British Journal of Nutrition 104, 40–47. https://doi.org/10.1017/S0007114510000243

Grajek, W., Olejnik, A., Sip, A., 2005. Probiotics, prebiotics and antioxidants as functional foods, in: Acta Biochimica Polonica. pp. 665–671. https://doi.org/10.1093/brain/awt108

Han, K.H., Enomoto, M., Pelpolage, S., Nagata, R., Fukuma, N., Fukushima, M., 2020. In vitro fermentation potential of the residue of Korean red ginseng root in a mixed culture of swine faecal bacteria. Food Funct 11, 6202–6214. https://doi.org/10.1039/d0fo00423e

Hidalgo-Cantabrana, C., Delgado, S., Ruiz, L., Ruas-Madiedo, P., Sánchez, B., Margolles, A., 2017. Bifidobacteria and Their Health-Promoting Effects. Microbiol Spectr 5, 1–19. https://doi.org/10.1128/microbiolspec.BAD-0010-2016

Hon, D.N.-S., 1995. Chemical Modification of Lignocellulosic Materials. Taylor & Francis.

Hu, F., Ragauskas, A., 2012. Pretreatment and Lignocellulosic Chemistry. Bioenergy Res 5, 1043–1066. https://doi.org/10.1007/s12155-012-9208-0

Hu, J., Arantes, V., Pribowo, A., Saddler, J.N., 2013. The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. Biotechnol Biofuels 6, 112. https://doi.org/10.1186/1754-6834-6-112

Jayapal, N., Samanta, A.K., Kolte, A.P., Senani, S., Sridhar, M., Suresh, K.P., Sampath, K.T., 2013. Value addition to sugarcane bagasse: Xylan extraction and its process optimization for xylooligosaccharides production. Ind Crops Prod 42, 14–24. https://doi.org/10.1016/j.indcrop.2012.05.019

Kabel, M.A., Kortenoeven, L., Schols, H.A., Voragen, A.G.J., 2002. In vitro fermentability of differently substituted xylo-oligosaccharides. J Agric Food Chem 50, 6205–6210. https://doi.org/10.1021/jf020220r

Kajihara, M., Kato, S., Konishi, M., Yamagishi, Y., Horie, Y., Ishii, H., 2000. Xylooligosaccharide decreases blood ammonia levels in patients with liver cirrhosis. Am J Gastroenterol 95, 2514–2514. https://doi.org/10.1111/j.1572-0241.2000.02712.x

Kimura, I., Ozawa, K., Inoue, D., Imamura, T., Kimura, K., Maeda, T., Terasawa, K., Kashihara, D., Hirano, K., Tani, T., Takahashi, T., Miyauchi, S., Shioi, G., Inoue, H., Tsujimoto, G., 2013. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4, 1829. https://doi.org/10.1038/ncomms2852

Kiziltas, A., Kiziltas, E.E., Boran, S., Gardner, D.J., 2013. Micro- and nanocellulose composites for automotive applications. 13th annual SPE Automotive Composites Conference and Exhibition 1–13.

Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66, 10–26. https://doi.org/10.1007/s00253-004-1642-2

Kumar, V., Bahuguna, A., Ramalingam, S., Kim, M., 2021. Developing a sustainable bioprocess for the cleaner production of xylooligosaccharides : An approach towards lignocellulosic waste management. J Clean Prod 316, 128332. https://doi.org/10.1016/j.jclepro.2021.128332

Lara-Flores, A.A., Araújo, R.G., Rodríguez-Jasso, R.M., Aguedo, M., Aguilar, C.N., Trajano, H.L., Ruiz, H.A., 2018. Bioeconomy and Biorefinery: Valorization of Hemicellulose from Lignocellulosic Biomass and Potential Use of Avocado Residues as a Promising Resource of Bioproducts. Energy, Environment, and Sustainability 141–170. https://doi.org/10.1007/978-981-10-7431-8_8

Lee, H.L., Shen, H., Hwang, I.Y., Ling, H., Yew, W.S., Lee, Y.S., Chang, M.W., 2018. Targeted approaches for in situ gut microbiome manipulation. Genes (Basel) 9. https://doi.org/10.3390/genes9070351

