UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(MICROBIOLOGIA APLICADA)

PRODUCTION OF XYLO-OLIGOSACCHARIDES BY DIFFERENT TREATMENTS OF BANANA PSEUDOSTEM AND THEIR PREBIOTIC EVALUATION VIA PROBIOTIC BACTERIA GROWTH AND THEIR METABOLITES

CAROLINE DE FREITAS

Rio Claro – SP
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Orientador: Prof. Dr. Michel Brienzo

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RESUMO

Xilo-oligossacarídeos (XOS) são oligômeros derivados da xilana, uma hemicelulose encontrada em muitos tipos de plantas. Sua estrutura é composta por unidades de xilose ligadas por ligações β-1,4, tornando-os não digeríveis pelo sistema digestivo humano, mas fermentáveis por microrganismos probióticos presentes na flora intestinal. Os XOS podem ser produzidos por hidrólise ácida ou enzimática de biomassa proveniente de resíduos agroindustriais, como pseudocaule de banana, ou de xilana solubilizada desses resíduos. Nesse contexto, este estudo comparou a produção de XOS por esses diferentes métodos, analisou a estrutura da xilana após a hidrólise e a tolerância de microrganismos probióticos aos compostos utilizados e produzidos no processo de hidrólise ácida. Em seguida, o crescimento dos microrganismos foi avaliado na presença de diferentes concentrações de XOS e dos metabólitos produzidos por sua fermentação. Na hidrólise enzimática da biomassa, utilizando estratégias de deslignificação parcial e moagem, o rendimento de XOS aumentou em 47% em comparação com a biomassa que não passou por esses dois processos. Além disso, o peso molecular da xilana analisado após dois ciclos de hidrólise indica que o substrato complexo restante limitou a ação da enzima. A produção de XOS por hidrólise ácida diluída da biomassa do pseudocaule de banana também é uma alternativa, uma vez que a conversão foi boa e houve baixa produção de xilose e subprodutos de degradação do açúcar. O crescimento de bactérias probióticas foi influenciado por ácidos orgânicos, furfural e HMF em diferentes concentrações, também pelo pH resultante do meio. Quanto maior a concentração desses compostos, menor o crescimento de *Lactobacillus*; no entanto, as bifidobactérias utilizadas neste estudo foram mais resistentes. Concentrações de ácido acético, láctico e fórmico aumentaram, em alguns casos, após o crescimento das bifidobactérias, indicando que também produzem esses ácidos orgânicos. Geralmente, quanto maior a concentração de furfural e HMF, menor o crescimento. Para o *Lactobacillus*, concentrações de 10, 7,5 e 5 g/L de HMF e 10 g/L de furfural inibiram o crescimento. Todos os microrganismos testados cresceram quando cultivados usando XOS produzidos por hidrólise ácida como fonte de carbono. Para a maioria dos microrganismos, a concentração ótima de XOS para o crescimento foi de 20 g/L de fermentação. As concentrações de metabólitos produzidos variaram dependendo da fonte de carbono e do microrganismo utilizado; no entanto, o lactato e o acetato foram os mais produzidos.

Palavras-chave: xilooligossacarídeos, endoxilanase, ácidos orgânicos, subprodutos, probióticos, prebióticos

ABSTRACT

Xylooligosaccharides (XOS) are oligomers from xylan, a hemicellulose found in many types of plants. Their structure is composed of xylose units linked through β-1,4 bonds, which makes them non-digestible by the human digestive system but fermentable by probiotic microorganisms present in the gut flora. XOS can be produced by acid or enzymatic hydrolysis of biomass from agro-industrial residues, such as banana pseudostem, or from solubilized xylan from these residues. In this context, this study compared the production of XOS by these different methods, analyzed the xylan structure after hydrolysis, and the tolerance of probiotic microorganisms by compounds used and produced in the acid hydrolysis process. After that, the growth of the microorganisms was evaluated in the presence of different concentrations of XOS and the metabolites produced by its fermentation. In the enzymatic hydrolysis of biomass, using both strategies of partial delignification and milling, the XOS yield went up by 47% compared to the biomass that has not gone through these two processes. In addition, the xylan molecular weight analyzed after two hydrolysis cycles indicate that the remaining complex substrate limited enzyme action. The production of XOS by diluted acid hydrolysis of banana pseudostem biomass is also an alternative since the conversion was good and there was low production of xylose and sugar degradation by-products. Probiotic bacteria growth was influenced by organic acids, furfural, and HMF in different concentrations, also by the consequent pH of the medium. The higher the concentration of these compounds, the lower the *Lactobacillus* growth, however, the bifidobacteria used in this study were more resistant. Concentrations of acetic, lactic, and formic acid increase, in some cases, after bifidobacteria growth, indicating that they also produce these organic acids. Generally, the higher the concentration of furfural and HMF, lower the growth. For *Lactobacillus*, concentrations of 10, 7.5 and 5 g/L HMF and 10 g/L furfural inhibited growth. All tested microorganisms grew when cultured using XOS produced via acid hydrolysis as their carbon source. For most microorganisms, the optimal XOS concentration for growth was 20 g/L fermentation. Concentrations of metabolites produced varied depending on the carbon source and microorganism used, however, lactate and acetate were the most produced.

Keywords: xylooligosaccharides, endoxylanase, organic acids, by-products, probiotics, prebiotics

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CHAPTER 1: INTRODUCTION

Xylooligosaccharides (XOS), oligomers composed of xylose units linked by β-1,4-glycosidic bonds, are reported to have several biological activities. They can present antioxidant activity, anti-inflammatory and immunomodulatory effects, among other properties, depending on their degree of polymerization that can vary from 2 to 10 (YAN et al., 2022c). XOS present properties of interest for the food industry, such as low-calorie sweetness, no off-taste, non-cariogenic, and stability over a wide range of pH and temperature (FREITAS; CARMONA; BRIENZO, 2019). Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (GIBSON et al., 2017a). XOS can promote the proliferation of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria*, which are able to ferment this oligomer and produce beneficial metabolites that can be absorbed by the host and confer a health effect.

Prebiotics are not absorbed by the human digestive system, they remain intact until reaching the colon, where probiotic bacteria will ferment them (FERNÁNDEZ et al., 2016). The major metabolic products produced after prebiotic fermentation by probiotic bacteria are short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. These compounds are essential for colonic mucosal health and are unavailable from the diet. They are related to bowel function, calcium absorption, and lipid metabolism (YAN et al., 2022a). The ratio of SCFAs produced can vary with the fermenter microorganism and the fiber source. Acetate suppresses appetite and enhances lipogenesis, while propionate suppresses cholesterol synthesis, and both of them share anti-inflammatory action. Butyrate has a unique role as the chief energy source for the colonic mucosa. The consumption of prebiotics is also related to decreased protein fermentation, which could lead to the formation of potentially harmful metabolites (O’KEEFE, 2016).

XOS production can contribute to a sustainable generation of products with value-added, since this process occurs mainly using inexpensive lignocellulosic residue. It can be carried out by several methods, diluted acid and enzymatic hydrolysis are examples. Diluted acid hydrolysis has the advantage of using the biomass, without the previous need for xylan solubilization. However, it can release monosaccharides and sugar degradation by-products, which can interfere in the XOS yield. Enzymatic hydrolysis has to be carried out after xylan solubilization and with a purified enzyme (1,4-β-endoxylanase) to obtain a better XOS concentration, however, it’s a much cleaner process with no by-products and xylose release.

Because of the current high demand for functional foods, the interest in prebiotics is growing. XOS show great promise, as they can be produced from agricultural industry residues. The Global Info Research, in 2018, projected a growth of 94 million USD in 2017 to 130 million USD in 2025 of the international market of XOS (PALANIAPPAN; ANTONY; EMMAMBUX, 2021a). However, XOS are still more expensive to produce compared to other prebiotics. Therefore, studies on the most feasible way to produce them still need to be developed. Furthermore, since the metabolites produced can vary depending on the microorganism and XOS source, studies in this area are important to better evaluate this process.

The present study compared three types of XOS production: enzymatic hydrolysis using solubilized xylan and biomass, and acid hydrolysis using biomass. In addition, the molecular mass and structure of xylan after hydrolysis were evaluated, along with the effect of a second hydrolysis cycle with the addition of a new enzyme load. Subsequently, the XOS generated through two distinct processes (enzymatic hydrolysis of xylan and acid hydrolysis of biomass) were subjected to evaluation as a carbon source for probiotic bacteria. The study further examined the growth of probiotics in the presence of potential inhibitors produced during acid hydrolysis and analyzed the resulting metabolites produced after fermentation of enzymatically produced XOS.

CHAPTER 2: OBJECTIVES

The objective of this study was to produce XOS from enzymatic and acid hydrolysis, evaluating the effect of a second enzymatic hydrolysis cycle, analyzing the growth of probiotic bacteria using XOS produced by the different processes as carbon sources (including inhibitors compounds from treatment) and the metabolites produced after the fermentation.

2.1. Specific objectives

- Production of xylooligosaccharides by enzymatic hydrolysis of banana pseudostem biomass, evaluating different enzyme combinations.
- Production of xylooligosaccharides by enzymatic hydrolysis of xylan from banana pseudostem, evaluating the efficiency of adding new enzyme load and the influence of the reaction volume on XOS concentration, verifying xylan molecular structure change over the hydrolysis.
- Production of xylooligosaccharides by dilute acid hydrolysis, evaluating the formation of xylose and sugar degradation by-products (furfural and hydroxymethylfurfural).
- Evaluation of the inhibitory effect of dilute acid hydrolysis by-products, such as acetic, lactic, and formic acids; furfural and hydroxymethylfurfural on the growth of probiotic bacteria.
- Evaluation of probiotic bacteria growth using XOS produced from enzymatic hydrolysis and dilute acid hydrolysis as carbon source, compared to glucose, xylose, and fructooligosaccharides.
- Evaluation of metabolites produced by probiotic bacteria after fermenting XOS produced from enzymatic hydrolysis and dilute acid hydrolysis as carbon source, compared to glucose, xylose and fructo-oligosaccharides.

2.2. Thesis format and organization

This study was organized into chapters “for the qualification exam”, which were designed considering independent publications such as book chapters and papers. The important sections of the thesis were separated into chapters, for example, the bibliographic review was compiled in a book chapter. The results were organized into two papers, respecting the scientific journal structure.

The organization was as follows: Chapter 1 - General introduction and novelty statement; Chapter 2 - Objectives; Chapter 3: Bibliographic review - Oligosaccharides from lignocellulosic biomass and their biological and physicochemical properties, that correspond to a book chapter already published in the Hemicellulose Biorefinery: A Sustainable Solution for Value Addition to Bio-Based Products and Bioenergy, published in Springer; Chapter 4: Enzymatic hydrolysis applied to banana pseudostem biomass compared to solubilized xylan for xylooligosaccharides production with high substrate concentration, that correspond to a scientific paper published at BioEnergy Research; Chapter 5: Production of xylooligosaccharides by dilute acid hydrolysis and the effect of inhibitors on probiotic growth, a scientific paper under elaboration; Chapter 6: Short-chain fatty acid production from the fermentation of xylan oligosaccharides by probiotic bacteria, a scientific paper under elaboration; Chapter 7: Conclusion

2.3. Thesis rational research line

Chapter 3 presents a literature review about oligosaccharides produced from lignocellulosic biomass, especially XOS, their physicochemical and biological properties and production methods.

Chapter 4 is composed of a published scientific paper presenting enzymatic hydrolysis of banana pseudostem biomass using different combinations of enzyme (xylanase and cellulase) to produce XOS; enzymatic hydrolysis of banana pseudostem xylan, evaluating the influence of reaction volume on the concentration of XOS produce, besides the addition of a new enzyme load after 24h. In addition, the xylan structure was evaluated after hydrolysis of different substrate concentrations.

Chapter 5 presents a scientific paper that shows the growth of probiotic bacteria in the presence of inhibitors that can be produced by dilute acid hydrolysis. The concentration of inhibitors after growth, production of XOS and sugar degradation by-products after dilute acid hydrolysis of banana pseudostem; and growth of probiotic bacteria in media containing the XOS produced by acid hydrolysis.

Chapter 6 presents a scientific paper that evaluates the growth of probiotic bacteria using different types of carbon sources (glucose, xylose, XOS and FOS) in different concentrations. After growth, the organic acids produced by the microorganisms were quantified.

CHAPTER 3: BIBLIOGRAPHY REVIEW - OLIGOSACCHARIDES FROM LIGNOCELLULOSIC BIOMASS AND THEIR BIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES

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Abstract

Hemicellulose present in lignocellulosic biomass can be converted into higher value-added products such as xylooligosaccharides and mannan oligosaccharides. These oligosaccharides have physicochemical and biological properties of great interest to several industry sectors, which has made them emerging molecules. They can be produced through chemical or enzymatic hydrolysis, focusing on a high efficiency and income to fulfill market needs. The most well-known characteristic of these oligosaccharides is their action as prebiotics, since they are not digested by the human digestive system and can be used by the intestinal flora, however they present several other health benefits and technological uses. Nowadays, besides of industrial production, research on novel production strategies is increasing, since these oligosaccharides have several advantageous properties and can be produced from a low-cost substrate.

Keywords: lignocellulose; hemicellulose; oligosaccharides; xylooligosaccharides; prebiotics

1. Introduction

The concept of biorefining is aligned with the search for new products through sustainable processes. The lignocellulosic biomass, being composed of three main classes of polymers (cellulose, hemicellulose and lignin), can be converted into a wide spectrum of compounds (AJAO et al., 2018). The commercial potential of hemicellulose is large, however it can be improved with the evolution of production processes. For this reason, many researches are being carried out in recent years with the objective of exploring hemicellulose from biomass for use in various industries, such as fuel, food, pharmaceutical and cosmetics (AHMAD; ZAKARIA, 2019; NAIDU; HLANGOTHI; JOHN, 2018a).

Due to increased public awareness of the role of food in promoting human health, consumers have preferred foods with additional attributes beyond their nutritional properties. Thus, scientific research has also begun to investigate the biological activity of food in human health. In the context of functional foods, biocomponents added to foods as supplements, prebiotics are promising ones. Many fermentable carbohydrates have been reported to have prebiotic effects, but the most widely documented prebiotics to cause human health benefits are non-digestible oligosaccharides (SINGH; BANERJEE; ARORA, 2015).

Xylooligosaccharides (XOS), mannan oligosaccharides (MOS) and arabinooligosaccharides (AOS) are non-digestible oligosaccharides (NDOs) that can be obtained by hemicellulose bioconversion through chemical and/or biological processes, including pretreatments and fermentation (MUSSATTO; MANCILHA, 2007). These oligosaccharides are water-soluble, usually composed of 3-10 monosaccharides and considered as food components with prebiotic properties, due to its selective potential to stimulate the growth of beneficial microorganisms in the intestinal tract (SOPHONPUTTANAPHOCA et al., 2018).

XOS and MOS are compounds that have β-linkages in their structure, which are resistant to hydrolysis by human digestive enzymes. Therefore, they reach the intestinal tract intact and can be used by the intestinal flora. XOS and MOS fermentation by gut bacteria produces short chain fatty acid (SCFA) such as acetate, propionate and butyrate that are related to the improvement of several intestinal functions, such as absorption of minerals and regulation of lipid and glucose metabolism (SINGH; BANERJEE; ARORA, 2015). In addition, the growth of beneficial bacteria is favoured by the consumption of prebiotics, making it difficult to pathogenic bacteria to establish, which in turn helps to prevent infections and allergies (FARIAS et al., 2019).

A prebiotic can be defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”. Compounds that affect the composition of microbiota through mechanisms that do not involve their selective use by host microorganisms for their growth, such as antibiotics, vitamins and minerals, are not considered prebiotics. The health effects of prebiotics are evolving, but currently include benefits for the gastrointestinal tract, cardiometabolism, mental health and bone (GIBSON et al., 2017b). Many fermentable carbohydrates have been reported to have prebiotic effects, but the most widely documented prebiotics to cause human health benefits are non-digestible oligosaccharides (RASTALL; GIBSON, 2015).

Prebiotics have been increasingly used by the food industry as functional ingredients in various preparations such as in the production of dairy products, confectionery, infant formulas, cereal bars, chocolate, and meat products, among others. The use of hemicellulose from lignocellulosic biomass as a source of raw material for the production of these compounds is a viable and sustainable alternative (FARIAS et al., 2019).

This chapter focused in to present the oligosaccharides chemical structure, physicochemical and biological properties, and production strategies considering chemistry and enzymatic. Application of oligosaccharides were related to the industrial production and future perspectives

2. Chemical Structure and Composition

Oligosaccharides are a group of polymeric carbohydrates that can be found in free or combined forms. They are several types of oligosaccharides, differing from each other in their nature of monomeric sugars and named accordingly. Oligosaccharides are defined by IUB-IUPAC nomenclature as oligomers composed of 2 to 10 monosaccharides. Hemicellulose have a random combination of monosaccharides, such as D-glucose, D-mannose, D-xylose and L-arabinose, therefore there are three main types of oligosaccharides that can be derived from hemicellulose: xylooligosaccharides (XOS), mannan oligosaccharides (MOS) and arabinooligosaccharides (AOS) (BELORKAR; GUPTA, 2016; MUSSATTO; MANCILHA, 2007).

XOS, oligosaccharides derived from xylan, are oligosaccharides composed of xylose chains linked by glycosidic β-1,4 bonds (Fig 1). Their degree of polymerization can vary from 2 to 10, depending on the number of xylose residues, and are known as xylobiose, xylotriose, xylotetraose, xylopentaose and so on. Besides xylose residues, xylan is commonly found in combination with other side groups such as arabinofuranosyl, glucopyranosyl uronic acid or acetyl groups, resulting in branched XOS with diverse properties and stability (CARVALHO et al., 2013; GUPTA, 2012; IBRAHIM, 2018; MEYER et al., 2015).

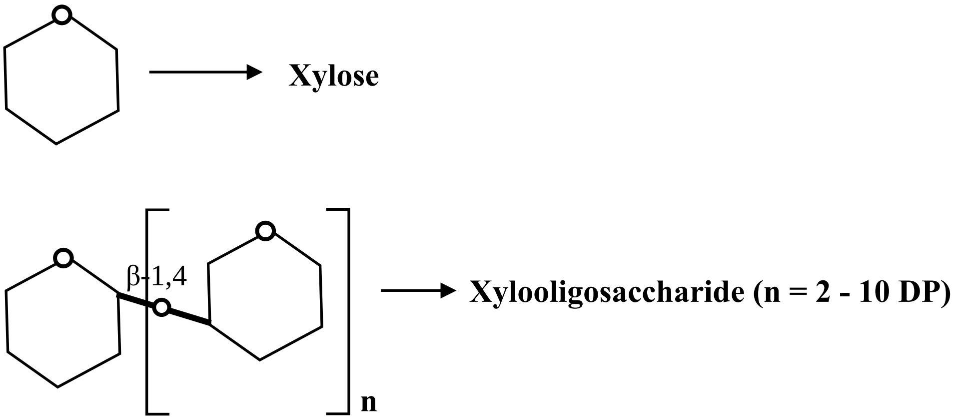


Fig 1. Xylooligosaccharide structure

Source: Author

Arabinooligosaccharides (AOS) are another class of oligosaccharides whose natural sources are arabinans present in plants cell wall components such as hemicellulose. They are branched pectic oligosaccharide comprised of α-1,5 linked backbone of L-arabinose residues, which can be substituted with α-1,2 and/or α-1,3 linked L-arabinose residues, the type of linkage depending on the source (Fig 2). Also, from this group are the arabinoxylooligosaccharides (AXOS), isolated from xylan chains with a substitution of arabinose side chains (BELORKAR; GUPTA, 2016; WESTPHAL et al., 2010).