Lee, H. v, Hamid, S.B. a, Zain, S.K., 2014. Conversion of Lignocellulosic Biomass to Nanocellulose: Structure and Chemical Process. The Scientific World Journal 2014, 1–20. https://doi.org/10.1155/2014/631013

Li, X., Wu, X., Ma, W., Chen, W., Zhao, F., 2022. Effects of dietary xylooligosaccharides supplementation on the intestinal morphology, nitrogen metabolism, faecal ammonia release, antioxidant capacity, and immune organ indices of broilers. Ital J Anim Sci 21, 1352–1361. https://doi.org/10.1080/1828051x.2022.2113747

Lima, D.U., Oliveira, R.C., Buckeridge, M.S., 2003. Seed storage hemicelluloses as wet-end additives in papermaking. Carbohydr Polym 52, 367–373. https://doi.org/10.1016/S0144-8617(03)00008-0

Lin, N., Dufresne, A., 2014. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J 59, 302–325. https://doi.org/10.1016/j.eurpolymj.2014.07.025

Liu, C.G., Xiao, Y., Xia, X.X., Zhao, X.Q., Peng, L., Srinophakun, P., Bai, F.W., 2019. Cellulosic ethanol production: Progress, challenges and strategies for solutions. Biotechnol Adv 37, 491–504. https://doi.org/10.1016/j.biotechadv.2019.03.002

Louis, P., Hold, G.L., Flint, H.J., 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 12, 661–672. https://doi.org/10.1038/nrmicro3344

Lutz, W.K., Schlatter, J., 1992. Chemical carcinogens and overnutrition in diet-related cancer 13, 2211–2216.

Lynd, L.R., Liang, X., Biddy, M.J., Allee, A., Cai, H., Foust, T., Himmel, M.E., Laser, M.S., Wang, M., Wyman, C.E., 2017. Cellulosic ethanol: status and innovation. Curr Opin Biotechnol 45, 202–211. https://doi.org/10.1016/j.copbio.2017.03.008

M. Kamm, B. Kamm, 2004. Principles of biorefineries. Appl Microbiol Biotechnol 64, 137–145. https://doi.org/10.1007/s00253-003-1537-7

Mäkeläinen, H., Forssten, S., Saarinen, M., Stowell, J., Rautonen, N., Ouwehand, A., 2010a. Xylo-oligosaccharides enhance the growth of bifidobacteria and *Bifidobacterium* lactis in a simulated colon model. Benef Microbes 1, 81–91. https://doi.org/10.3920/BM2009.0025

Mäkeläinen, H., Saarinen, M., Stowell, J., Rautonen, N., Ouwehand, A.C., 2010b. Xylo-oligosaccharides and lactitol promote the growth of *Bifidobacterium* lactis and Lactobacillus species in pure cultures. Benef Microbes 1, 139–148. https://doi.org/10.3920/BM2009.0029

Manisseri, C., Gudipati, M., 2010. Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides. LWT 43, 421–430. https://doi.org/10.1016/j.lwt.2009.09.004

Marcondes, W.F., 2018. Integração da produção de xilo-oligossacarídeos como co-produto de alto valor agregado ao processo de produção de celulose nanofibrilada a partir do bagaço de cana-de-açúcar (Dissertation). Escola de Engenharia de Lorena, Universidade de Sao Paulo, Lorena.

Marcondes, W.F., Milagres, A.M.F., Arantes, V., 2020. Co-production of xylo-oligosaccharides, xylose and cellulose nanofibrils from sugarcane bagasse. J Biotechnol 321, 35–47. https://doi.org/10.1016/j.jbiotec.2020.07.001

Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., Sierro, F., di Yu, Schilter, H.C., Rolph, M.S., Mackay, F., Artis, D., Xavier, R.J., Teixeira, M.M., Mackay, C.R., 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461, 1282–1286. https://doi.org/10.1038/nature08530

Megazyme, 2020. Ammonia (rapid) assay procedure.