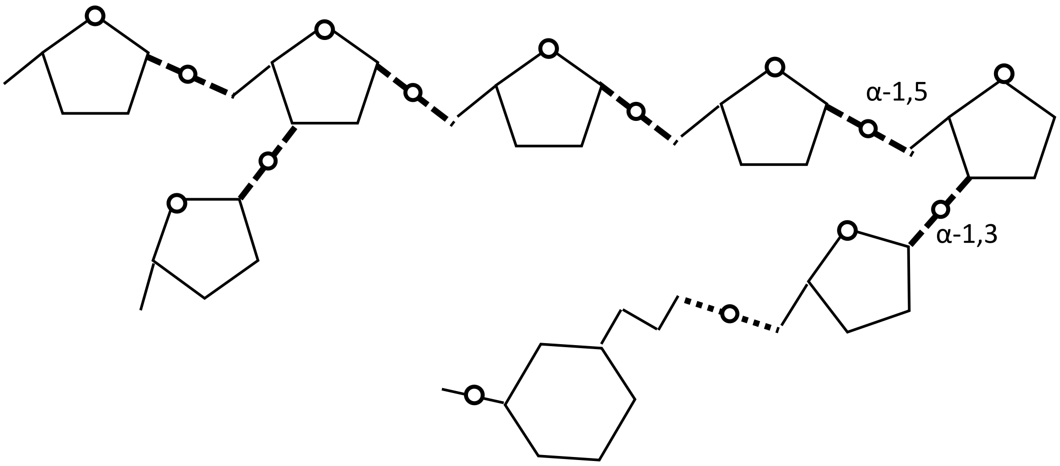


Fig 2. Arabinooligosaccharide structure

Source: Author

Mannan is a major constituent of hemicellulose from softwood. Mannan oligosaccharides can present a linear form, consisting of a C6 sugar, mannose, backbone linked by β-1,4 glycosidic bonds, or it can present side chains of D-glucose or D-galactose linked to the main chain by α-1,6 linkages (Fig 3), characterizing glucomannan oligosaccharides and galactomanannoligosaccharides, respectively (BHATIA et al., 2019; SINGH; SINGH; ARYA, 2018).

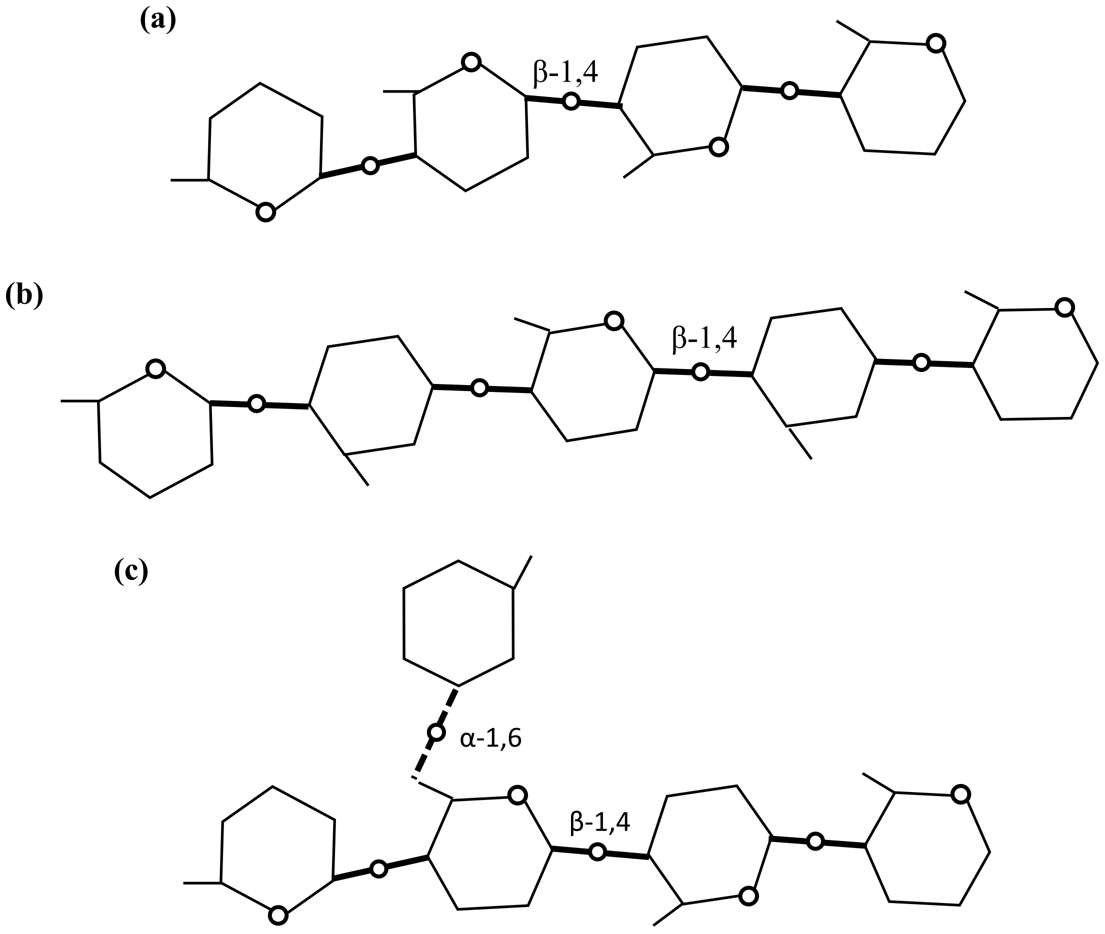


Fig 3. Mannan oligosaccharides structure. (a) linear mannanooligosaccharide; (b) glucomannanooligosaccharide; (c) galactomannanooligosaccharide

Source: Author

3. Properties of oligosaccharides

The physicochemical and physiological properties of each oligosaccharide can differ depending on the sugar residues present in the main chain, anomeric configuration, side groups and linkage types. Usually, polymer backbone of hexoses are more strongly bound than that of pentoses. Also, β-linkages are stronger than α-linkages, that often generate the branches (GULLÓN et al., 2014; OTIENO; AHRING, 2012a).

3.1. Physicochemical properties

Oligosaccharides are water soluble and usually about 0.3 to 0.6 times as sweet as sucrose. Sweetness depends on the chemical structure and degree of polymerization, usually it decreases with longer oligosaccharide chains. Relative sweetness of XOS is 30% when compared to sucrose, while MOS and AOS can present 60% sucrose sweetness and none of them have an off-taste. This attribute is highly exploited in food formulations as a replacement of sucrose (HIRAYAMA, 2002; SAMANTA et al., 2015).

In some food production, oligosaccharides relatively low sweetness is desirable when a bulking agent is needed to enhance other food flavors. In the formulation of very sweet foods, for example, they can be used together with artificial sweeteners to mask the aftertastes produced by some of them. Besides, their higher molecular weight increases viscosity comparing with monosaccharides, which leads to an improved mouthfeel. They also can be used as humectants because of their high moisture-retaining capacity, which reduces water activity, convenient for microbial contamination control (MUSSATTO; MANCILHA, 2007).

Oligosaccharides are stable over a wide range of pH (2.0 to 8.0) and temperatures (up to 135 ºC), which is a good feature for incorporations into foods, however higher pH and temperature then those can impair their physicochemical properties and nutritional values. The stability in acid conditions, most likely to be found in the gastrointestinal tract, is also favorable for its use as prebiotics. Melting temperature was reported a 134 ºC for XOS, 132 ºC for MOS and 164 ºC for AOS (BELORKAR; GUPTA, 2016; CHACHER et al., 2017; GULLON et al., 2017; GUPTA, 2012; HU et al., 2016; SAMANTA et al., 2015; SAVILLE; SAVILLE, 2018).

Table 1. Physicochemical properties of oligosaccharides (xylooligosaccharides, mannan oligosaccharides and arabinooligosaccharides) derived from hemicellulose.

Property	XOS	MOS	AOS
Molecular formula	$C_{5n}H_{8n+2}O_{4n+1}$; n = 2 to 10	$C_{6n}H_{10n+2}O_{5n+1}$; n = 2 to 10	$C_{5n}H_{8n+2}O_{4n+1}$; n = 2 to 10
Molecular weight (g/mol)	282 to 1338	342 to 1638	282 to 1338
Relative sweetness	30% of sucrose	60% of sucrose	60% of sucrose
pH stability	2.5 to 8.0	2.0 to 7.0	2.0 to 7.0
Temperature stability	up to 135 ºC	up to 120 ºC	up to 135 ºC
Melting temperature	134 ºC	132 ºC	164 ºC
Energy value (kcal/g)	1.5	3.75	1.5

Oligosaccharides can alter the freezing temperature of frozen foods. The antifreezing activity of xylobiose on water at temperatures higher than –10 ºC is greater than that of glucose, sucrose and maltose. They are also able to control the intensity of Maillard reactions in heat-processed foods. The caloric value of oligosaccharides can usually vary from 1.5 to 2.0 kcal/g and are 40-50% lower than sucrose, which allows their use in anti-obesity diets, in exception mannan oligosaccharides present a similar caloric value to sucrose. In addition, oligosaccharides present an acceptable odor and are non-cariogenic (HU et al., 2016; PATEL; GOYAL, 2011; SAVILLE; SAVILLE, 2018).

3.2. Biological/Physiological properties

XOS and MOS are known as non-digestible oligosaccharides (NDO), which means that they cannot be digested by humans since the human body lacks the enzymes required to hydrolyze the β-linkages present in these molecules. Because of this property, these oligosaccharides can be considered potential prebiotics, as they reach the gastrointestinal tract without being degraded and are available for the intestinal flora (BHATIA et al., 2019; SWENNEN; COURTIN; DELCOUR, 2006).

For the metabolism of NDOs, bacterias present in the intestinal tract have to produce glycolytic enzymes to hydrolyze them into mono or disaccharides. These smaller molecules can be transported into the cell where they will be further metabolized to short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate. The acidification of the colon environment is beneficial for the development of probiotic bacteria such as bifidobacteria and lactobacilli (FARIAS et al., 2019).

XOS, MOS and AOS have similar effects to dietary fiber due to its indigestible quality, and thus can prevent constipation. The SCFAs produced by the fermentation of these oligosaccharides by the colonic bacteria are efficiently absorbed and used by the human epithelial cells, stimulating their growth and water absorption, which consequently improves the intestinal mobility. In addition, the high concentration of SCFAs lowers the colon pH enhancing bioavailability of minerals, mainly calcium and magnesium. Besides, the increase in water absorption increases the solubility of some minerals (MORENO et al., 2017; MUSSATTO; MANCILHA, 2007; SINGH; BANERJEE; ARORA, 2015).

Non-digestible oligosaccharides may also have an effect on lipid regulation, since acetate and propionate influence cholesterol production, decreasing lipid and cholesterol blood levels. They also can influence on glycemia by altering the absorption of macronutrient by delaying gastric emptying, thus prevent peak levels of blood glucose. Plasmatic glycemia is also affected by the bacterial production of SCFAs (AL-SHERAJI et al., 2013).

NDOs can affect the mucosal immune system of the intestine, but their beneficial effects are not only confined to the intestinal tract. They can modulate both innate and adaptive immune mechanisms by incrementing the number of *Bifidobacteria*, which results in production of anti-inflammatory metabolites and induces phagocytoses, protecting the organism against infections (ALTHUBIANI et al., 2018).

The use of NDOs in the animal feed industry, mainly for livestock, is increasing. Many factors can cause stress to the animals and compromise its normal microbiota, causing susceptibility to enteric diseases. Addition of antibiotics in feed often causes side effects and sometimes are even legally banned. However, some oligosaccharides, specialty MOS, can prevent colonization of pathogenic bacteria more than stimulate beneficial microorganisms, therefore they can be a promising alternative for the use of antibiotics. In animals, NDOs are also able to enhance growth and feed conversion, as well as prevent early mortality (CARVALHO et al., 2013; SWENNEN; COURTIN; DELCOUR, 2006).

Several human and animal studies have been carried out to identify the potential health benefits of these oligosaccharides and to assess their possible intolerance and safety for consumption. For humans, studies have shown that the best daily intake for oligosaccharides is between 2-5 g. In animal models, the administration of a dose ranging between 7-10% of the diet did not showed any adverse effects (AL-SHERAJI et al., 2013; SAMANTA et al., 2015).

In summary, the consumption of NDOs such as XOS, MOS and AOS, in the appropriate dosage, results in improvement of human and animal health, controlling the gut microbiota balance through the stimulation of beneficial bacteria growth, which also increases the production of short-chain fatty acids. Thus, this results in improvement of intestinal membrane integrity and minerals absorption, as well as lower glycemic levels and improved immunity (FARIAS et al., 2019).

4. Production of prebiotic oligosaccharides

Xylooligosaccharides, mannan oligosaccharides and arabinooligosaccharides are obtained by the hydrolysis of a natural polysaccharide, hemicellulose. However, due to the lignocellulosic biomass recalcitrance, a pre-treatment for hemicellulose solubilization and its separation from the other structural components, cellulose and lignin, is required. To isolate hemicellulose, physical, thermal or chemical methods can be used but despite of the method, the final purpose is to obtain a hemicellulose with desirable conditions for oligosaccharides production (Freitas et al. 2019; Kruschitz and Nidetzky 2020).

4.1. Chemical hydrolysis

Chemical pretreatments are widely used to obtain high sugar yields at low cost (LOOW et al., 2016). It is possible to produce oligosaccharides of interest to the food, pharmaceutical, and chemical industries. This interest is due to their numerous health benefits, from lignocellulosic materials by methods such as acid hydrolysis, autohydrolysis, and alkaline extraction of polysaccharide followed by acidic or enzymatic hydrolysis (Fig 4) (BRIENZO et al., 2016a). In chemical pretreatments in addition to the oligosaccharides of interest, unwanted and toxic substances such as monosaccharides and sugar degradation products can be produced, requiring a purification process (IBRAHIM, 2018; KHAT-UDOMKIRI et al., 2018; WANG et al., 2018a).

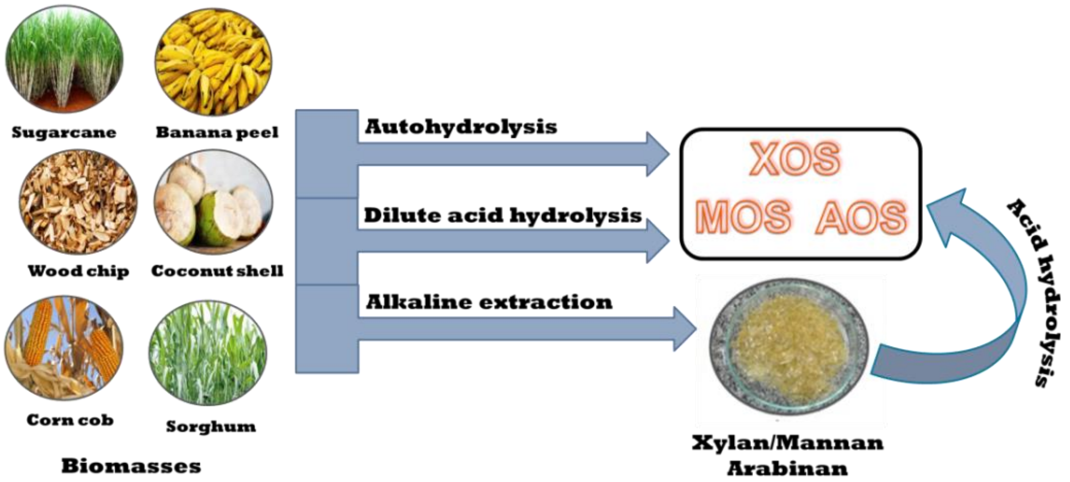


Fig 4. Chemical pretreatments of biomass for the production of xylooligosaccharides (XOS), mannooligosaccharides (MOS), and arabinooligosaccharides (AOS).

Source: Author

4.1.1. *Dilute Acid Hydrolysis*

Dilute acid hydrolysis (Fig 5) has the potential for large-scale oligomer production and is commonly used for biomass fractionation because it is efficient, cheaper, and faster than other methods. In addition, it can recover approximately 80 % of sugars from hemicellulose. Due to its glycosidic bonds and shorter chain, hemicellulose is the first hydrolyzed component of biomass. During hemicellulose breakdown, oligosaccharides are released and solubilized in the medium, which composition can vary according to the used biomass. Raw materials rich in xylan, such as sugarcane bagasse and corncob, are used for the production of xylooligosaccharides (XOS); biomasses that have a greater amount of glucomannans or galactomannans, such as coffee grounds, release mannooligosaccharides (MOS) and materials containing arabinan, such as rice straw are hydrolyzed to obtain arabinooligosaccharides (AOS). The characteristics and degree of polymerization of the oligosaccharides formed during this process cannot be predicted, as they depend on the type of substrate used, conditions and severity of the reaction (LIU et al., 2019; NOPVICHAI et al., 2019; QING et al., 2013; YU, 2007).

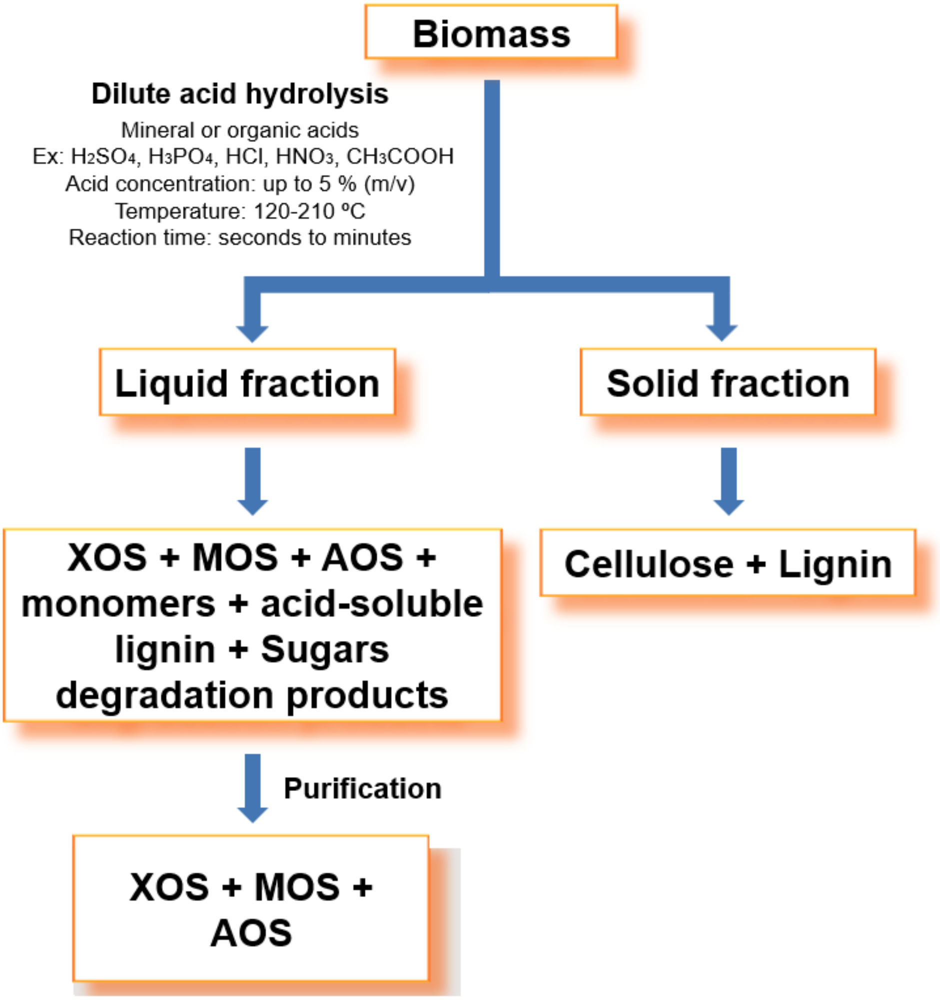


Fig 5. Schematic representation of lignocellulosic biomass acid hydrolysis for oligosaccharides production

Source: Author

In acid hydrolysis, monomers such as xylose, glucose, arabinose, mannose, and galactose are also released, as well as the acetyl and uronic groups of the hemicellulose chain (WILLFÖR et al., 2009). Cellulose undergoes few modifications under mild conditions due to its tight structure, high crystallinity, and degree of polymerization. After hydrolysis with dilute acid, cellulose is more accessible to cellulolytic enzymes, facilitating a further process of enzymatic hydrolysis (SHIMIZU et al., 2020a). Lignin, like cellulose, remains in the solid phase almost intact, only acid-soluble lignin, corresponding to approximately 5 to 10 % of the total lignin, is solubilized (BRIENZO et al., 2017; MUSSATTO, 2016; ORIEZ; PEYDECASTAING; PONTALIER, 2019; SCHMATZ et al., 2020; ŚWIĄTEK et al., 2020).