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31, 426–428. https://doi.org/10.1021/ac60147a030

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96, 673–686. https://doi.org/10.1016/j.biortech.2004.06.025

Moura, P., Barata, R., Carvalheiro, F., Gírio, F., Loureiro-Dias, M.C., Esteves, M.P., 2007. In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by *Bifidobacterium* and

Lactobacillus strains. LWT - Food Science and Technology 40, 963–972. https://doi.org/10.1016/j.lwt.2006.07.013

Mussatto, S.I., Bikaki, N., 2016. Technoeconomic Considerations for Biomass Fractionation in a Biorefinery Context, Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802323-5.00025-6

Nikodinovic-Runic, J., Guzik, M., Kenny, S.T., Babu, R., Werker, A., O'Connor, K.E., 2013. Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoate) production using bacteria, in: Advances in Applied Microbiology. Academic Press Inc., pp. 139–200. https://doi.org/10.1016/B978-0-12-407673-0.00004-7

NutraSource, I., 2013. GRAS Notice 000458: Xylooligosaccharides.

Nygaard, I., Dembelé, F., Daou, I., Mariko, A., Kamissoko, F., Coulibaly, N., Borgstrøm, R.L., Bruun, T.B., 2016. Lignocellulosic residues for production of electricity, biogas or second generation biofuel: A case study of technical and sustainable potential of rice straw in Mali. Renewable and Sustainable Energy Reviews. https://doi.org/10.1016/j.rser.2016.03.023

O'Brien, J., Wilson, I., Orton, T., Pognan, F., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267, 5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x

O'Callaghan, A., van Sinderen, D., 2016. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.00925

Öhgren, K., Bura, R., Saddler, J., Zacchi, G., 2007. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour Technol 98, 2503–2510. https://doi.org/10.1016/j.biortech.2006.09.003

Okazaki, M., Fujikawa, S., Matsumoto, N., 1990. Effects of xylooligosaccharide on growth of bifidobacteria. Nippon Eiyo Shokuryo Gakkaishi 43, 395–401. https://doi.org/10.4327/jsnfs.43.395

Otieno, D.O., Ahring, B.K., 2012. The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides

(XOS), arabinooligosaccharides (AOS), and mannooligosaccharides (MOS). Carbohydr Res 360, 84–92. https://doi.org/10.1016/j.carres.2012.07.017

Palaniappan, A., Antony, U., Emmambux, M.N., 2021. Current status of xylooligosaccharides: Production, characterization, health benefits and food application. Trends Food Sci Technol 111, 506–519. https://doi.org/10.1016/j.tifs.2021.02.047

Palframan, R.J., Gibson, G.R., Rastall, R.A., 2003. Carbohydrate preferences of *Bifidobacterium* species isolated from the human gut. Curr Issues Intest Microbiol 4, 71–75.

Pandey, K.B., Rizvi, S.I., 2009. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. Oxid Med Cell Longev 2, 270–278. https://doi.org/10.4161/oxim.2.5.9498

Pereira, B., Marcondes, W.F., Carvalho, W., Arantes, V., 2021. High yield biorefinery products from sugarcane bagasse: Prebiotic xylooligosaccharides, cellulosic ethanol, cellulose nanofibrils and lignin nanoparticles. Bioresour Technol 342. https://doi.org/10.1016/j.biortech.2021.125970

Pérez-Burillo, S., Pastoriza, S., Jiménez-Hernández, N., D'Auria, G., Francino, M.P., Rufián-Henares, J.A., 2018. Effect of Food Thermal Processing on the Composition of the Gut Microbiota. J Agric Food Chem 66, 11500–11509. https://doi.org/10.1021/acs.jafc.8b04077

Pineiro, M., Asp, N.-G., Reid, G., Macfarlane, S., Morelli, L., Brunser, J.O., Tuohy, K., 2008. FAO Technical Meeting on prebiotics. J Clin Gastroenterol 42, 156–159.

Pokusaeva, K., Fitzgerald, G.F., van Sinderen, D., 2011. Carbohydrate metabolism in Bifidobacteria. Genes Nutr 6, 285–306. https://doi.org/10.1007/s12263-010-0206-6

Poul, E. le, Struyf, S., Springael, J., Lannoy, V., Decobecq, M., Dupriez, V., Vassart, G., Damme, J. van, Parmentier, M., Detheux, M., 2003. Functional Characterization of Human Receptors for Short Chain Fatty Acids and Their Role in Polymorphonuclear Cell Activation * 278, 25481–25489. https://doi.org/10.1074/jbc.M301403200

Qing, Q., Li, H., Kumar, R., Wyman, C.E., 2013. Xylooligosaccharides Production, Quantification, and Characterization in Context of Lignocellulosic Biomass Pretreatment, in: Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals. John Wiley & Sons, Ltd., pp. 391–415.