Indeed, a recent study by Marcondes et al. (2020) showed that hemicellulose-derived sugars yields is higher if the acid pretreatment is carried out at two stages. The first stage is performed with the objective of extracting the fast hemicellulose, while the second stage is performed to extract the slow hemicellulose. Thereby, the authors evaluated the acid hydrolysis of sugarcane bagasse at different conditions to maximize the solubilization and recovery of the hemicellulose in sugarcane bagasse as XOS with high selectivity. However, the results showed that the first stage yielded a XOS-rich, high purity hydrolysate and in the second stage only a xylose-rich hydrolysate could be obtained at high purity. The authors conclude that one strategy to reduce the high xylose production would be to identify the most suitable pretreatment for the target biomass with the aim to improve the XOS yield.

Diluted acid is preferred over concentrated acid for the production of XOS, MOS, and AOS because, although the rate of breakdown of the polysaccharide chain is lower and slower, there is a greater formation of oligosaccharides. Therefore, less amount of monosaccharides and undesirable products are formed. Moreover, diluted acids are generally less corrosive and toxic and, consequently, operating costs are lower. Another disadvantage of concentrated acid is the difficulty of recycling it after use and the risks involved (BARUAH et al., 2018; OTIENO; AHRING, 2012b).

In more severe processes, hemicellulose or cellulose can be degraded, with the formation of degradation products such as furfural, hydroxymethylfurfural, aldehydes, and phenolic acids that can be separated by osmotic filtration and used in industry. For example, furfural is used in the production of plastics, nylon, and lubricants. Levulinic acid can be used in solvents, polymers, pesticides, sedatives, and anxiolytics (JIANG et al., 2016; ZABOT et al., 2019). Fig 6 shows the products formed during the acid-catalyzed hydrolysis of xylan.

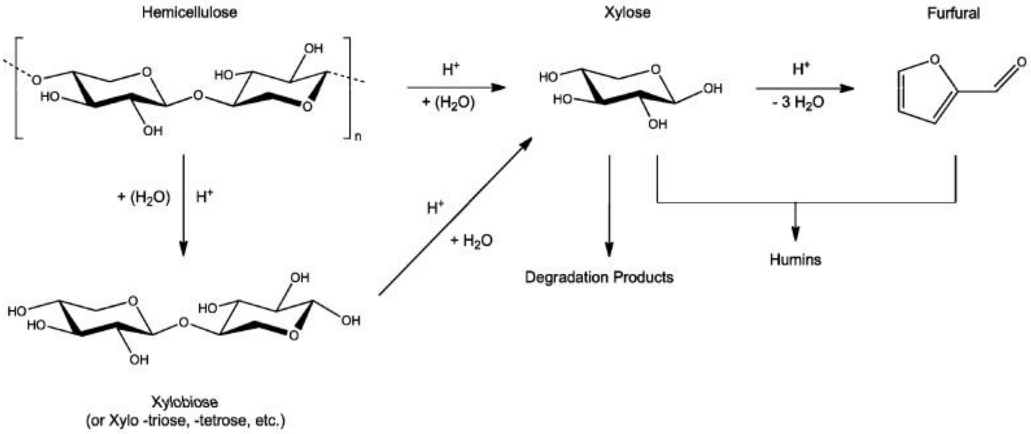


Fig 6. Production of xylooligosaccharides by xylan acid hydrolysis and formation of degradation products such as furfural and humins.
Source: Enslow and Bell (2012)

In pretreatment with diluted acid, the acid usually at a concentration below 5 % (m/v) is added to the biomass and the mixture is heated between 120 and 210 ºC, with pressure up to 10 atm and time-varying from seconds to minutes (Fig 5). The sulfuric acid (H_2SO_4) is generally used in this type of pretreatment because it hydrolyzes biomass more efficiently. It is possible to recover high concentrations of hemicellulosic sugars with low concentrations of acid, reducing process costs (LOOW et al., 2016).

Beyond sulfuric acid other mineral acids like hydrochloric (HCl), nitric (HNO_3) or phosphoric acid (H_3PO_4) are also studied. In comparison with sulfuric acid, it is possible to state that hydrochloric acid is more expensive and less efficient, its more corrosive and impacts the environment, factors that limit its use (ORIEZ; PEYDECASTAING; PONTALIER, 2019). Nitric acid has the advantage of being less corrosive to stainless steel but is more expensive and some studies suggest that under optimized conditions there was less conversion of hemicellulose into sugars (YANG et al., 2015). Regarding phosphoric acid, there are not many studies about the fractionation of biomass, but its use for this purpose seems to be promising because of its ability to efficiently hydrolyze hemicellulose, besides being a safer and more beneficial alternative to the environment, however it’s the most expensive one (DE VASCONCELOS et al., 2013; FONTANA; TIBONI; KOOP, 2017; LOOW et al., 2016; ORIEZ; PEYDECASTAING; PONTALIER, 2019).

In addition to mineral acids, another alternative for the production of oligosaccharides are organic acids such as acetic, gluconic, oxalic, maleic, and citric, among others, which are advantageous due to the higher formation of oligosaccharides, lower concentration of xylose, and degradation products such as furfural (LIN et al., 2017; ZHOU; XU, 2019).

4.1.2. Autohydrolysis

The main purpose of autohydrolysis (Fig 7) is to solubilize hemicellulose, decrease the formation of unwanted products and increase the accessibility of cellulose to cellulolytic enzymes. In Autohydrolysis, also called liquid hot water (LHW) the biomass is hydrolyzed only using water under high temperature and pressure, between 150 and 230 ºC and approximately 50 bar, respectively. Reaction time can vary between minutes and hours, and usually occurs in reactors, however recently microwave and steam explosion systems are being used for heating (Fig 8) (BARUAH et al., 2018; JEONG; LEE, 2015; KAPOOR et al., 2018; WANG et al., 2019).

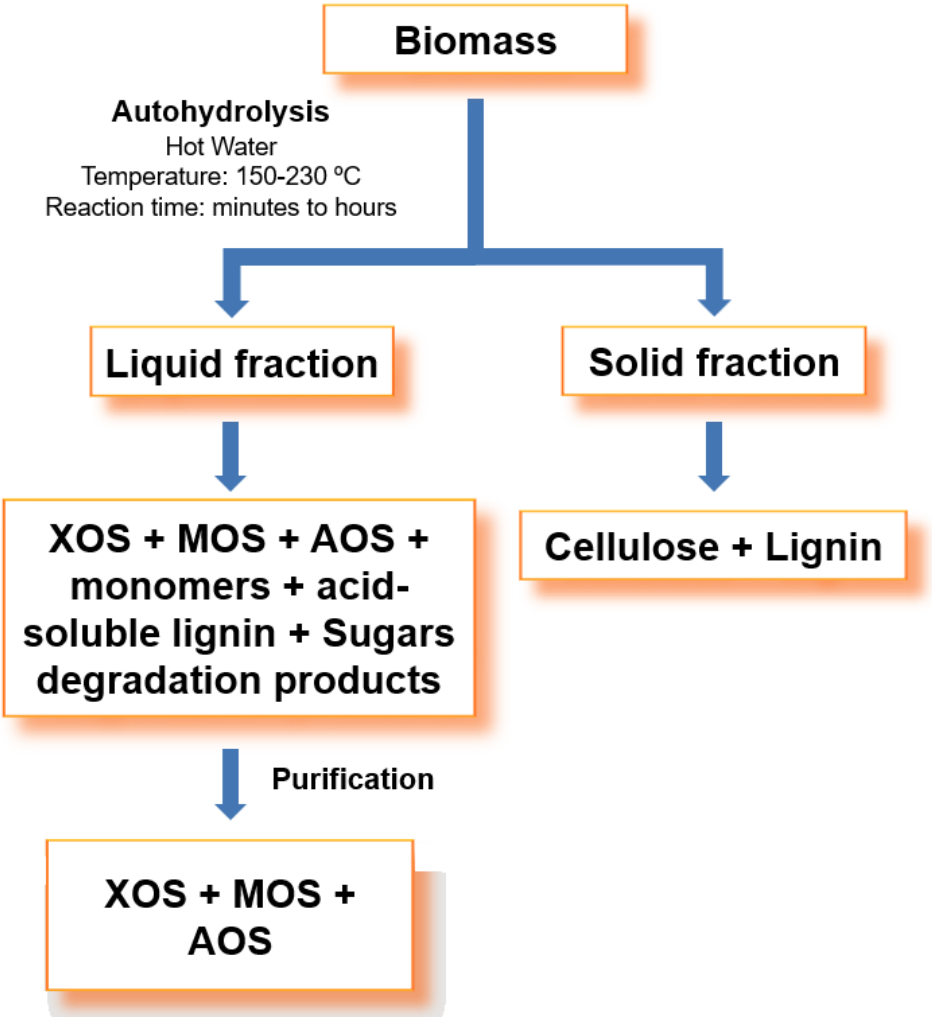


Fig 7. Schematic representation of lignocellulose biomass autohydrolysis for the production of oligosaccharides

Source: Author

The high temperature promotes hydrolysis of acetyl and uronic groups present in the xylan releasing acetic and uronic acid in the medium, that act as catalysts by increasing xylan hydrolysis rate (CANO et al., 2020; SINGH; KALIA, 2017). The reaction (Fig 9) is similar to what occurs in hydrolysis with dilute acid.

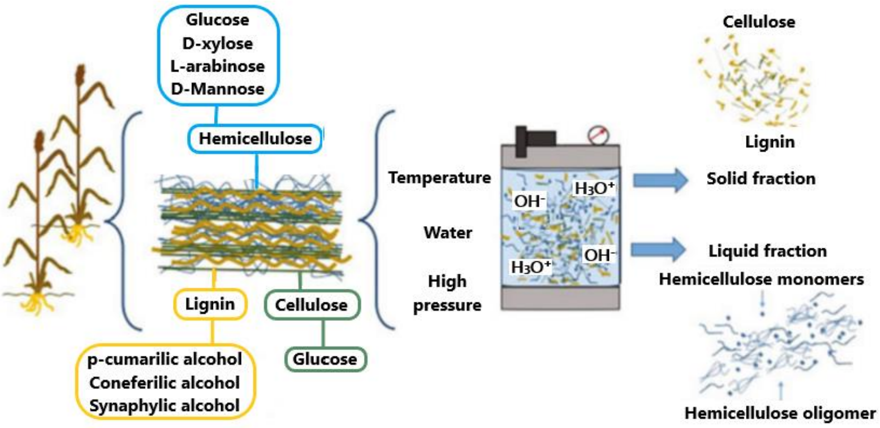
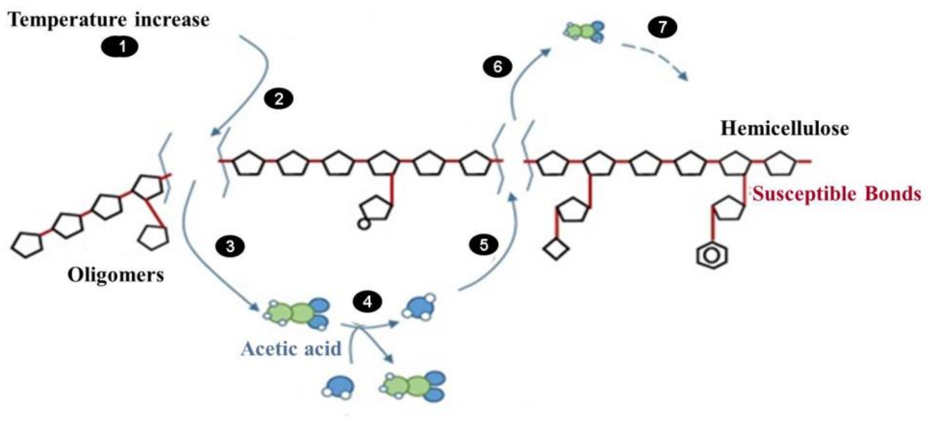


Fig 8. Autohydrolysis of lignocellulosic materials

Source: Kapoor et al. (2018)

Hemicellulose is more susceptible to hydrolysis in this method due to its amorphous structure. In mild temperatures and short periods, hemicelluloses rich in xylan, mannan, and arabinan are hydrolyzed into high molecular weight oligosaccharides that are soluble in the liquid fraction along with other hemicellulose sugars, such as xylose and arabinose. At higher temperatures, oligosaccharides are hydrolyzed to monosaccharides and sugar degradation products. In the temperature range usually used in autohydrolysis, there are few changes in cellulose and lignin structure. They remain in the solid phase, which can be recovered at the end of the process and hydrolyzed by enzymes. Above 230 ºC the breakdown of cellulose chain into oligosaccharides and glucose is intensified and at 300 ºC the conversion to glucose is almost complete (GULLÓN et al., 2014; KAPOOR et al., 2018; PIELHOP et al., 2015; RUIZ et al., 2013; SARIP et al., 2016; VALLEJOS et al., 2015). After the reaction, the hydrolysate is separated from the solid fraction and purified by removing the by-products to obtain high purity oligosaccharides (CHEN, 2015).



1-Temperature increase
2-Hydrolysis reactions
3-Breaking the ether bonds and formation of oligomers and acetic acid
4-Ionization of acetic acid
5-Degradation of polysaccharide catalyzed by hydronium ion
6-Breaking other ether bonds and formation of different oligomers and acetic acid
7-Repetition of stages 4, 5 and 6

Fig 9. Hemicellulose chain breakdown in autohydrolysis
Source: Adapted from Amiri and Karimi 2018).

It is possible to obtain high yields of hemicellulose sugar oligomers, mainly from agricultural residues and herbaceous. Regarding woods, hardwoods are more susceptible to autohydrolysis than softwoods because they have more acetyl groups in the hemicellulose chain, delignification is more efficient and there is less condensation of lignin. For the hydrothermal processes of softwoods, more severe conditions are necessary, due to the greater amount of lignin (XIAO; SONG; SUN, 2017).

This pretreatment is advantageous since it does not require the use of chemical, corrosive, and toxic reagents, preventing equipment corrosion and the need for chemical recycling. Only water is used, an environmentally friendly and low cost solvent, reducing the need to use neutralizing chemicals. The costs of this pretreatment are lower compared to others. However, high temperature and pressure are conditions that are difficult to control and that can cause thermal degradation of hemicellulose (Fig 10). This process, called pyrolysis, generates compounds that are not of interest such as furfural, derived from pentoses, uronic acids, and hydroxymethylfurfural (HMF) derived from hexoses, acetic, formic, levulinic acid, and lignin degradation products (CHEN, 2015; PIELHOP et al., 2015; TYAGI et al., 2019; VALLEJOS et al., 2015; WANG et al., 2019).

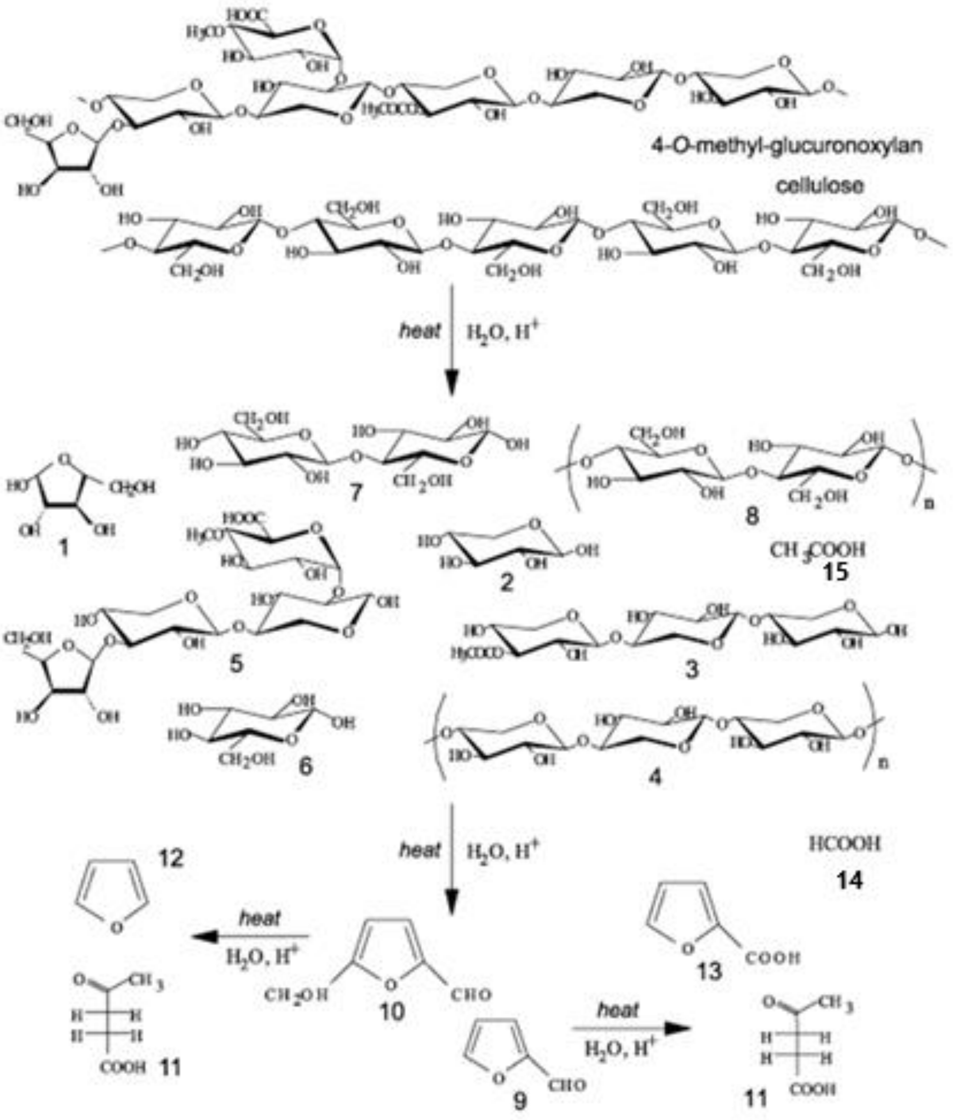


Fig 10. Products formed in the hydrothermal processes of 4-O-methylglucuronoxylan and cellulose: (1) arabinose; (2) xylose; (3) acetylated xylooligomers (DP of 3); (4) xylooligomers of higher molecular mass; (5) acidic, branched oligosaccharides; (6) glucose; (7) cellobiose; (8) cellooligomers; (9) furfural; (10) hydroxymethylfurfural; (11) levulinic acid; (12) furan; (13) 2-furoic acid (pyromucic acid); (14) formic acid; (15) acetic acid

Source: Adapted from Pereira Ramos 2003).

4.1.3. Alkaline Solubilization

Alkaline solubilization (Fig 11) is a method to separate hemicellulose from the other lignocellulose components and used it for the production of oligosaccharides. First, the polysaccharide is solubilized with alkaline reagents and in the second step, it is hydrolyzed with dilute acid or enzymes (Loow et al. 2016; Figueiredo et al. 2017).

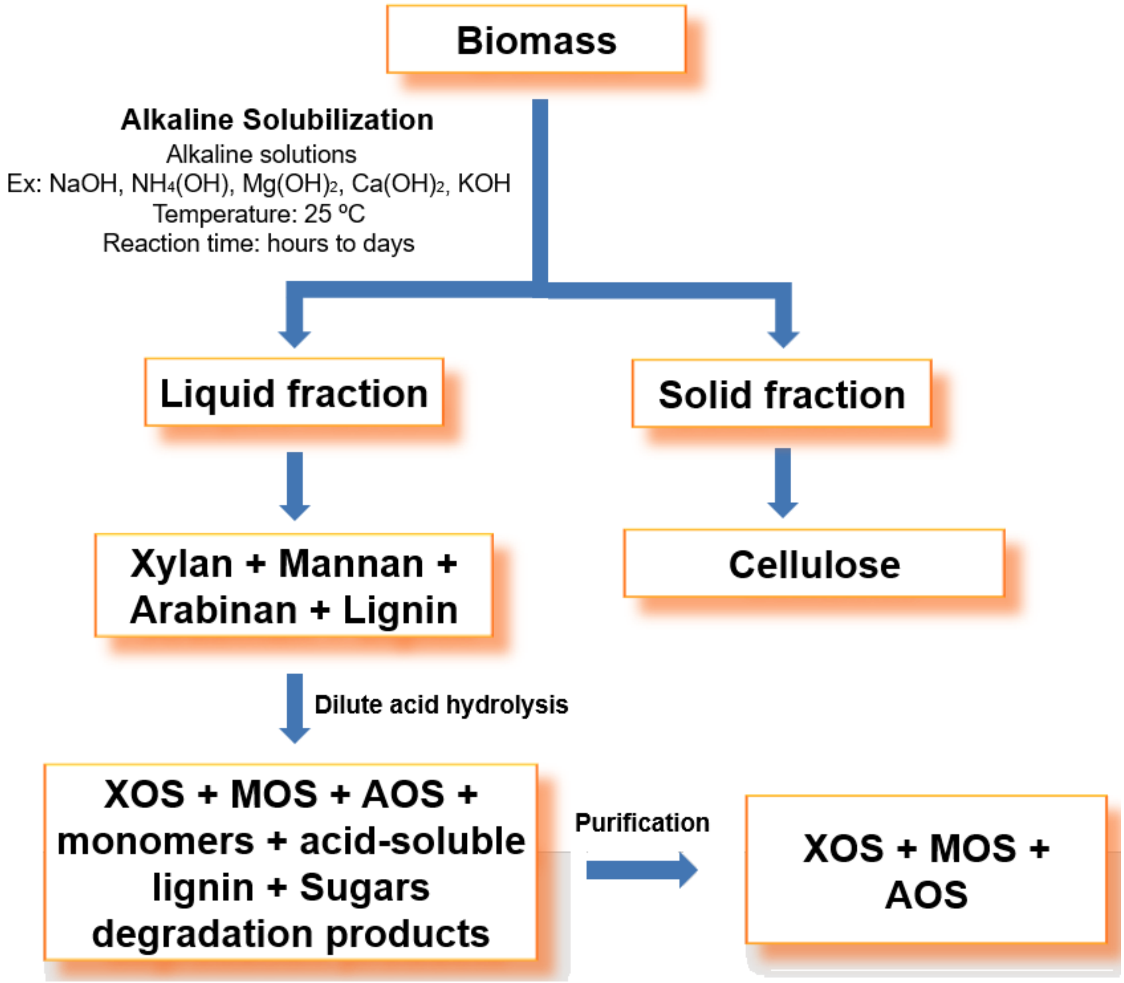


Fig 11. Schematic representation of lignocellulosic biomass alkaline extraction followed by acid hydrolysis for production of oligosaccharides

Source: Author

This pretreatment is more effective for agricultural residues and herbaceous crops due to their lower amount of lignin, unlike woody biomasses. Alkaline reagents are used in the process such as sodium hydroxide ($NaOH$), ammonium ($NH_4(OH)$), magnesium

($Mg(OH)_2$), calcium ($Ca(OH)_2$), and potassium (KOH). A saponification reaction of ester bonds between lignin and hemicellulose occurs resulting in the removal and solubilization of lignin, removal of acetyl groups from hemicellulose, decrease in the degree of polymerization and crystallinity of cellulose, and swelling of its fibers, increasing its porosity and surface area, without its degradation. All of these factors facilitate access to carbohydrates by enzymes. Under certain conditions, hemicellulose is also removed from the biomass and solubilized allowing subsequent chemical or enzymatic processes to obtain XOS, MOS e AOS (BARUAH et al., 2018; BOCHMANN, 2019; DEVI; DHAKA; SINGH, 2016; HARMSEN; LIPS; BAKKER, 2013).

NaOH is the most commonly used reagent due to its efficiency and when combined with oxidizing agents such as hydrogen peroxide (H_2O_2) and peracetic acid ($C_2H_4O_3$) the delignification process is increased, generating a solid fraction with greater enzymatic digestibility (LOOW et al., 2016). The hydrogen peroxide in alkaline medium has been extensively studied for the production of bleached hemicellulose. In this method the hydroxyl reacts with H_2O_2 forming hydroperoxy anion (HOO^-) and superoxide ($O^-{}_2$). The anion reacts with H_2O_2 that has not been dissociated to form hydroxyl radicals and superoxide oxidize and discolor lignin and break the ester bonds between hemicellulose and lignin, solubilizing them (Brienzo et al. 2016; Rabetafika et al. 2014).

The main advantage in alkaline methods is that the reactions occur under room temperature and pressure and process conditions are less severe compared to acid hydrolysis, causing less damage to equipment. In addition, polysaccharides are separated from other biomass components, increasing the efficiency of the enzyme during oligosaccharides production. Solid waste generated in the process is mainly composed of cellulose that can be used in the production of paper and cellulose derivatives or hydrolyzed into glucose by acids or enzymes and used in the production of biofuels. Another advantage is the possibility of recovering some bases such as calcium hydroxide that can be recovered as insoluble calcium carbonate after adding carbon dioxide in the aqueous medium. In this method, however, the sugar yield is low, there may be decomposition by alkali, formation of undesirable products, reagent needs to be neutralized and recovered at the end of reaction and the process is slow, taking hours or days. High concentrations of alkali increase production costs and are a concern for the environment (CHEN; LEE; ONG, 2019; DEVI; DHAKA; SINGH, 2016; KHAT-UDOMKIRI et al., 2018; LOOW et al., 2016).

4.2. Enzymatic hydrolysis

In the last decades, enzymatic hydrolysis for oligosaccharide production and many other processes, have been used as a substitute of chemical hydrolysis due their work features, such as mild temperature (40 - 60ºC) and pH (4.5 – 6.0), high product yields (%), higher specificity, absence of corrosive effect and for being environmentally friendly (AMORIM; SILVÉRIO; RODRIGUES, 2019; KHAT-UDOMKIRI et al., 2018; SATARI; KARIMI; KUMAR, 2019; WANG et al., 2018b).

Xylan and mannan are chemically complex and their depolymerization requires a set of enzymes called hemicellulases, among which there are specific enzymes for each glycosidic bond in the carbohydrate chain (HENRISSAT, 1991). As well as for all hydrolase enzymes, hemicellulases catalyze the breakdown of glycosidic bonds using water as a proton transfer mediator to the free ends of the carbohydrates separated (Henrissat 1991; Nordberg Karlsson et al. 2018; Houfani et al. 2020).

Enzymes can be used in their free state in the reaction medium or immobilized in some adsorbent material. They can be immobilized on different supports as in alginate beads, di-vinylbenzene copolymer, mesoporous silica, glyoxal agarose resin, polyethylene glycol hydrogel, among others (DE OLIVEIRA et al., 2018; TERRASAN; DE MORAIS JUNIOR; CONTESINI, 2018). The use of immobilized enzymes instead of free enzymes has the main advantage of allowing the reuse and recycling of them, and can reduce the costs of oligosaccharide production process. In addition, immobilization can increase enzyme stability, improves activity and selectivity, decreases inhibition by the product or other factors and facilitates the purification process of oligosaccharides (DE OLIVEIRA et al., 2018; SUKRI; MIMI SAKINAH, 2018; TERRASAN; DE MORAIS JUNIOR; CONTESINI, 2018).

Many microorganisms, mainly fungi and eubacteria, produce hemicellulases but they can be also produced by archaea and plants being founded, in this case, in seeds and fruits (ANDRADE; AGUIAR; ANTRANIKIAN, 2001; KIMURA et al., 2002; LISBOA et al., 2006; TERRONE et al., 2018, 2020; TÖRRÖNEN et al., 1992; ZHAO; ZHENG; ZHOU, 2011). Fungi are the most explored source of hemicellulases due their ability to secrete metabolic products to the environment. For the industrial production of enzymes, mainly fungi and some species of bacteria are used (JAGTAP et al., 2017; SATARI; KARIMI; KUMAR, 2019). The main microbial producing hemicellulases used commercially are those of the genera *Trichoderma, Aspergillus, Bacillus, Penicillium.*

The enzyme systems of these microbe are reported to be inducible for the production of hemicellulases when specific components are present in the reaction medium (AMORIM; SILVÉRIO; RODRIGUES, 2019; JAGTAP et al., 2017; SONI; KANGO, 2013; TERRONE et al., 2020; TÖRRÖNEN et al., 1992). Microorganisms' hemicellulases-producers in general are mesophilic, but studies with extremophiles species have been carried out in search of enzymes with greater stability and properties such as very high or very low optimum pH and temperature (ANDRADE; AGUIAR; ANTRANIKIAN, 2001; KUMAR; SATYANARAYANA, 2015).

For industrial production of enzymes, in general, submerged fermentation methods are used because they allow better recovery of enzymes in the liquid medium, which are in an environment controlled in temperature and pH that preserves enzymes molecular properties (ÁLVAREZ et al., 2019; JAGTAP et al., 2017; SATARI; KARIMI; KUMAR, 2019). The cost of enzyme production is still high compared to chemical products, which is still the bottleneck in the use of enzymes in most large-scale processes (SATARI; KARIMI; KUMAR, 2019). In enzyme production, several factors must be considered and evaluated to obtain the highest amount of enzyme concentration possible released. The main factors that must be controlled are pH, temperature, substrate composition and concentration, agitation and aeration (BATTISTA; BOLZONELLA, 2018; KHAT-UDOMKIRI et al., 2018; TERRONE et al., 2020). Another factor that must be controlled during the production of hemicellulases is the product concentration, because in many natural microorganisms genetic systems the presence of monosaccharides or even some oligosaccharides represses the production of the enzyme of interest, reducing productivity (BATTISTA; BOLZONELLA, 2018; SATARI; KARIMI; KUMAR, 2019).

Although the use of wild microorganisms has been carried out for a long time in the field of enzyme production and is still a viable technology in some cases (JAGTAP et al., 2017). The use of genetically modified microorganisms has expanded in research centers and enzyme production industries, through techniques such as that of recombinant DNA (PETROVA; PETROV, 2017; SONI; KANGO, 2013; ZHAO; ZHENG; ZHOU, 2011). The heterologous expression of hemicellulases through cloning, provides a cheaper and simpler production, with higher amount of enzyme recovered, higher yield, besides facilitating the reaction conditions and their control, allowing more efficient recovery at the end of production (KUMAR; SATYANARAYANA, 2015; PETROVA; PETROV, 2017; ZHAO; ZHENG; ZHOU, 2011).

Hemicellulases are a multi-enzyme system of depolymerizing and debranching enzymes, that act synergistically in the hemicellulose hydrolysis and they are classified and named based on their substrate specificity and sometimes on their molecular mechanism (HOUFANI et al., 2020; NORDBERG KARLSSON et al., 2018; SATARI; KARIMI; KUMAR, 2019). The specificity of enzymes for a single substrate occurs due to its complex three-dimensional conformation, which shapes to an active site responsible for catalyze. In the active site, enzymes perform hydrolysis catalysis by two acid amino acid residues. One acts protonating the substrate, while the second performs a nucleophilic attack (inversion). The carboxylate group can also functions as a general base, abstracting a proton from a nucleophilic water molecule which attacks the anomeric carbon rising a product with the retention of the configuration at the anomeric center (BATTISTA; BOLZONELLA, 2018; NORDBERG KARLSSON et al., 2018).

4.2.1. β-xylanases

These enzymes, also known as endo-1,4-β-D-xylanases or 1,4-β-D-xylan xylohydrolases (EC 3.2.1.8), cleave the glycosidic bonds in the xylan main chain releasing smaller non-substituted or branched xylooligossacharides (2 - ~7 DP) and reducing xylan DP (GLIBOWSKI; SKRZYPCZAK, 2017; WANG et al., 2019). Microbial β-xylanases (fungal or bacterial) vary in molecular weight from 6 kDa to 150 kDa (BATAILLON et al., 2000; KHANNA; GAURI, 1993; KURAMOCHI et al., 2016) and have pI values ranging from 1.7 to 9.5 (JALAL et al., 2009; KRISANA et al., 2005; TANAKA et al., 2005). Most β-xylanases have optimum acidic pH (DECELLE; TSANG; STORMS, 2004; JNAWALI et al., 2018; KNOB; CARMONA, 2010), but exceptions can be found (HWANG et al., 2010; MAMO et al., 2009). Almost all described have only one subunit and, in most cases, are glycosylated (glycoproteins) (NORDBERG KARLSSON et al., 2018; TERRONE et al., 2020).

β-xylanase are extremely dependent on the presence of accessory enzymes in the reaction medium, and they can differ from each other in relation to their specificity by bonds in the xylan backbone (BAJPAI, 2014; BRAGA et al., 2014; TERRONE et al., 2020). Some of these enzymes cut glycosidic bonds of the main chain randomly at unsubstituted regions of the substrate, but most of them select bonds for hydrolysis depending on the nature of the substrate, chain length, branched substituents, etc (BAJPAI, 2014; BRIENZO et al., 2016a; KHAT-UDOMKIRI et al., 2018; SUKRI;

MIMI SAKINAH, 2018). Their connection to the substrate also depends on the substituents on the xylose residues neighboring the attacked residues to guide themselves to the substrate binding, having little or no action against linear xylose backbone unsubstituted (BAJPAI, 2014; NORDBERG KARLSSON et al., 2018).

β-xylanase can be classified as specific or cross-specific. With specificity for the substrate, its action is restricted to β-1,4 bonds in xylans, and when it has cross specificity it can also catalyze the hydrolysis of β-1,4 bonds in other polymers such as carboxymethyl cellulose (BAJPAI, 2014; NORDBERG KARLSSON et al., 2018). Therefore, β-xylanases catalyze the hydrolysis of xylans and xylooligosaccharides (Fig 12) and its action results in β-D-xylopyranosyl oligomers with DP of more than 4 xylose residues with substitution groups or not, depending on the substrate, but also they can release xylose, xylobiose, and xylotriose (Sukri and Mimi Sakinah 2018; Wang et al. 2018a; Freitas et al. 2019; Houfani et al. 2020). The efficiency with which they hydrolyze oligosaccharides decreases with decreasing DP or with increasing substrate concentration (KHAT-UDOMKIRI et al., 2018; WANG et al., 2018b). Most β-xylanase works via retaining mechanism with a double-displacement (BRAGA et al., 2014; NORDBERG KARLSSON et al., 2018).

Endo-1,4-β-xylanases are to date classified under GH-families 5, 7, 8, 10, 11, 30, 43, 51 and 98. They differ in their physico-chemical properties, structure, mode of action and substrate specificities. Xylanases from family 10 generate more xylose than xylanases from family 11. Xylobiose and substituted xylotriose are the main products of family 10, while xylobiose, xylotriose and substituted xylotetraose are the main products of family 11. The GH30 family contains xylanase specialized in the cleavage of linear parts of xylan and requiring a MeGlcA substitution on the xylan chain as well, whereas xylanases found in GH5 family does not attack linear xylan, and depends on substitution in position 3 of xylose, but very little is known about the mode of action of this enzyme (BIELY; SINGH; PUCHART, 2016; KATSIMPOURAS et al., 2019).

β-xylosidase are enzymes very specific for xylooligosaccharides generated by the action of β-xylanase and other hydrolases, that are not substituted and have DP up to 4. This enzyme act from the non-reducing end, releasing D-xylose as the single product of its hydrolysis (Fig 12). Thus, for xylooligosaccharides production this enzyme is not desired due to the degradation of the oligosaccharides, decreasing the yield of XOS, and the inhibition effects that they may cause in XOS production (PETROVA; PETROV, 2017).

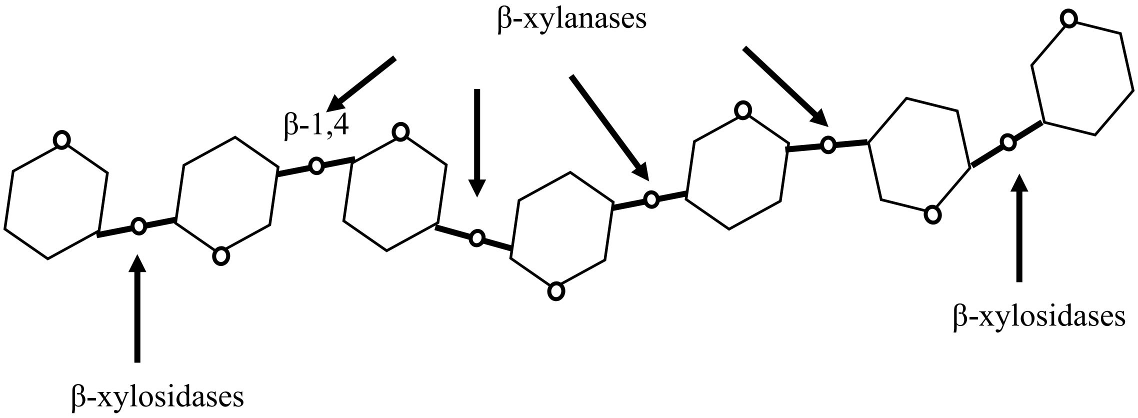


Fig 12. β-xylanases and β-xylosidases active sites in debranched xylan.

Source: Author

4.2.2. β-D-mannanases

Mannans are linear or branched chain polysaccharides, which can have a single type of sugar in their main chain or several types, a heteropolymer (BHATIA et al., 2019; SINGH; SINGH; ARYA, 2018). Mannan backbone degradation depends on the action of the depolymerizing β-mannanase that hydrolyses β-1,4-linked mannose residues of galacto(gluco)mannans, releasing mannan oligosaccharides (Fig 13) (NOPVICHAI et al., 2019; SONI; KANGO, 2013). The activity of these enzymes, also known as mannan endo-1,4-β-mannosidase or 4-β-D-mannan mannanohydrolase (EC 3.2.1.78), varies with the number and distribution of substituents in the carbohydrate chain, and with the glucose/mannose ratio. β-D-mannanases can vary in size from 18 kDa to 162 kDa. They have a wide range of pI and optimal pH (from acid to basic) (BLIBECH et al., 2010; CHEN et al., 2007; CHO et al., 2006; LISBOA et al., 2006; ZHOU; XUE; MA, 2018).

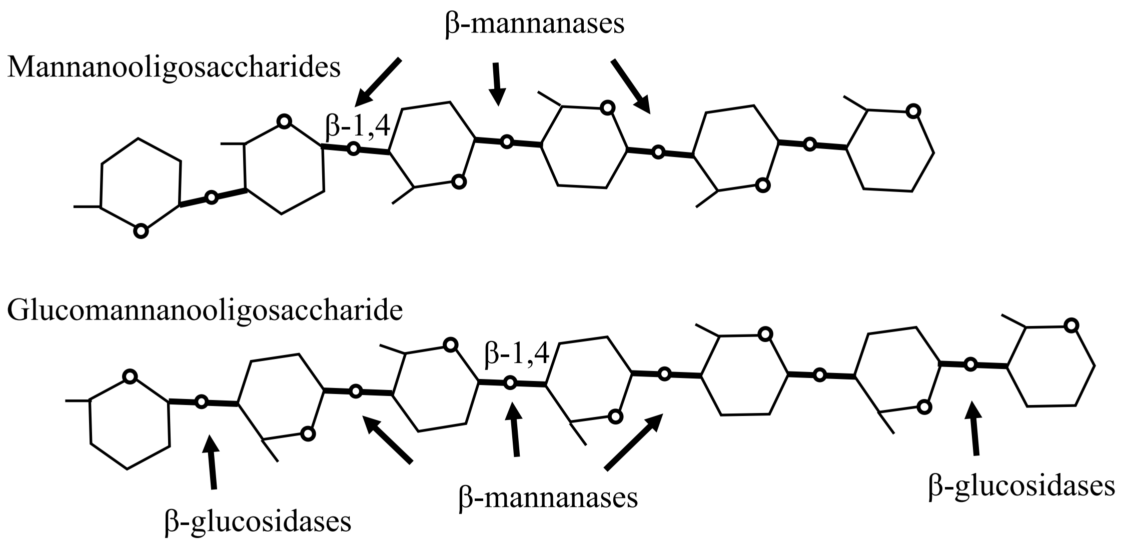


Fig 13. β-mannanases active sites in debranched mannanooligosaccharides and β-mannanases and β-glucosidases active sites in debranched glucomannan oligosaccharides.