QYReaseach, 2022. Market Analysis and Insights: Global Xylooligosaccharides (XOS) Market [WWW Document]. URL https://www.qyresearch.com/index/detail/4141252/global-xylooligosaccharides-xos-market-insights-and-forecast-to-2028 (accessed 7.16.22).

Ramos, L.P., Suota, M.J., Fockink, D.H., Pavaneli, G., da Silva, T.A., Łukasik, R.M., 2020. Enzymes and biomass pretreatment, in: Recent Advances in Bioconversion of Lignocellulose to Biofuels and Value-Added Chemicals within the Biorefinery Concept. Elsevier, pp. 61–100. https://doi.org/10.1016/B978-0-12-818223-9.00004-7

Rampersad, S.N., 2012. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. Sensors 12, 12347–12360. https://doi.org/10.3390/s120912347

Rastall, R.A., Gibson, G.R., 2015. Recent developments in prebiotics to selectively impact beneficial microbes and promote intestinal health. Curr Opin Biotechnol 32, 42–46. https://doi.org/10.1016/j.copbio.2014.11.002

Rettger, L.F., Cheplin, H.A., 1921. A treatise on the transformation of the intestinal flora: with special reference to the implantation of bacilus acidophilus. Yale University Press 13.

Rivière, A., Selak, M., Lantin, D., Leroy, F., de Vuyst, L., 2016. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.00979

Rocha, G.J. de M., Nascimento, V.M., Gonçalves, A.R., Silva, V.F.N., Martín, C., 2015. Influence of mixed sugarcane bagasse samples evaluated by elemental and physical–chemical composition. Ind Crops Prod 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Rocha, G.J.M., Gonçalves, A.R., Oliveira, B.R., Olivares, E.G., Rossell, C.E. v, 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Ind Crops Prod 35, 274–279. https://doi.org/10.1016/j.indcrop.2011.07.010

Rosales-Calderon, O., Arantes, V., 2019. A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol. Biotechnol Biofuels 12, 240. https://doi.org/10.1186/s13068-019-1529-1

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., Tuohy, K., 2018. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr 57, 1–24. https://doi.org/10.1007/s00394-017-1445-8

Santibáñez, L., Henríquez, C., Corro-Tejeda, R., Bernal, S., Armijo, B., Salazar, O., 2021. Xylooligosaccharides from lignocellulosic biomass: A comprehensive review. Carbohydr Polym 251, 117118. https://doi.org/10.1016/j.carbpol.2020.117118

Sanz, M.L., Gibson, G.R., Rastall, R.A., 2005. Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro. J Agric Food Chem 53, 5192–5199. https://doi.org/10.1021/jf050276w

Schell, D.J., Farmer, J., Newman, M., Mcmillan, J.D., 2003. Dilute-Sulfuric Acid Pretreatment of Corn Stover in Pilot-Scale Reactor: Investigation of Yields, Kinetics, and Enzymatic Digestibilities of Solids. Appl Biochem Biotechnol 105, 69–86. https://doi.org/10.1385/ABAB:105:1-3:69

Shapiro, H., Thaiss, C.A., Levy, M., Elinav, E., 2014. The cross talk between microbiota and the immune system: metabolites take center stage. Curr Opin Immunol 30, 54–62. https://doi.org/10.1016/j.coi.2014.07.003

Shima, M., Kimura, Y., Adachi, S., Matsuno, R., 1999. Recovery of caco-2 cell monolayers to normal from the transport-enhanced state induced by capric acid sodium salt and its monoacylglycerol. Biosci Biotechnol Biochem 63, 680–687. https://doi.org/10.1271/bbb.63.680

Sigma, n.d. Resazurin based toxicology assay - TOX 8.