Source: Author

As well as xylans, for the complete degradation of mannans, accessory enzymes are needed to act on the branches of the chain. These enzymes are β-mannosidase, β-glucosidase, α-galactosidase and acetyl esterase. β-mannanase are enzymes most active on mannans with few substitutions of galactose residues. This galactose residues blocks the access of β-mannanase to the mannose backbone especially if these residues are on alternate sides in the main chain. Once galactose substitutions are on the same side of the chain, the hindering effect of β-mannanase access to the main chain is reduced. The products of β-mannanase hydrolysis are mainly oligomannan or oligoglucomannan (4-5 DP) but also mannobiose and mannotriose can be released. The presence of galactose substitutions in the mannose residues adjacent to the ends hinders the action of these enzymes, reducing their activity (HOUFANI et al., 2020; NOPVICHAI et al., 2019; RAHMANI et al., 2017; SONI; KANGO, 2013).

In addition to the ability to hydrolyze glycosides, β-mannosidases also have transglycosylation activity, which is, in this case, the synthesis of mannan oligosaccharides and another specific oligosaccharides through the binding of β-glycosides (SCHRÖDER et al., 2006).

4.2.3. Debranching enzymes (accessory enzymes)

Contrasting enzymes that act on the polysaccharide main chain, the substituents are liberated by accessory enzymes, glycosidases and esterases, that hydrolyze the bonds between the main chain and its substituents or the side chains of these backbones. According to hydrolysis characteristics, the accessory enzymes can be divided into enzymes that attack the substituents whether they are present in polymers or oligomers; and enzymes that do not act on polymeric substrates, being active only in branched or substituted oligosaccharides, generally generated by the action of β-xylanase or β-mannanase.

4.2.3.1. α-Arabinofuranosidase

Enzymes that remove non-reducing α-L-arabinosyl residues of arabinans, arabinoxylans and arabinogalactans, with great specificity for the substrate, and can release arabinose from various substrate (Fig 14). α-Arabinofuranosidases can be monomeric, dimeric (2 subunits), tetrameric (4 subunits) and octameric (8 subunits) with molecular weights reaching from 49 to 530 kDa, isoelectric points from 3.5 to 9.7, and optimal pH values range from 2.3 to 7.4 (HESPELL; O'BRYAN, 1992; INÁCIO; LOPES CORREIA; DE SÁ-NOGUEIRA, 2008; MARGOLLES; DE LOS REYES-GAVILÁN, 2003; TERRONE et al., 2020). Some characteristics of the substrate that influence α-arabinofuranosidase activity are the substitutions of arabinose in the main chain and the DP. Most of these enzymes release only arabinose residues linked by α-1,3 bonds to the main chain. Others are able to act on bonds α-1,2, α-1,3 and α-1,5 (exo α-L-arabinofuranosidase EC 3.2.1.55). Some α-arabinofuranosidase release arabinose residues linked by α-1,2 or α-1,3 bonds specifically from xylose residues with a single substitution (HOUFANI et al., 2020).

Some of these enzymes release arabinose only from terminal xyloses or from xylooligosaccharides (4-5 DP), while others are able to release arabinose from nonterminal, from the intact polysaccharide (endo α-L-arabinofuranosidase EC 3.2.1.99). These characteristics mean that α-arabinofuranosidase can be divided into those involved in the degradation of arabinoxylans and those that are "arabinose-releasing" enzymes, which act on different substrates regardless of their composition. In a recent study reported by Terrone et al. (2020), the action of an α-arabinofuranosidase produced by

Aspergillus hortai on oat xylan (arabinoxylan) for the production of oligosaccharides was reported, and the release of arabinoses in the reaction medium was observed, mainly when purified α-arabinofuranosidase was associated with an endoxylanase. α-arabinofuranosidase that release arabinose residues from arabinoxylans in the absence of β-xylanases was reported by Kormelink et al. (1991) but they are rare.

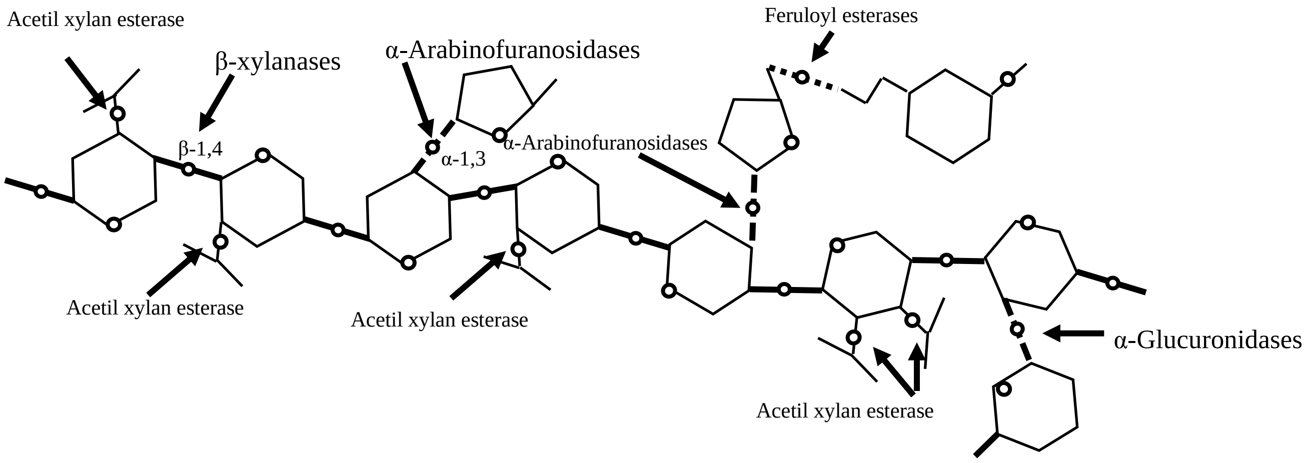


Fig 14. Xylan-degrading enzymes active sites in branched xylan.

Source: Author

4.2.3.2. α-Glucuronidase

These enzymes remove D-glucuronosyl residues or 4-O-methyl-D-glucuronosyl residues with α-1,2-glycosidic bond, from the xylan backbone (Fig 14). There are very few α-glucuronidases (EC 3.2.1.131) described in the literature in relation to β-xylanases for example. Despite this, the α-glucuronidases described have a diversified optimal pH rate, varying between 4 and 8 but most of them prefer acidic media (ROGOWSKI et al., 2014; ROSA et al., 2013; SEPTININGRUM et al., 2015; XUE et al., 2008). α-Glucuronidases have activity mainly in glucuronoxylans and arabinoglucuronoxylans and most of those described have a preference for smaller oligosaccharides (2 - 6 DP) with little activity in polysaccharides and oligosaccharides with DP greater than 10 (HOUFANI et al., 2020; LEE et al., 2012; ROGOWSKI et al., 2014), but some exceptions have been described (YAN et al., 2017). The activity of these enzymes is highly inhibited by the presence of other adjacent substituents in the oligomer, especially acetyl groups (CHONG et al., 2015).

4.2.3.3. Galactosidase

Galactosidase are a group of enzymes that hydrolyze the D-galactoses binding of oligosaccharides. They are classified into α-galactosidase (EC 3.2.1.22) that act on α-1,6 glycosidic bonds of galactose branching linked to galacto(gluco)mannan main chain (Fig 15), releasing D-galactopyranosyl residues (HOUFANI et al., 2020; JINDOU et al., 2002; SONI; KANGO, 2013), and β-galactosidase (EC 3.2.1.23), that catalyze the hydrolysis of β-galactosyl from the non-reducing end of several oligosaccharides especially pectin galactans, or synthetized oligosaccharides by transgalactosylation of lactose dimers (CHO; SHIN; BUCKE, 2003; KANEKO; KOBAYASHI, 2003; PETROVA; PETROV, 2017). The use of α-galactosidase is more relevant in the mannan oligosaccharide production process due to its action on hemicellulose. α-Galactosidases have a wide range of optimal pH and isoelectric point, varying between 3 and 8 (KING et al., 2002; SHIBUYA et al., 1997; WANG et al., 2010).

For the production of mannan oligosaccharides, the presence of α-galactosidase in the reaction medium is important because this enzyme acts on the main chain releasing galactose residues and preparing the substrate for mannanases. Some enzymes in this group release galactose from long polymer chains of larger galactomannans and oligosaccharides while others show greater affinity for small branched oligosaccharides (WANG et al., 2010).

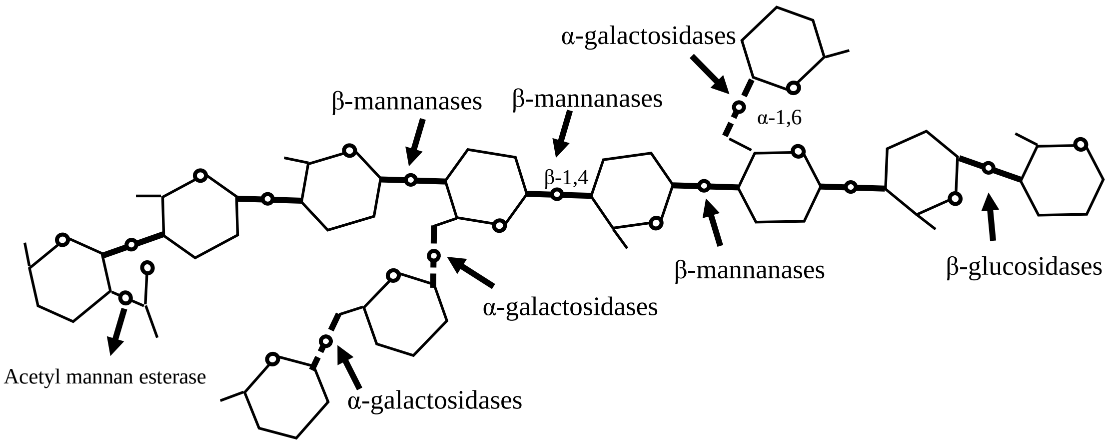


Fig 15. β-mannanases, β-glucosidases and α-galactosidases active sites in branched galactoglucomannan.

Source: Author

4.2.4. Synergy and enzymatic cocktails for the production of xylo/mannan oligosaccharides

The production of oligosaccharides via enzymatic hydrolysis, from branched polymeric substrates, such as xylans and mannans, depends on a complex of cooperative or synergistic enzymes. This complex presents a variety of chain and side chain-cleaving enzymes of different specificities that are responsible for cleaving the different bonds present in those substrates (HOUFANI et al., 2020). For both depolymerization of xylans and mannans, the set of enzymes consists of a main enzyme, which will attack the bonds of the polysaccharide backbone, and two or three debranching enzymes that create new sites on the main chain, thus helping major enzymes in accessing their substrate. At the same time, the action of main-chain cleavage enzyme facilitates the activity of accessory enzymes, since they reduce solution viscosity and increase diffusion of the substrate in the medium by decreasing degree of polymerization (ÁLVAREZ et al., 2019). The synergy phenomenon can be observed either when purified main-chain or debranching enzymes are used (JAGTAP et al., 2017).

The synergy between two or more enzymes occurs when the presence of one enzyme in the reaction medium enables or enhances the action of the other enzymes (TERRONE et al., 2020; VAN DYK; PLETSCHKE, 2012). According to Soni and Kango (2013), the synergy between two or more enzymes can be classified into homosynergy and heterosynergy. Homosynergy is observed when the mixture of two (or more) enzymes that catalyze main chain bonds, or two (or more) debranching enzymes with different specificities, results in a greater quantity of products than the sum of products released by the enzymes applied separately (KUMAR; WYMAN, 2009; VAN DYK; PLETSCHKE, 2012). This effect can occur between two main-chain cleavage enzyme for example, a β-mannanase and a β-mannosidase, or between two or more debranching enzymes (AMORIM et al., 2019; SONI; KANGO, 2013). In some cases, a substituent can hinder the action of removing another substituent in the neighborhood by accessory enzymes. In this type of synergistic interaction, it is considered that the action of one enzyme provides substrate for the other enzyme or allows the other enzyme access to its substrate (BATTISTA; BOLZONELLA, 2018).

However, some main-chain cleavage enzymes may require the prior action of an accessory enzyme. This is called heterosynergy. An example of this is when an enzyme that attacks the polysaccharide backbone is unable to access its substrate in the presence of substituted glycoside residues. The presence of these substituents decreases the catalytic efficiency of the enzyme by blocking access. In this case, the accessory enzymes act by releasing the substituents from the main chain, allowing an increase in enzyme activity that reduces degree of polymerization (TERRONE et al., 2020). Therefore, heterosynergy is when the mixture of one (or more) degrading enzyme from the main chain and one (or more) debranching enzyme is more efficient in releasing hydrolysis products than when these enzymes are used individually (SONI; KANGO, 2013).

Synergistic studies between enzymes and also studies on the sequence with which the enzymes work more efficiently, have increased in recent years to obtain greater production of xylooligosaccharides (ÁLVAREZ et al., 2019; TERRONE et al., 2020; WANG et al., 2010). In these studies, it is possible to evaluate the physical barriers that the enzymes encounter to access their substrate and also the characteristics of the substrate recalcitrance. In addition to the synergy of the enzymes, the proportions of each enzyme and the sequence that each enzyme will be applied in the reaction medium is also relevant. The results may differ when the enzymes are used in sequence, and not simultaneously. That is why sequential studies about performance of each enzyme are important for the synergistic effect between them to generate an amount of product as largest as possible. This strategy reduces cost of reaction by reducing the required enzyme load, which is often wasted, and allows obtaining more efficient enzymatic cocktails for the hydrolysis of polysaccharides (SATARI; KARIMI; KUMAR, 2019).

For the production of xylooligosaccharides, the synergistic action usually occurs between endoxylanases, α-arabinofuranosidase and glucuronidases, for the majority of (glucuronoarabino)xylans, especially if these substrates were treated with some type of pretreatment that removes other substituents such as ferulic acids and acetyl groups (ÁLVAREZ et al., 2019; BRAGA et al., 2014; KHAT-UDOMKIRI et al., 2018; TERRONE et al., 2020). Generally, the pretreatments used for the production of XOS, which precede enzymatic hydrolysis, are the alkaline extractions in the presence of potassium hydroxide (KOH), sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) or ammonia, because they separate hemicelluloses from other lignocellulosic material components, preserving their characteristics and making them more available for the action of hemicellulases (CHEN et al., 2018; JNAWALI et al., 2018; KHAT-UDOMKIRI et al., 2018; PETROVA; PETROV, 2017; SATARI; KARIMI; KUMAR, 2019). Using β-xylanases alone in the reaction medium release several types of heterogeneous xylan fragments, containing xylose oligomers in their composition substituted with arabinose, uronic acids or acetylations (AMORIM et al., 2019; BAJPAI, 2014). Associated with debranching enzymes, the variety of fragments decreases and xylanases are able to release more homogeneous xylose oligomers.

Regarding the production of mannan oligosaccharides from galactomannans or galactoglucomannans, the presence of at least one debranching enzyme, like α-galactosidases or acetylmannanesterases, are required to act together with β-mannanases, which are strongly blocked by the presence of galactose substitutions and acetyl groups in the main chain (SONI; KANGO, 2013; WANG et al., 2010).

Due to the synergistic use of different enzymes in the reaction medium, it is crucial to combine catalytic and physical-chemical properties of each enzyme before their use in the industrial oligosaccharide production processes. Properties such as temperature, reaction time, sequence of addition of each enzyme and especially optimal pH of activity must be combined and controlled for efficient obtaining of xylo/mannan oligosaccharides.

4.3. Industrial production

The market for oligosaccharides has grown in recent years, mainly in countries such as China and Japan but also in Europe, however few companies produce these compounds industrially nowadays. Xylooligosaccharides are the oligosaccharides from hemicellulose most produced worldwide, with China as the main consumer country. Since 2003, the use of XOS as a feed additive in the livestock industry is approved by the Ministry of Agriculture of the People's Republic of China, which has caused the increased consumption of the oligosaccharide in the country. In 2015 the consumption of XOS as additives in animal feed in China was 1.600 tons (CCM DATA & BUSINESS INTELIGENCE, 2016).

Longlive Bio-Technology Company Ltd, located in China, occupies a leading position worldwide with regard to the production capacity of xylo-oligosaccharides. They are able to produce 4.000 tons per year and another production line is being constructed with a capacity of 6.000 tons per year. The raw material used for the production of XOS is the corn cob, however the company also uses it in the manufacture of several other products such as dextrose, maltose, maltodextrin, sorbitol, xylitol, xanthan gum, etc (CHINA FOOD ADDITIVES & INGREDIENTS ASSOCIATION, 2010).

Henan Heagreen Bio-technology Co., Ltd., founded in 2014, is another functional oligosaccharide producer in China. The main business of Heagreen is xylooligosaccharides production and sales as well as related services. It is the only company specializing in the production, research and development of XOS in the world. Enzymatic hydrolysis, using a multi enzymatic complex, is the technology used by this company to transform the raw material (corn cobs) into oligosaccharides. For the purification and concentration of the molecules, a membrane integration technology is used, a low-energy, environmentally friendly method. Finally, the product is dried through a vacuum low temperature drying technology, which makes the product have the characteristics of high density and strong anti-hygroscopic performance (HENAN HEAGREEN BIO-TECHNOLOGY, 2015).

Pilot-scale production of xylooligosaccharides from *Miscanthus* was conducted using steam explosion pretreatment and enzymatic hydrolysis. To evaluate XOS generation, parameters such as temperature and reaction time were varied, considering the effects on sugar recovery and formation of fermentation inhibitors. XOS yields up to 52% (w/w of initial xylan) were obtained under the steam explosion conditions studied (200 ºC; 15 bar; 10 min). A commercial endoxylanase was responsible for the production of xylobiose from XOS-rich hydrolysate (380-500 g/kg of initial xylan), which is the XOS with highest prebiotic potential. Under the conditions which yielded the highest amount of XOS, the production of degradation compounds such as HMF and furfural was low, 0.2 g/L and 0.3 g/L respectively. Another pilot-plant production of xylooligosaccharides from corncob was carried out by steaming and enzymatic hydrolysis. It was able to obtain 74.5% of XOS in the final product, mainly comprised by xylobiose and xylotriose. This information shows that this method was successful in producing xylooligosaccharides with prebiotic action and has potential to be applied on an industrial scale (BHATIA et al., 2020; YUAN et al., 2004).

Considering the situation in Brazil, where 140 thousand tons of sugarcane bagasse are produced from ethanol production, this would be a raw material with high potential for the production of xylooligosaccharides. If a hydrothermal treatment were used to solubilize sugarcane bagasse xylan in the form of XOS, with a yield of 43.6%, it would be possible to produce 14,000 tons of XOS per year (CARVALHO et al., 2018).

The increased yields of XOS may lead to new uses for the xylan-derivatives, besides the benefits human/animal health. For example, adsorption studies of XOS on kraft pulp fibers revealed positive effects on pulp yield (CORNETTI; FERRAZ; MILAGRES, 2020), beating and pulp strength properties (BAN et al., 2011). These results support the theory that XOS enter most pores in the fibre to a greater extent than high-molecular-weight xylan does. To some extent, these properties can help to meet various needs in the large pulp and paper market.

Conclusions and Future Perspectives

The development of biorefineries is becoming progressive in the last years because of the ideal to use renewable resources to obtain food, chemicals and fuels. Therefore, biomass has been considered a resource of upmost importance. Polysaccharide related residues are a great source for bioactive compounds, oligosaccharides being one of them. Oligosaccharides derived from hemicellulose are recognized as competitive emerging prebiotics and the use of lignocellulosic biomass as raw material for their production is considered a potential strategy to reduce production costs (AMORIM et al., 2019; CANO et al., 2020).

The global prebiotics market reached US$ 2.90 billion in 2015 and US$ 4.24 billion in 2017. This market is expected to grow approximately 12.7% by 2025 profiting around US$ 10.55 billion (BHATIA et al., 2020; HURTADO-ROMERO et al., 2020; MANO et al., 2018). From 1997 to 2008, about 32 products containing oligosaccharides from hemicellulose, mostly XOS, were lunched. However, XOS market price (1-200 US$ / kg) is yet higher when compared to other oligosaccharides such as fructooligosaccharides (1-20 US$ / kg), galactooligosaccharides (5-100 US$ / kg) and inulin (10-100 US$ / kg). Therefore, several researches are being developed in order to produce a pure form and high yield of these oligosaccharides from different types of hemicellulose-containing substrates in a cost-effective way. These novel production strategies can be further integrated on biorefinery processes (SINGH; BANERJEE; ARORA, 2015).

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CHAPTER 4: ENZYMATIC HYDROLYSIS APPLIED TO BANANA PSEUDOSTEM BIOMASS COMPARED TO SOLUBILIZED XYLAN FOR XYLOOLIGOSACCHARIDES PRODUCTION WITH HIGH SUBSTRATE CONCENTRATION

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Abstract

Xylan is a polysaccharide present in lignocellulosic biomass, which is obtained in large quantities as agricultural and agroindustrial waste. In this study xylooligosaccharides (XOS) were produced by the enzymatic hydrolysis of xylan, using biomass itself or solubilized xylan as substrate. In addition, enzymatic hydrolysis of solubilized xylan was performed with two cycles in a higher volume and higher substrate concentrations. XOS and xylan molecular weight were evaluated. After partial delignification, the hemicellulose in the material increased by 6 percentual points due to the lignin removal. Milling and partial delignification of biomass helped to improve the enzymatic hydrolysis since conversion went up by 47.13% after the use of both strategies together. Regarding the solubilized xylan enzymatic hydrolysis, the increase of substrate concentration led to a decrease in the yield of around 13%, however, the amount of XOS produced went from 14.68 g/L to 50.40 g/L. The hydrolysis 2nd cycle led to an increase of only around 10% conversion for all substrate concentrations. After hydrolysis, the molecular weight of the xylan decreased, as did the polydispersity. However, after the second hydrolysis cycle both the molecular weight and polydispersity increased, since probably the enzyme acted in the easing xylan fraction and the remaining complex substrate limited enzyme action. Comparatively, the enzymatic hydrolysis of solubilized xylan resulted in a higher yield and concentration than enzymatic hydrolysis of the partial delignified biomass.

Keywords: hemicellulose, endoxylanase, molecular weight, prebiotic, *Aspergillus versicollor*.

1. Introduction

Xylan is a heteropolysaccharide composed of xylose residues linked by β-1,4 glycosidic bonds, which backbone can present small groups such as acetyl, phenolic, ferulic, and coumaric acid linked to it (DEUTSCHMANN; DEKKER, 2012; NAIDU; HLANGOTHI; JOHN, 2018b). Because of the hemicellulosic matrix organization and the strong association of hemicellulose with other cell wall components, xylans are related to secondary wall composition and structure associated with biomass recalcitrance. The recovery of hemicellulose fraction is interesting for several integrated bioprocesses, allowing their utilization for the production of value-added molecules and by-products (QASEEM; SHAHEEN; WU, 2021; SCAPINI et al., 2021). There are several possible applications for hemicellulose, however, xylooligosaccharides (XOS) production is one of the potential due to the health aspects. XOS are non-digestible carbohydrates considered as prebiotics and one of the value-added products. XOS can be produced from lignocellulosic biomass, specifically by the hydrolysis of xylan, the major hemicellulose to occur in nature (GULLÓN et al., 2014; POLETTO et al., 2020).

Banana is a fruit largely consumed and appreciated in the world and they are grown in tropical and subtropical regions (NAPOLEÃO; RODRIGUES DE JESUS; LEONEL, 2021). It is the world's oldest farmed crop and one of the tallest herbaceous plants with a pseudostem (GUPTA et al., 2022a; SAWARKAR et al., 2022). It's estimated that around 130 countries have banana crops with a production of almost 120 million tons in 2020. Brazil is the 3rd largest banana producer in the world, where the production reached 6.64 million tons in 2020 as reported by the Food and Agriculture Organization of the United Nations (FAO) (FAOSTAT, 2020). For each ton of bananas obtained, 3 tons of pseudostem are produced (FERNANDES et al., 2013). Banana agricultural waste has a wide range of applications such as in the energy sector, paper and pulp sector, fiber and film, bioplastic, environmental sector, and animal feed (AHMAD; DANISH, 2018; INGALE; JOSHI; GUPTE, 2014; JAMAL; K. SAHEED; ALAM, 2011; REDDY MARELLA; MADIREDDY; MARIPI, 2014; TAMBUWAL; OKOH; OGBIKO, 2020), however little is known about the use of this residue to produce xylooligosaccharides by the enzymatic hydrolysis of this biomass.

Enzymatic hydrolysis of lignocellulosic materials is a widely proposed process since it usually presents higher yields compared to other types of hydrolysis due to its reaction specificity and absence of by-products formation (BRIENZO et al., 2016b).

Besides that, the mild temperature and pressures in which this reaction occurs are another important characteristic of enzymatic hydrolysis (SELVARAJAN; VEENA, 2017). Recent studies reported direct enzymatic hydrolysis of biomass, however, is a difficult task due to the highly complex structure and low porosity of lignocellulose that prevents effective enzyme attack. Therefore, the extraction of xylan or at least a biomass pretreatment collaborates for better enzyme action and biomass conversion, mainly for XOS production (BRIENZO et al., 2016b; FREITAS et al., 2022; NORDBERG KARLSSON et al., 2018; ZHOU; TIAN, 2022).

After xylan solubilization or extraction from lignocellulose, XOS can be produced by the enzymatic hydrolysis of the central backbone by endo-1,4-β-xylanases (E.C. 3.2.1.8), which breaks the β-1,4 xylose bonds in the main chain (FREITAS; CARMONA; BRIENZO, 2019; ZAMORA ZAMORA et al., 2021; ZERVA et al., 2021). XOS are a substrate for β-xylosidase, enzymes that act in the non-reducing end releasing xylose. In order to obtain a higher yield of XOS, the activity of β-xylosidase must be little or non-existent. Besides that, the xylose production must be maintained as low as possible to avoid any inhibitory effect on endoxylanase activity. The amount of XOS produced will differ mainly accordingly to the enzyme load, substrate concentration, and reaction time (DE FREITAS et al., 2021; SANTIBÁÑEZ et al., 2021).

Xylooligosaccharides, oligomers composed of xylose units linked by β-1,4-glycosidic bonds, have recently gained attention in the food research and industry due to their prebiotic potential and positive impact on both human and animal health. These oligomers can present different degrees of polymerization, ranging from 2 to 10 monomeric units. They display higher stability than other similar prebiotic molecules such as inulin and fructooligosaccharides because of their thermostability up to 100 ºC and at a wide range of pH (from 2.5 up to 8.0) (FREITAS et al., 2022; KUMAR et al., 2021; MANO et al., 2017).

The biological properties of XOS depend on their structure and degree of polymerization. Usually, a lower degree of polymerization XOS present higher prebiotic activity, however, studies show that a mixture of XOS with a degree of polymerization ranging from 2 to 6 was used as a carbon source for the growth of probiotics (DE FREITAS et al., 2021; POLETTO et al., 2020). As non-digestible oligosaccharides, XOS are not degraded by the enzymes of the human digestive system, reaching the gastrointestinal tract (GI). They are consumed by the probiotics and these microorganisms will produce short-chain fatty acids as by-products of XOS fermentation, molecules that influence the GI cells integrity, help in the immune function modulation, and glucose regulation (KOH et al., 2016a; PALANIAPPAN; ANTONY; EMMAMBUX, 2021b).

Technologies have been evolving in order to facilitate the production of high value-added compounds from lignocellulosic agricultural wastes efficiently and cleanly. In view of the increasing demand and consumption of functional foods, the production of XOS from these residues is a good alternative and different methods should be applied to find the most advantageous way of production. The present study evaluated XOS production from xylan solubilized from banana pseudostem, with two hydrolysis cycles. Comparatively, XOS were produced by enzymatic hydrolysis applied directly to the partial delignified biomass (banana pseudostem). The molecular weight of the xylan was monitored over the enzymatic hydrolysis.

2. Material and Methods

2.1. Biomass partial delignification

Ten grams of milled banana pseudostem were added by 200 mL of 20% (m/m) hydrogen peroxide (H_2O_2) solution with pH 11.6 (BRIENZO; SIQUEIRA; MILAGRES, 2009b). The solution was stirred at 150 rpm at 25ºC for 4 h. The solid fraction had its pH neutralized, dried at 50ºC, and later used for enzymatic hydrolysis. Hydrogen peroxide was applied here with a lower charge compared to the process of xylan solubilization.

2.2. Xylan solubilization

Ten grams of milled banana pseudostem partial delignified were added to 200 mL of hydrogen peroxide (H_2O_2) solution 6% (w/v). The pH was adjusted to 11.6 and the solution was stirred at 150 rpm for 24 h at 25 ºC [31]. The samples were filtered after the reaction, and the pH of the liquid fraction, rich in hemicellulose, was adjusted to 6 and concentrated to 1/3 of the original volume in an oven at 60 ºC. Xylan was precipitated by the addition of 3 volumes of 96% (v/v) ethanol. This process was repeated with 70% (v/v) ethanol until a transparent/clear supernatant. The material was dried at 45 ºC and stored for later use in the enzymatic hydrolysis.

2.3. Biomass chemical characterization

An acid hydrolysis was performed by adding 3 mL of 72% (w/w) sulfuric acid to a sample containing 300 mg of extractive-free banana pseudostem. After 1 h at 30ºC, 84 mL of distilled water was added to stop the reaction. This suspension was autoclaved at 121 ºC for 1 h. The samples were filtered through a No. 4 sintered glass filter, previously tared. The material retained in the filter was weighed, quantifying the insoluble lignin. The absorbance of the liquid fraction at 215 nm and 280 nm was used to quantify the soluble lignin. The content of glucose, xylose, and arabinose was quantified by high performance liquid chromatography (HPLC) on WATERS equipment using Aminex HPX-87H BIO-RAD column (300 x 7.8 mm); temperature of 50 °C; sulfuric acid 0.005 mol/L with flow 0.6 $mL.min^{-1}$ as eluent; 20 µL of sample volume and detector of refractive index (Waters 2414) (BRIENZO; SIQUEIRA; MILAGRES, 2009b)

2.4. Biomass enzymatic hydrolysis for XOS production

Untreated and partial delignified banana pseudostem was submitted to enzymatic hydrolysis. Before the hydrolysis, the biomasses were ball milled for 30 min. The enzymatic hydrolysis reaction occurred in Falcon tubes containing 5 mL of 0.05 mol/L sodium phosphate buffer (pH 6) in a shaker at 55 ºC with the agitation of 120 rpm. The reaction was conducted with *A. versicolor* endoxylanase previously purified (DE FREITAS et al., 2021), and the endoglucanase used was Celluclast (Novozymes). The initial activity of the endoxylanase was 1314 UI/mL and of the endoglucanase was 2910 UI/mL. The substrate concentration used was 2% and 24 h reaction time (DE FREITAS et al., 2021). Four different enzyme configurations were used: 50 UI/g of endoxylanase for 24 h, 50 UI/g of endoglucanase and 50 UI/g of endoxylanase for 24 h, 50 UI/g of endoglucanase for 6 h followed by 50 UI/g of endoxylanase for 18 h and 50 UI/g of endoglucanase for 24 h followed by 50 UI/g of endoxylanase for 24 h. To stop the reaction, the tubes were boiled in water for 5 min. The hydrolysate was filtered with a 0.22 µm syringe filter prior to HPLC analysis. The experiments were performed in triplicate, and the average showed and performed Tukey test, 95% of confidence level (p< 0.05).

2.5. Xylan enzymatic hydrolysis with a high substrate load and in two cycles

The solubilized xylan from partial delignified xylan was submitted to enzymatic hydrolysis. The hydrolysis was performed in flasks containing 25 mL of 0.05 mol/L sodium phosphate buffer (pH 6) in a shaker at 55 ºC with the agitation of 120 rpm. The reaction was conducted with *A. versicolor* endoxylanase previously purified [25] and Celluclast endoglucanase (Novozymes). Each hydrolysis had variations in hemicellulose concentration (2, 4, 6, 8, and 10%). The enzyme load used was 50 UI/g and 24 h reaction. The flasks were heated in boiling water for 5 min to stop the enzyme reaction. After the first hydrolysis cycle, xylan was precipitated from the solution using 3 volumes of 92% (v/v) ethanol. The ethanol was removed by volume evaporation in an oven at 40 ºC.

The xylan precipitated from the first enzymatic cycle was submitted to new enzymatic hydrolysis using the same conditions. The hydrolysate from the first and second cycles were filtered with a 0.22 µm syringe filter prior to analysis of XOS and xylan molecular weight.

2.6. Xylooligosaccharides quantification

To determine XOS concentration, samples were submitted to high performance liquid chromatography (HPLC) on WATERS equipment using Aminex HPX-87C BIO-RAD column (300 x 7.8 mm); temperature of 80 °C; ultrapure water with flow 0.6 mL.min^{-1} as eluent; 20 µL of sample volume and detector of refractive index (Waters 2414). For standards, were used different concentration solutions of xylose (Sigma), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), and xylohexaose (X_6) (Megazyme) (DE FREITAS et al., 2021).

2.7. Xylan molecular weight

To determine xylan molecular weight, samples were submitted to Ultra Performance Liquid Chromatography (UPLC) on WATERS equipment using the following parameters: Acquity UPLC BEH SEC column (4.6 x 150 mm); temperature of 40 ºC; eluent: ultrapure water with flow 0.5 mL.min^{-1}; sample volume: 20 µL; detector: refractive index at 35 ºC. Pullulan presenting molecular weights of 6300 Da, 9800 Da, 22000 Da, 49400 Da, 107000 Da, 201000 Da, 334000 Da, and 739000 Da (Shodex-Waters) were used as standards.

2.8. FTIR analysis of xylan

Hemicellulose solubilized from banana pseudostem by hydrogen peroxide and residue from the enzymatic hydrolysis was analyzed by Infrared (FTIR), using FTIR-IRAffinity-1S Shimadzu spectrophotometer (MARTINS et al., 2021).

2.9. Statistical Analysis

To evaluate the significance of differences among the mean values, an analysis of variance (ANOVA) was performed at 95% of significance level ($p < 0.05$) using the statistical software Statistica 10 for Windows.

3. Results and Discussion

3.1. Biomass Chemical Characterization

Chemical characterization of banana pseudostem was performed before and after partial delignification (Table 1) in order to evaluate chemical modification. Cellulose, hemicellulose, and lignin content in the raw biomass were 41.9%, 14.8%, and 14.02%, respectively. The results of cellulose and hemicellulose were similar to the one obtained by (DÍAZ et al., 2021), which demonstrated that untreated banana pseudostem contains 44.59% and 14.98% of cellulose and hemicellulose, respectively. Lignin content obtained by them was 7.92%, similar to the one obtained in the present study after the partial delignification. However, other authors reported a higher amount of these macromolecules in the banana pseudostem raw biomass, especially cellulose (BARUAH et al., 2022; SHIMIZU et al., 2018).

Table 1. Chemical characterization of banana pseudostem (BP) before and after partial delignification.

Biomass	Composition (%, dry base)		
	Cellulose/Glucan	**Hemicellulose**	**Lignin**
Raw BP	41.9 ± 1.18	14.8 ± 0.47	14.02 ± 0.17
Partial delignified BP	47.7 ± 0.89	16.3 ± 0.23	8.06 ± 0.33

The chemical composition of the macromolecules was different after partial delignification. The cellulose and hemicellulose percentage increased due the lignin content decreased in 6 percentual points. This indicates that the pretreatment removed part of the lignin, which can collaborate for enzymatic hydrolysis since lignin is a physical barrier to the action of enzymes because it prevents them to access the substrate (ZAMORA ZAMORA et al., 2021). In another study, after pretreatment of banana pseudostem with H_2O_2, cellulose amount increased as the reagent concentration increased, however, the opposite happened to hemicellulose and lignin, which had its content reduced by the increase in H_2O_2 concentration (SHIMIZU et al., 2018).

It can be noticed that raw banana pseudostem presents a low lignin content when compared with the chemical characterization of other raw lignocellulose biomasses. The amount of lignin found in corn stalk, sugarcane bagasse, guava bagasse, and orange bagasse were 25.3%, 20.0%, 33.75%, and 22.74%, respectively (BRIENZO et al., 2016b; PEREIRA et al., 2021; ZHAO et al., 2016). The low lignin content is a characteristic of the banana pseudostem since its structure does not present high rigidity. This is interesting because a simple and not severe pretreatment could help to remove lignin, transforming the biomass in order to obtain a higher yield of enzymatic hydrolysis.

3.2. Biomass enzymatic hydrolysis for XOS production

The enzymatic hydrolysis was performed using banana pseudostem (BP) in four different conditions: untreated, untreated and milled (Raw-BP), partial delignified (PD-BP), partial delignified and milled (PD-BM-BP). The milling and partial delignification were performed in order to improve the accessibility of the enzyme to the material. The milling process reduces particle size and increases the exposed surface area, while alkaline pretreatments reduce the lignin content (MELATI et al., 2019; SHIMIZU et al., 2020b; ZAMORA ZAMORA et al., 2021). In addition, four enzyme configurations were used: endoxylanase for 24 h, single reaction with endoxylanase and endoglucanase for 24 h, endoglucanase for 6 h followed by endoxylanase for 18 h, endoglucanase for 24 h followed by endoxylanase for 24 h. The combination of enzymes was applied to evaluate the joint action of the enzymes in a possible positive influence on biomass hydrolysis.

The milling process improved the enzymatic hydrolysis, however, the combined condition of partial delignification and milling doubled the conversion when compared to the hydrolysis of the raw material (Table 2). Regards the enzyme configuration, there was not much difference between the combined action of the enzymes and only using endoxylanase. In fact, in some cases, the conversion was lower using both enzymes. It is known that xylanase can be used as an auxiliary enzyme for the enzymatic hydrolysis of cellulose into glucose, and that this synergistic interaction shows significant improvement in cellulose accessibility by increasing fiber swelling and porosity (HU; ARANTES; SADDLER, 2011). However, the use of a cellulase as an auxiliary for enzymatic hydrolysis of xylan is still not a well-elucidated effect.

Table 2. Enzymatic hydrolysis of raw and partial delignified banana pseudostem for XOS production.