Sigma-Aldrich, n.d. Lactate Dehydrogenase Activity Assay Kit (MAK066) - Technical Bulletin.

Siró, I., Plackett, D., 2010. Microfibrillated cellulose and new nanocomposite materials: a review. Cellulose 17, 459–494. https://doi.org/10.1007/s10570-010-9405-y

Sluiter, a, Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of Sugars , Byproducts , and Degradation Products in Liquid Fraction Process Samples - NREL/TP-510-42623. NREL 1–14.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly-Y, M., Glickman, J.N., Garrett, W.S., 2013. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. Science (1979) 341, 569–573. https://doi.org/10.1126/science.1241165

Söderling, E., Hirvonen, A., Karjalainen, S., Fontana, M., Catt, D., Seppä, L., 2011. The Effect of xylitol on the composition of the oral flora: A pilot study. Eur J Dent 5, 24–31. https://doi.org/10.1055/s-0039-1698855

Statista, 2022. Major sugar cane producers worldwide, 2020 | Statista [WWW Document]. URL https://www.statista.com/statistics/267865/principal-sugar-cane-producers-worldwide/ (accessed 10.24.22).

Svetlov, S.A., 2019. Design of Sodium Carboxymethyl Cellulose Processing Hardware. Chemical and Petroleum Engineering 55, 439–443. https://doi.org/10.1007/s10556-019-00643-4

Takeuchi, T., Ishii, D., Mori, S., 1983. Separation of oligomers by high-performance micro gel permeation chromatography. J Chromatogr A 257, 327–335. https://doi.org/10.1016/S0021-9673(01)88188-7

Tan, L.U.L., Mayers, P., Saddler, J.N., 1998. Purification and characterization of a thermostable xylanase from Bacillus amyloliquefaciens. Enzyme Microb Technol 22, 42–49. https://doi.org/10.1016/S0141-0229(97)00102-6

Tanaka, M., Taniguchi, M.A., Matsumo, R., Kamikubo, T., 1981. Purification and properties of cellulases from Eupenicillium javanicum. Journal Fermentation Technology 59, 177–183.

Tian, D., Hu, J., Bao, J., Chandra, R.P., Saddler, J.N., Lu, C., 2017. Lignin valorization: Lignin nanoparticles as high-value bio-additive for multifunctional nanocomposites. Biotechnol Biofuels 10, 1–11. https://doi.org/10.1186/s13068-017-0876-z

Tomé, D., 2021. Protein digestion and bioavailability, in: Reference Module in Food Science. Elsevier. https://doi.org/10.1016/B978-0-12-821848-8.00034-2

Uebanso, T., Kano, S., Yoshimoto, A., Naito, C., Shimohata, T., Mawatari, K., Takahashi, A., 2017. Effects of consuming xylitol on gut microbiota and lipid metabolism in mice. Nutrients 9. https://doi.org/10.3390/nu9070756

Vacilotto, M.M., Pellegrini, V.O.A., Sepulchro, A.G.V., Capetti, C.C. de M., Curvelo, A.A.S., Marcondes, W.F., Arantes, V., Polikarpov, I., 2022. Paludibacter propionicigenes GH10 xylanase as a tool for enzymatic xylooligosaccharides production from heteroxylans. Carbohydr Polym 275. https://doi.org/10.1016/j.carbpol.2021.118684

van Craeyveld, V., Swennen, K., Dornez, E., van de Wiele, T., Marzorati, M., Verstraete, W., Delaedt, Y., Onagbesan, O., Decuypere, E., Buyse, J., de Ketelaere, B., Broekaert, W.F., Delcour, J. a, Courtin, C.M., 2008. Structurally Different Wheat-Derived Arabinoxylooligosaccharides Have Different Prebiotic and Fermentation Properties in Rats. J Nutr 138, 2348–2355. https://doi.org/10.3945/jn.108.094367

Vázquez, M.J., Alonso, J.L., Domínguez, H., Parajó, J.C., 2000. Xylooligosaccharides: manufacture and applications. Trends Food Sci Technol 11, 387–393. https://doi.org/10.1016/S0924-2244(01)00031-0