Biomass	Enzymatic hydrolysis	XOS (g/L)					
		X2	X3	X4	X5+X6	Total	Conversion (%)
Raw BP	**Ex**	-	1.06	-	0.18	1.24	41.82 b
	Ex + Eg	0.04	0.76	-	0.23	1.02	34.61 a, b
	Eg 6h + Eg	0.01	0.62	-	0.21	0.84	28.48 a
	Eg 24h + Ex	0.91	0.37	-	-	1.28	43.10 b
Raw milled BP	**Ex**	-	2.01	-	0.14	2.15	72.63 c, d, e
	Ex + Eg	0.03	1.86	-	0.12	2.01	67.77 c, d, e
	Eg 6h + Eg	0.03	1.59	-	0.05	1.67	56.48 b, c, d
	Eg 24h + Ex	0.85	1.16	-	-	2.01	67.90 c, d, e
Partial delignified BP	**Ex**	0.05	1.98	-	0.51	2.53	77.66 d, e, f
	Ex + Eg	0.07	1.23	-	0.51	1.81	55.37 b, c, d
	Eg 6h + Eg	0.07	1.74	-	0.44	2.25	69.11 c, d, e
	Eg 24h + Ex	1.72	0.61	-	-	2.33	71.24 c, d, e
Partial delignified and milled BP	**Ex**	0.07	2.03	-	0.75	2.85	87.31 e, f
	Ex + Eg	0.11	1.84	-	0.76	2.71	83.19 d, e, f
	Eg 6h + Eg	0.12	1.63	-	0.86	2.61	80.21 d, e, f
	Eg 24h + Ex	0.62	2.32	-	-	2.94	90.23 f

Different letters indicate significant differences (Tukey test, 95% of confidence level, $p < 0.05$) between treatments.

X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose.

Ex: single endoxylanase for 24 h; Ex + Eg: both endoxylanase and endoglucanase for 24 h; Eg 6h + Eg: single endoglucanase for 6 h followed by single endoxylanase for 18 h; Eg 24h + Ex: single endoglucanase for 24 h followed by single endoxylanase for 24 h.

The endoglucanase action did not collaborate to improve xylan hydrolysis, since there was no significant difference between the conversion in hydrolysis using only endoxylanase and both enzymes in any of the biomasses. Hemicellulose forms a network on the cellulose surface, and hydrolysis with cellulases and endoxylanase did not contribute to the process of XOS production. On the other hand, it is recognized the positive effect of hemicellulose removal/hydrolysis previously to cellulose hydrolysis (ZHOU; TIAN, 2022). However, there was a significant difference between the conversion of the raw BP and the raw milled BP, also between raw BP and pretreated BP, suggesting that both the milling process and the partial delignification were able to modify the lignocellulose structure, improving enzyme accessibility.

The digestibility of xylan in the untreated sugarcane bagasse (composed of 21.65% lignin and 22.95% xylan) after enzymatic hydrolysis using both enzymes (endoglucanase and endoxylanase) was only 7.55% (HUANG et al., 2018). Differently, in the present study 41.82% of xylan was hydrolyzed using a biomass with only 14% lignin. After partial delignification, sugarcane bagasse lignin content was also reduced to 8% and xylan digestibility after hydrolysis with both enzymes went up to 63.54%, a similar result to the one obtained in the present study, of 55.37%.

The result of total XOS produced by the hydrolysis with the combined action of endoxylanase and endoglucanase represents the total amount of oligosaccharides produced, since the molecular weight of oligomers from cellulose (COS) and from xylan is similar and their retention time in the chromatogram can overlap. The amount of COS produced after the hydrolysis with only endoglucanase for 24 h previously to the addition of endoxylanase was 0.35 g/L, 0.43 g/L, 1.10 g/L, and 1.26 g/L, resulting in a conversion of 4.29%, 5.27%, 11.73% and 13.37% for the raw BP, raw milled BP, pretreated BP, and pretreated milled BP, respectively (results not shown).

The highest conversion using only endoxylanase was 87.31% obtained by the enzymatic hydrolysis of pretreated and milled banana pseudostem. Although this is a high conversion, the concentration of XOS obtained was low when compared to the concentration of XOS achieved with xylan previously solubilized from banana pseudostem, in a similar hydrolysis condition. After the biomass hydrolysis, 2.85 g/L of XOS was obtained, while after xylan hydrolysis, 10.48 g/L of XOS was produced with a conversion, an increase of almost 4 times, with a conversion of 58.0 % (DE FREITAS et al., 2021). Thus, it can be stated that for large-scale XOS production, the extraction of xylan from biomass is advantageous.

3.3. Xylan enzymatic hydrolysis for XOS production with high substrate concentration in two cycles

Substrate concentration influences the conversion rate and concentration of XOS produced by enzymatic hydrolysis. Moreover, for an industrial scale is important to produce a high concentration of products. As the substrate concentration increased, the XOS concentration also increased but the conversion decreased (Table 2). XOS with a higher degree of polymerization such as xylotetraose, xylopentose, and xylohexose were mainly produced. In contrast with biomass hydrolysis, there was xylotetraose production. The solubilized xylan certainly is more exposed to enzyme action compared to the biomass. Probably, this is also related to the difference in the XOS yield, due to the better enzyme action in the solubilized xylan.. Water is an important element when it comes to enzymatic hydrolysis since it’s the reaction medium in which the enzymes, substrate, and the products resulting from hydrolysis will be present. The interaction between water and substrate influences the mass transfer during hydrolysis and by increasing the substrate concentration, also the medium viscosity is increased, limiting the mass transfer (SANT’ et al., 2020).

The decrease in XOS production yield with the increase in substrate concentration was already expected since the same result was obtained in a previous study. In addition, using the same conditions and increasing reaction medium volume by 5 times, the conversion obtained in the present study increased by 33.42%, 31.46%, 27.82%, 19.15%, and 25.48% in the substrate concentration of 2%, 4%, 6%, 8%, and 10%, respectively (DE FREITAS et al., 2021). This indicates that the reaction volume is also an important parameter that must be considered in order to improve the enzymatic hydrolysis.

Table 3. Xylan enzymatic hydrolysis in two cycles, with diverse substrate concentration for XOS production.

Enzymatic hydrolysis	Xylan[1] Concentration (%)	XOS (g/L)					
		X2	X3	X4	X5+X6	Total	Conversion (%)
1st hydrolysis	**2**	0.25	0.52	8.98	4.94	14.68 a	73.41
	4	0.44	1.06	12.09	11.10	24.70 a, b	61.74
	6	0.80	0.34	16.24	13.02	30.40 b	50.66
	8	1.19	0.38	19.11	15.31	35.99 b	44.99
	10	1.28	0.62	25.72	22.78	50.40 c	50.39
2nd hydrolysis	**2**	0.05	1.57	-	1.01	2.63 a	13.14
	4	0.1	1.34	-	1.99	3.43 a	8.58
	6	0.12	0.98	-	3.51	4.61 a, b	7.68
	8	0.27	1.30	-	7.83	9.40 b	12.70
	10	0.23	0.20	-	7.83	8.26 b	10.74
Total	**2**	0.30	2.09	8.98	5.95	17.31 a	86.55
	4	0.54	2.40	12.09	13.09	28.13 a, b	70.32
	6	0.92	1.32	16.24	16.53	35.01 b, c	58.35
	8	1.46	1.68	19.11	23.14	45.39 c, d	57.69
	10	1.51	0.82	25.72	30.61	58.66 d	61.13

Different letters indicate significant differences (Tukey test, confidence level of 95%, $p < 0.05$) between treatments. X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose. In the second hydrolysis the liquid fraction was removed and another dose of enzyme as added. 1. Xylan extracted from banana pseudostem

A second cycle of hydrolysis was performed removing the liquid fraction and a new dose of the enzyme (in buffer) was added. There was no production of xylotetraose and the conversion was lower with all substrate concentrations, an indication that the majority of hemicellulose was already hydrolyzed in the first hydrolysis. The main XOS produced were xylopentose/xylohexose. Looking at the total conversion, after the two hydrolysis cycles, the conversion with 6%, 8%, and 10% substrate was similar and acceptable. However, the amount of XOS produced with 10% substrate concentration was higher and statistically different, then more interesting to scale up the process.

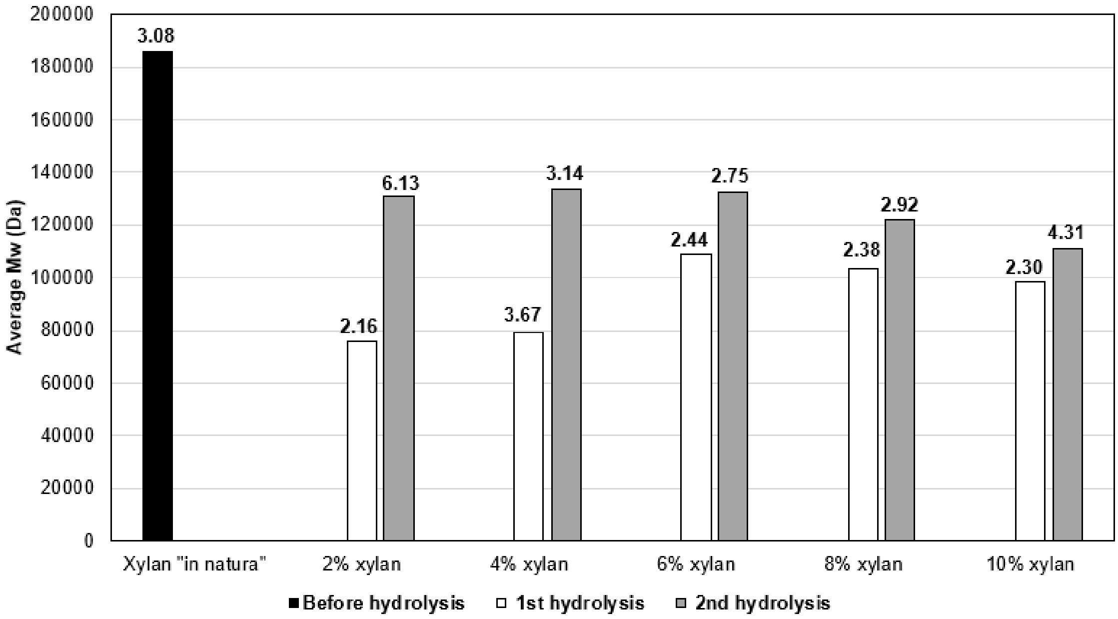
3.4. Xylan molecular weight over the enzymatic hydrolysis

The average molecular weight of xylan extracted from banana pseudostem before hydrolysis was 185 kDa (Figure 1). This result was higher than the ones reported for alkali-soluble birchwood, water-soluble, and alkali-soluble sugarcane bagasse xylan, which were 16.9 kDa, 31.5 kDa, and 75.9 kDa, respectively (JACOBS; DAHLMAN, 2001; XIE et al., 2020). However, the molecular weight of a commercial arabinoxylan from corn bran was reported as 253 kDa, higher than the result obtained in the present study (KALE et al., 2018). Xylan molecular weight will vary depending on its source and the process used for its solubilization. Perhaps, the molecular weight could influence several of the xylan application, and these properties deserve more attention.

In polymers, polydispersity refers to the variation in the length of molecular chains. A polydisperse polymer will have molecular chains of different lengths. This is often quantified using the Polydispersity Index (PDI), which is the ratio of the average molecular weight to the number average molecular weight. In Figure 1, polydispersity is indicated above each bar of the graph.

The analysis of the average molecular weight of residual xylan, after the hydrolysis aimed to evaluate whether the enzyme action would be different depending on the substrate concentration in the reaction medium. After the first hydrolysis, with all substrate concentrations, the average molecular weight decreased as expected due to the enzyme action breakdown of the xylan chain. The average molecular weight of residual xylan after hydrolysis with 2% and 4% substrate concentration was lower than that of hydrolysis with higher substrate concentration, indicating that the enzyme action was better in the lowest substrate concentrations. This could be due to the water content in the reaction mixture that can be reduced by a high concentration of substrate and an increase in the viscosity can cause enzyme inhibition (DE FREITAS et al., 2021). Therefore, probably a higher concentration of substrate, by increasing the medium viscosity, could hinder the enzymatic action and consequently, the production of XOS.

Figure 1. Molecular weight (Mw) and polydispersity of xylan after 24 h of enzymatic hydrolysis



Source: Author

After enzymatic hydrolysis, a decrease in arabinoxylan molecular weight was reported by the work of Kale et al. (KALE et al., 2018), however with the increase of enzyme load after 24 h reaction using a commercial endoxylanase. After the second hydrolysis, the average xylan molecular weight was higher than in the first hydrolysis for all substrate concentrations, which suggests that the enzyme hydrolyzed most of the small xylans present in the substrate and probably was not able to hydrolyze the larger ones. There are different mechanisms of action for a xylan-degrading enzyme to promote the breakdown of the xylan chain and one of them is an initial decrease in the degree of polymerization with the formation of short and soluble oligosaccharides (MOREIRA; FILHO, 2016).

A study reported sweet gum xylan (hardwood biomass) alkali solubilized and precipitated after glycerol thermal processing. Two xylan portions were isolated: a precipitated xylan (water insoluble xylan portion) and a water-soluble xylan. The degree of polymerization of the water insoluble xylan was over 3 times higher than of the water-soluble xylan (ZHANG et al., 2016). In the present study, the higher xylan molecular weight after the second hydrolysis was the insoluble xylan fraction, which remained unhydrolyzed, probably due to the difficult of enzymes act in its structure.

The concentration of wood xylan was reported to affect solubility. Comparing solutions of 1 g/L and 20 g/L, the most concentrated one presented a higher degree of heterogeneity (CARLSSON, 2022). In the present study, the 10% xylan solution probably corresponds to 20 g/L of the paper cited. Perhaps, an increase in the heterogeneity could be provoked by the increase in xylan concentration. Moreover, the enzyme action could be affected by the medium heterogeneity.

In the present study, the difficulty to completely hydrolyze the substrate is not related to enzyme factor since a new charge of enzyme showed low contribution in to improve the XOS yield (Table 4). The analysis of the substrate, on the other hand, showed that the xylan kept its structure after the first stage hydrolysis, with no evidence of recalcitrance increase. The molecular weight increased over the reaction, meaning that the enzyme acted first in the easiest part of the substrate. The increase in the molecular weight could be related to an increase in the insoluble fraction of the xylan, hindering the enzymatic hydrolysis.

Table 4. Distribution of xylan molecular weight after the first hydrolysis with different substrate concentrations.

Residual xylan Mw after the 1st hydrolysis											
Xylan before hydrolysis		**2% xylan**		**4% xylan**		**6% xylan**		**8% xylan**		**10% xylan**	
Mw (kDa)	**Fraction (%)**	**Mw (kDa)**	**Fraction (%)**	**Mw (kDa)**	**Fraction (%)**	**Mw (kDa)**	**Fraction (%)**	**Mw (kDa)**	**Fraction (%)**	**Mw (kDa)**	**Fraction (%)**
439	18.47	351	4.45	284	6.48	351	7.37	351	6.61	351	5.9
166	14.99	121	7.25	92	13.44	121	15.29	121	14.56	121	14.16
82	17.73	59	17.97	46	14.47	59	21.24	59	21.19	59	21.26
50	15.59	38	20.43	32	11.96	38	18.75	38	18.83	38	19.04
36	11.73	30	15.06	26	12.67	30	14.16	30	14.38	30	14.84
30	8.47	25	11.88	21	12.36	25	10.31	25	10.57	25	10.64
26	6.02	22	9.59	16	12.01	22	6.82	22	7.3	22	7.5
23	4.02	17	7.38	10	9.1	17	3.66	17	3.88	17	3.97
20	2.67	12	4.84	4	5.44	12	1.84	12	2.03	12	2.02
16	0.31	6	1.15	1	2.07	6	0.56	6	0.65	6	0.67

The initial xylan polydispersity was 3.08, and it decreases after the first hydrolysis for the most substrate concentrations (Figure 1). A different result was reported by Kale et al. (2018), observing an increase in xylan polydispersity after enzymatic hydrolysis. In the present study, a Mw can be observed with more details regards to the amount of xylan per Mw fraction. Observing Table 4, which shows the distribution of xylan molecular weight after the first hydrolysis, it can be noticed that xylan before hydrolysis presented more chains with different molecular weights, whereas a fraction of 18.47% consisted of a molecular weight of approximately 439 kDa. After the first hydrolysis, xylan with molecular weights between 25-120 kDa were obtained in higher quantity and the highest molecular weight obtained was only a 7.37 % fraction of 350 kDa xylan in 6% substrate concentration. The lowest molecular weight fraction found in xylan before hydrolysis was 16 kDa, while after hydrolysis, for almost all substrate concentrations, the lowest molecular weight fraction found was 6 kDa, a reduction of more than half in the Mw.

After the second cycle hydrolysis, the polydispersity increased since a wide range of molecular weight xylan was obtained, mainly ranging from 27 kDa to 306 kDa (Table 5). Also, the fraction of the higher molecular weight xylan found in each of the substrate concentrations increased when compared to the first hydrolysis. This is another indication that the enzyme is breaking down the xylan into lower molecular weight fractions, but the higher the molecular weight of the xylan, the more difficult it is for the enzyme to perform this process. The first hydrolysis enzymes worked resulting in a decrease of the Mw, and also producing short-chain oligomers. However, in the second hydrolysis, more XOS were produced probably by enzyme action on the easiest fraction of the xylan. The complex substrate for hydrolysis were remaining as a substrate with limited enzyme action. This high Mw xylan perhaps could contain pending groups that hindered the endoxylanase action. Another possible explication could be related to residual lignin, which could difficult enzyme action. For lignocellulose hydrolysis lignin is enriched over the cellulases action, resulting in a material more recalcitrant after the enzymatic process (WALLACE et al., 2016).

Table 5. Distribution of xylan molecular weight after the second cycle hydrolysis with different substrate concentrations.

2nd hydrolysis											
Xylan before hydrolysis		**2% xylan**		**4% xylan**		**6% xylan**		**8% xylan**		**10% xylan**	
Mw (Da)	**Fraction (%)**	**Mw (Da)**	**Fraction (%)**	**Mw (Da)**	**Fraction (%)**	**Mw (Da)**	**Fraction (%)**	**Mw (Da)**	**Fraction (%)**	**Mw (Da)**	**Fraction (%)**
439	18.47	254302	18.16	306	14.93	351	11.74	284	11.83	261	11.07
166	14.99	79786	19.38	101	19.42	121	18.2	92	22.02	82	21.9
82	17.73	41243	19.03	50	19.6	59	20.66	46	23.6	42	23.5
50	15.59	29449	15.15	34	14.77	38	16.61	32	16.34	30	15.89
37	11.73	24354	11.14	27	11.07	30	12.13	26	10.94	25	10.75
30	8.47	19558	7.82	23	8.34	25	8.82	21	7.33	20	7.01
26	6.02	12787	5.16	19	6.39	22	6.36	16	4.83	14	4.65
23	4.02	5707	2.12	13	3.48	17	3.64	10	1.66	7	2.68
20	2.67	1458	0.94	6	1.87	12	1.14	4	1.13	2	1.4
16	0.31	179	1.1	2	0.13	6	0.7	1	0.32	0.2	1.15

A FTIR analysis was applied for a structural evaluation of the xylan after the enzymatic hydrolysis, with the aim of observe structural modification that could hinder the enzyme action. Xylan structure remain similar after hydrolysis and the different substrate concentration did not alter the xylan structure (Figure 2). The main bands observed were typical for this polysaccharide, for example, the band at 900 cm^{-1}, which represents the β-glycosidic linkages between the xylose units. This band appeared in low intensity only in hydrolyzed xylans. The band at 1043 cm^{-1} is assigned to C-O stretching in C-O-C linkages (KONG et al., 2018). The band at 1175 cm^{-1} could be attributed to arabinosyl side chains, suggesting that the pending groups are still present in the enzymatic hydrolysis residual xylan. Lignin presence (residual) normally is observed at 1510 cm^{-1}, however, this band was not observed in the xylan. On the other hand, a band at the region of 1340 cm^{-1} was observed, and it can be attributed to syringil ring present in the lignin structure. An intense band was observed at 1650 cm^{-1} that can be assigned to C=O groups. Hydrogen peroxide in alkaline medium can provoke oxidation of the xylan chain, generating hexenuronica acids (BRIENZO; SIQUEIRA; MILAGRES, 2009b). The absorption in the region of 1500-1800 cm^{-1} would not be noticiable in a polymeric chain consisting of pure xylopyranose units. Therefore, the absorption in this region was due to another substituent in the main ou side chain of the xylan (hexenuronica acid), or water adsorbed (VENA et al., 2013). Perhaps, an intensity increase at 1650 cm^{-1} could suggest the presence of a recalcitrant fraction of the xylan chain.