Vinoth Kumar, R., Pugazhenthi, G., Pakshirajan, K., 2016. Petroleum Versus Biorefinery-Based Platform Chemicals. Platform Chemical Biorefinery: Future Green Chemistry 33–53. https://doi.org/10.1016/B978-0-12-802980-0.00003-1

Walker, A.W., Duncan, S.H., Carol McWilliam Leitch, E., Child, M.W., Flint, H.J., 2005. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol 71, 3692–3700. https://doi.org/10.1128/AEM.71.7.3692-3700.2005

Walzl, A., Unger, C., Kramer, N., Unterleuthner, D., Scherzer, M., Hengstschläger, M., Schwanzer-Pfeiffer, D., Dolznig, H., 2014. The resazurin reduction assay can distinguish cytotoxic from cytostatic compounds in spheroid screening assays. J Biomol Screen 19, 1047–1059. https://doi.org/10.1177/1087057114532352

Windey, K., de Preter, V., Verbeke, K., 2012. Relevance of protein fermentation to gut health. Mol Nutr Food Res 56, 184–196. https://doi.org/10.1002/mnfr.201100542

Xu, J., Liu, B., Wu, L., Hu, J., Hou, H., Yang, J., 2019. A waste-minimized biorefinery scenario for the hierarchical conversion of agricultural straw into prebiotic xylooligosaccharides, fermentable sugars and lithium-sulfur batteries. Ind Crops Prod 129, 269–280. https://doi.org/10.1016/J.INDCROP.2018.12.002

Yang, J., Tang, Q., Xu, L., Li, Z., Ma, Y., Yao, D., 2019. Combining of transcriptome and metabolome analyses for understanding the utilization and metabolic pathways of Xylo-oligosaccharide in *Bifidobacterium adolescentis* ATCC 15703. Food Sci Nutr 7, 3480–3493. https://doi.org/10.1002/fsn3.1194

Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G., 2016. Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. Renewable and Sustainable Energy Reviews 66, 751–774. https://doi.org/10.1016/j.rser.2016.08.038

Zhang, J., Siika-Aho, M., Tenkanen, M., Viikari, L., 2011. The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnol Biofuels 4. https://doi.org/10.1186/1754-6834-4-60

Zhang, K., Wang, N., Lu, L., Ma, X., 2020. Fermentation and Metabolism of Dietary Protein by Intestinal Microorganisms. Curr Protein Pept Sci 21, 807–811. https://doi.org/10.2174/1389203721666200212095902

SUPPLEMENTARY MATERIAL

Report of Enzymatic actives and bile acid concentration

Analyst: Wilian Fioreli Marcondes

Protocols: INFOGEST (Brodkorb et al., 2019)

Pepsin activity: 03/03/2022

Enzyme: Pepsin from porcine

Lot: SLCJ3493

Pepsin concentrations (µg/mL) = µg in assay	Test (A 280 nm)	Blank (A 280 nm)	Δ A Test (280 nm)	Activity (U/mg)
0.5	0.648	0.635	0.013	2600.0
1.5	0.68	0.639	0.041	2733.3
2	0.699	0.651	0.048	2400.0
2.5	0.702	0.642	0.060	2400.0
			Average	2533.3
			St dev	163.3
			% St der	6.4

Trypsin activity: 03/03/2022

Enzyme: Pancreatin from porcine pancreas

Lot: PCode 1003351804

Concentrations tested (mg/mL)	Volume of enzyme solution (mL)	0	Blank Slope	Enzyme Slope	Trypsin activity (U/mg)
0.400	0.100	0.04000	0	0.01541733	2.14130
0.667		0.06667		0.02611749	2.17646
0.200		0.02000		0.00633027	1.75841
				Average	2.025
				St dev	0.232
				% St dev	11.446

Bile acid concentration: 03/03/2022

Lot: BCBQ8807V

Stock [g/mL]	**0.01**					
Dilution rate	100	100	100			
		Fluorescence				
Blank	14254	15435	14685			
sample	18236	19593	19025			
Standart	67392	70253	75639			
[µM/mL]	162.015	164.1532	153.319	**Average**	**St der**	% St der
[mM/g]	16.2015	16.41532	15.3319	**16.20**	**0.574**	3.54