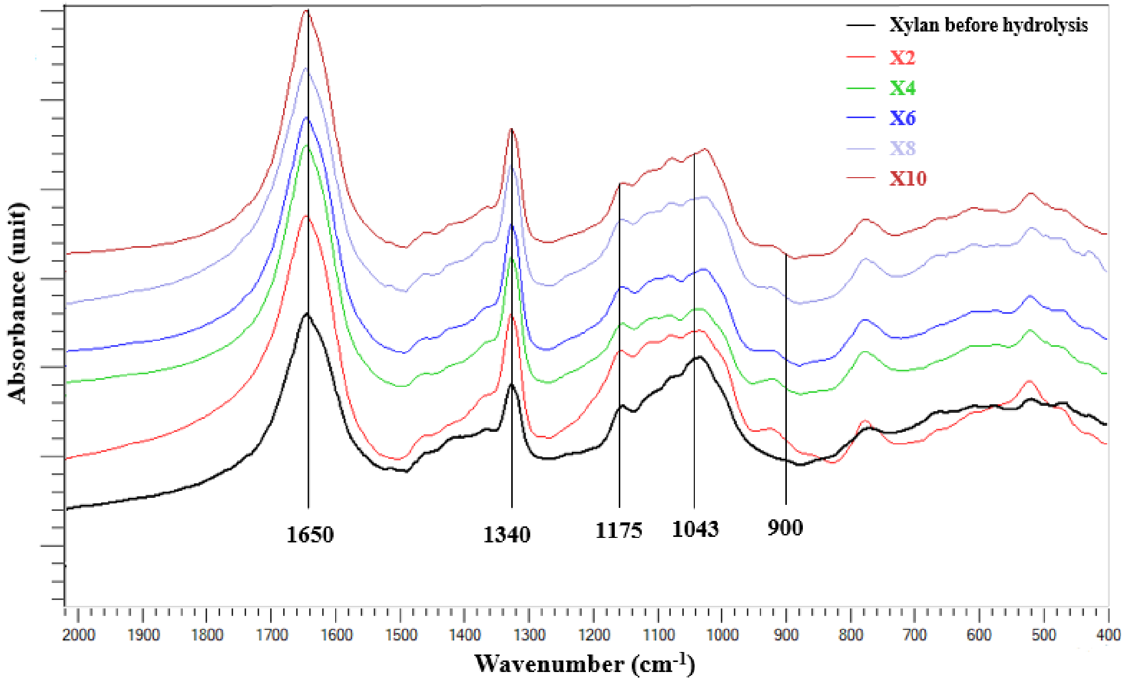


Figure 2. FT-IR spectra of xylan before and after hydrolysis. X2: 2% xylan, X4: 4% xylan, X6: 6% xylan, X8: 8% xylan and X10: 10% xylan

Source: Author

4. Conclusion

The partial delignification reduced the lignin content of banana pseudostem biomass almost by half. Both the milling process and delignification were important to improve the xylan hydrolysis yield. However, there was no significant difference between the use of only endoxylanase and its combination with endoglucanase for xylan hydrolysis applied to the biomass. Despite high yield conversion (percentage), the enzymatic hydrolysis of banana pseudostem biomass did not produce a high concentration of XOS.

The increase in the substrate concentration led to a decrease in yield but an increase in XOS concentration improved the conversion into XOS. After first enzymatic hydrolysis, xylan molecular weight and polydispersity decreased, producing XOS. However, in the second cycle hydrolysis, both molecular weight and polydispersity increased with a low increment in the XOS yield. The present study showed that xylan solubilization previous to the hydrolysis results in better yield and concentration compared to enzyme applied to the biomass.

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CHAPTER 5: EFFECT OF ACID HYDROLYSIS BY-PRODUCTS (PRESENT IN XYLOOLIGOSACCHARIDES SOLUTION) ON THE PROBIOTIC MICROORGANISMS GROWTH

Abstract

Xylooligosaccharides are xylose oligomers and potential prebiotics that can be produced from acid hydrolysis of lignocellulosic biomass. Probiotic bacteria are able to ferment them and produce metabolites beneficial for human health. The production of XOS from the acid hydrolysis of lignocellulosic biomass, as well as the viability of probiotic bacteria in a medium containing XOS as a carbon source, was investigated. XOS from banana pseudostem biomass were produced by dilute acid hydrolysis using 2% sulfuric acid. Compounds produced by pseudostem acid hydrolysis for XOS production were analyzed in culture of this XOS medium with probiotic bacteria. Other products like lactic acid, and acetic acid, produce by lactic acid bacteria in culture, also was analyzed. An amount of 14 g/L of XOS were produced with a yield of 88% with acid hydrolysis. The organic acids, in almost all concentrations, completely inhibited the growth of *Lactiplantibacillus plantarum* 1462 and *Lacticaseibacillus paracasei* 2218. Formic acid presented the most severe effect on *B. breve* and *B. longum,* only allowing growth with 1 g/L. There was a production of acetic, lactic, and formic acid by the bifidobacteria in the medium where they were able to grow similarly to the control, in which the concentration of organic acids increased. Both *Lactobacillus* were able to grow at high concentrations of furfural, on the other hand only at 1 g/L of HMF. The bifidobacteria grew in all concentrations, however, the growth was lower as the concentration increased, showing the HMF inhibitory effect. The concentration of furfural and HMF was reduced in all media the microorganisms were able to grow, indicating they could consume it. When cultured using XOS produced through acid hydrolysis as the carbon source, all investigated microorganisms exhibited growth. While the majority of them were unaffected by phenolic compounds removal from XOS solution, the removal of extractives prior to acid hydrolysis led to a significant enhancement in growth. The most remarkable growth was observed in *Lc. paracasei* 2218, which thrived best in a medium containing unfiltered XOS without extractives. This growth was equivalent to 79% of that observed in the same medium when using xylose as the carbon source. Considering these findings, XOS generated through acid hydrolysis could serve as effective prebiotics, stimulating the growth of probiotic microorganisms. **Keywords:** furfural, hydroxymethylfurfural, organic acids, by-products, *Lactiplantibacillus, Bifidobacterium*.

1. Introduction

Xylooligosaccharides (XOS) are xylose oligomers (2-10 sugar units) with prebiotic potential since they are oligosaccharides non-digestible by the human digestive system and can selectively increase and stimulate the growth of beneficial microorganisms, such as *Bifidobacterium* and *Lactobacillus* (FREITAS; CARMONA; BRIENZO, 2019; PALANIAPPAN; ANTONY; EMMAMBUX, 2021c). They can be applied in food for fiber content and viscosity improvement (LEDDOMADO et al., 2021), in pharmaceutical industry as antioxidants, cholesterol-lowering, anti-hyperglycemic and to balance intestinal microbiota (LIM et al., 2018; SALEH et al., 2021), and in the livestock industry as dietary supplementation for farming animals (ZHOU et al., 2021). Such technological and nutritional applications increase XOS global demand, promoting more research on different and efficient ways to produce them (CORIM MARIM &GABARDO, 2021).

Xylan is the substrate used for XOS production and can be found in different lignocellulosic materials, such as agroindustrial residue. Their use to produce XOS is environmentally and economically advantageous (PINALES-MÁRQUEZ et al., 2021). An interesting lignocellulosic biomass obtained from fruit farming is the banana pseudostem. Produced after banana harvesting, for each ton of bananas obtained, 3 tons of pseudostem are generated (FERNANDES et al., 2013). As reported by the Food and Agriculture Organization of the United Nations (FAO), Brazil's banana production reached 6.64 tons in 2020, being the 3rd largest banana producing country in the world (FAOSTAT, 2020). A large amount of waste is generated from the production of banana, which is largely consumed in the world, however, there are few studies on the use of this residue (FREITAS; BRIENZO, 2022; PEREIRA et al., 2022).

XOS production can be carried out by physical-chemical and/or enzymatic treatments. Since the use of enzymes can be a drawback because of its cost, diluted acid for xylan hydrolysis is an interesting way to produce XOS (PALANIAPPAN; ANTONY; EMMAMBUX, 2021c). Xylan chain breakage by acid hydrolysis can occur through random chain attack, reducing the degree of polymerization and releasing oligomers, or through depolymerization, which depends on chain size and releases monosaccharides. The acids most commonly used in this process are sulfuric, hydrochloric, acetic, and phosphoric acids. Through direct acid hydrolysis of the biomass, the hemicellulose is solubilized, generating oligomers and pentose in the liquid fraction, and an insoluble fraction, rich in cellulose and lignin. The acid concentration should be low, in order to avoid the hydrolysis of hemicellulose into monomers, such as xylose, and consequently the release of compounds from its degradation (BRIENZO et al., 2016b; FORSAN et al., 2021). However, as this method can also produce xylose and lignocellulose biomass pretreatment can release by-products, such as acetic acid, furfural, and hydroxymethylfurfural (HMF), an optimal condition of acid concentration, reaction time and temperature must be used to increase the amount of XOS produced without increasing the amount of the unwanted products (FORSAN et al., 2021; FREITAS; CARMONA; BRIENZO, 2019).

Furfural and HMF are by-products genearated during the acid hydrolysis of lignocellulosic materials, and they are considered the most toxic compounds among fermentation inhibitors in lignocellulosic hydrolysates (BECERRA et al., 2022; CANDIDO et al., 2021). These unwanted by-products can induce several effects such as a reduction of cell growth rate, interference with fermentative enzymes, diminishment of cell membrane permeability, breakdown of DNA, among others (MUÑOZ-PÁEZ et al., 2019). However, studies have shown that some bacteria, including probiotics, are resistant to these compounds, and can even consume and transform them into other bioproducts (MATOS et al., 2016; VAN NIEL et al., 2012). In addition, acids used for the reaction can be toxic to the cells. Acetic acid is a weak organic acid whose toxic effect results mainly from its dissociation inside microbial cells, causing a decrease in intracellular pH, while formic acid, formed from the degradation of furfural, can inhibit the enzyme cytochrome c oxidase, an important protein of the electron transport chain of microbial cells (TRČEK; MIRA; JARBOE, 2015).

The by-products generated from the diluted acid process for XOS production, as well as the acid itself, can be inhibitors of probiotic growth, preventing XOS prebiotic action. Considering the increase in the demand for XOS as a market product and the importance of research to improve its production, this study aimed to evaluate if the XOS produced by diluted acid could be used as a prebiotic solution. For that, the use of XOS by the probiotics in the presence of acids and by-products that can be generated from the process was evaluated. The inhibitory role of by-products compounds on the probiotics was evaluated with a wide range of concentration.

2. Material and Methods

2.1. Dilute acid hydrolysis for XOS production

The dilute acid hydrolysis assay was performed using 5 g of banana pseudostem biomass in 50 mL of sulfuric acid 2% (m/v) at 80 ºC for 35 min, according to optimized condition (FORSAN et al., 2021). The hydrolysis was performed with and without the previous removal of biomass extractives (8 h ethanol + 8 h water in Soxhlet). After the reaction time, the heating was stopped by adding the samples in an ice bath. The hydrolysate was separated from the solid residue by vacuum filtering with filter paper (FORSAN et al., 2021). In the liquid fraction were quantified XOS and by-products generated.

2.2.Probiotics growth on media with inhibitors

2.2.1. Microorganisms

Lactiplantibacillus plantarum (CBMAI 1462) and *Lacticaseibacillus paracasei* (CBMAI 2218) were obtained from the Brazilian Collection of Environmental and Industrial Microorganisms – CBMAI (State University of Campinas). Microorganisms were maintained as stock culture in a solid MRS medium at 4 ºC.

Bifidobacterium longum BB-05 Danisco and *Bifidobacterium breve* BB-03 Danisco were obtained from the Laboratory of Industrial Biotechnology (São Paulo State University – Assis, São Paulo, Brazil). Microorganisms were maintained as stock culture in solid MRS medium with 0.05% cysteine at 4ºC.

2.2.2. Growth medium and microorganisms growth

Formulated MRS medium (DE MAN; ROGOSA; SHARPE, 1960) was prepared and autoclaved for 20 min at 121 ºC. Cysteine 0.05% was added to the medium for *Bifidobacteria*. Each of the compounds considered inhibitors in this study (lactic acid, acetic acid, formic acid, furfural, and hydroxymethylfurfural) was added to the medium in different concentrations (10, 7.5, 5, 2.5 and 1 g/L). The strains were grown at 37 ºC for 72 h in MRS media in a 96 well-plate, with agitation for *Lactobacilli* and without agitation for *Bifidobacteria*. Triplicates were performed for each concentration. Optical density (OD) at 630 nm was measured every 15 min in the Tecan Sunrise™absorbancemicroplate reader.

2.3. Microorganisms growth using XOS produced by diluted acid hydrolysis as carbon source

To evaluate the prebiotic action of XOS produced by dilute acid hydrolysis, the microorganisms were inoculated in MRS media added with XOS (filtered and non-filtered) as carbon source. Glucose and xylose were used as controls. The strains were grown at 37 ºC for 96 h in a 96 well-plate, with agitation for *Lactobacilli* and without agitation for *Bifidobacteria*. Quadruplicates were performed for each concentration. Optical density (OD) at 630 nm was measured every 15 min in the Tecan Sunrise™absorbancemicroplate reader.

2.4. Compounds quantification

2.4.1. Determination of inhibitors concentration

To determine the concentration of lactic acid, acetic acid, and formic acid (medium formulation after microorganism growth) a BIO-RAD Aminex HPX-87H column was used in High Performance Liquid Chromatography (Waters Equipment). The following conditions were used: temperature of 50 ºC; 0.6 mL/min flow of sulfuric acid 0.05 mol/L and refractive index detector.

For furfural and hydrolymethylfurfural (HMF) determination (medium formulation after microorganism growth) a XBridge C18 3.5µm (4.6 x 50mm) column was used in Ultra Performance Liquid Chromatography (UPLC – Waters Equipment). The conditions used were: 35 ºC temperature; 0.8 mL/min flow of water/acetonitrile (8:1) with 1% acetic acid and PDA detector at 274 nm.

2.4.2. Determination of XOS and by-products concentration

Xylooligosaccharides content from the hydrolysate and microorganisms growth medium (after 72 h) was determined by HPLC (Waters) using BIO-RAD Aminex HPX-87C (300 x 7.8 mm) column at temperature of 80 ºC; 0.6 mL/min flow of deionized water and index refractor detector. Production of by-products (furfural and HMF) was determined using the same method as described in item 2.2.

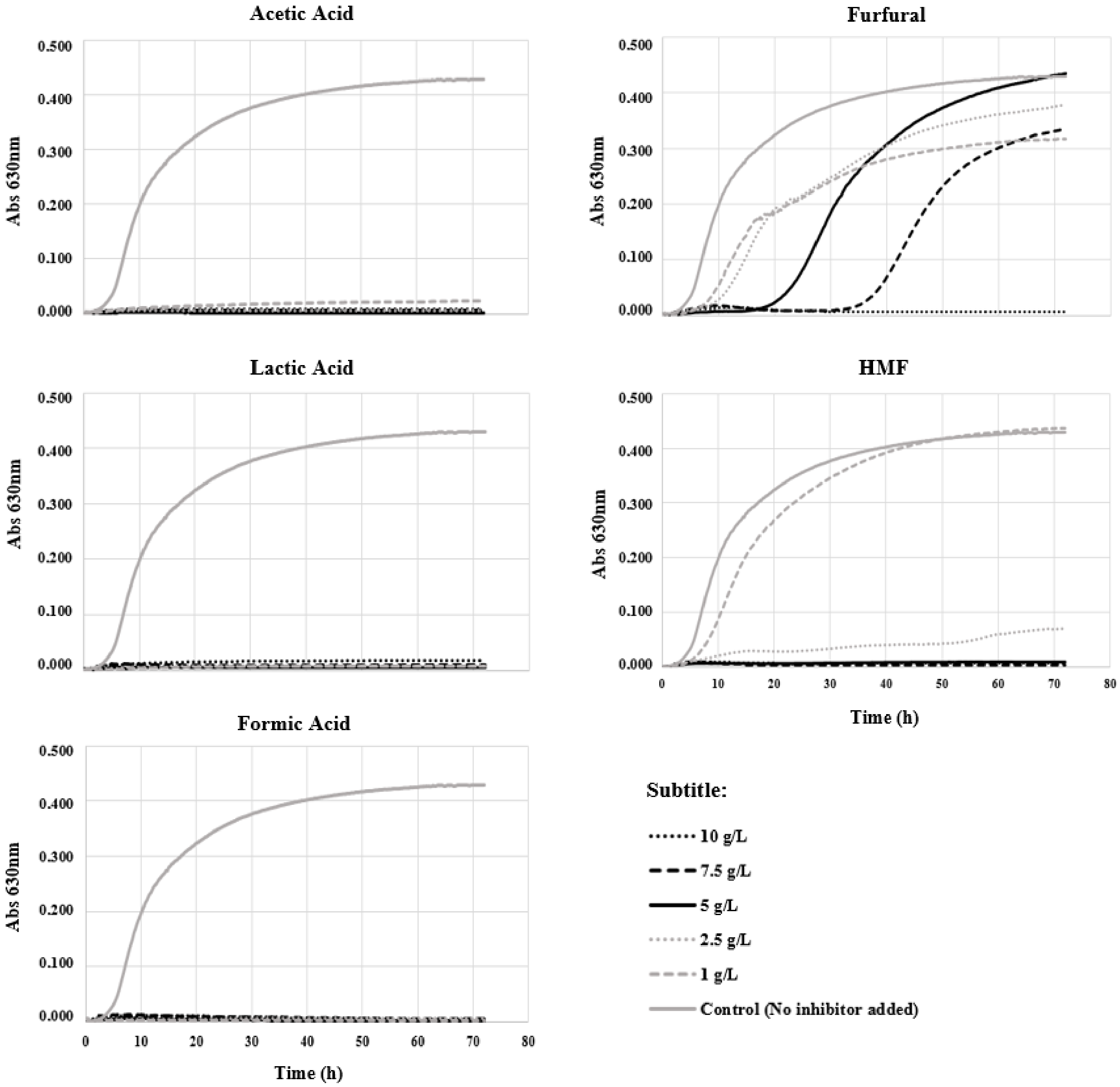
3. Results and Discussion

3.1. Probiotics growth on media with compounds related to acid hydrolysis of biomass

The inhibitory effect of five different compounds on the growth of four probiotic bacteria was evaluated. Furfural and HMF are sugar degradation by-products that can be generated in the production of XOS by dilute acid hydrolysis of biomass. Formic acid can be formed by furfural degradation, and acetic acid, for being a xylan pendant group, can be released in the biomass acid hydrolysis. Besides that, the acetic acid also can be used for biomass hydrolysis, replacing sulfuric acid, with a high production of oligomers and low formation of sugar degradation by-products. The lactic acid is a metabolic by-product, produced by the microorganism itself, which can also be inhibited by it depending on the concentration.

Lc. plantarum 1462 was not able to grow in media containing lactic, formic, and acetic acids even with the lowest concentrations of 1 g/L evaluated (Fig 1). At all concentrations tested, furfural slowed the bacteria growth, with total inhibition at 10 g/L. The higher the furfural concentration, the longer it took for the growth to achieve the exponential phase. At 5 g/L growth took about 20 h to begin the exponential phase, however, it equaled the control (no inhibitor) after 72 h. The only tested concentration of HMF in which the bacteria were able to grow was 1g/L, matching the growth of the control after 72 h. The medium with 2.5 g/L of HMF provoked a growth of around 20% related to the control assay.

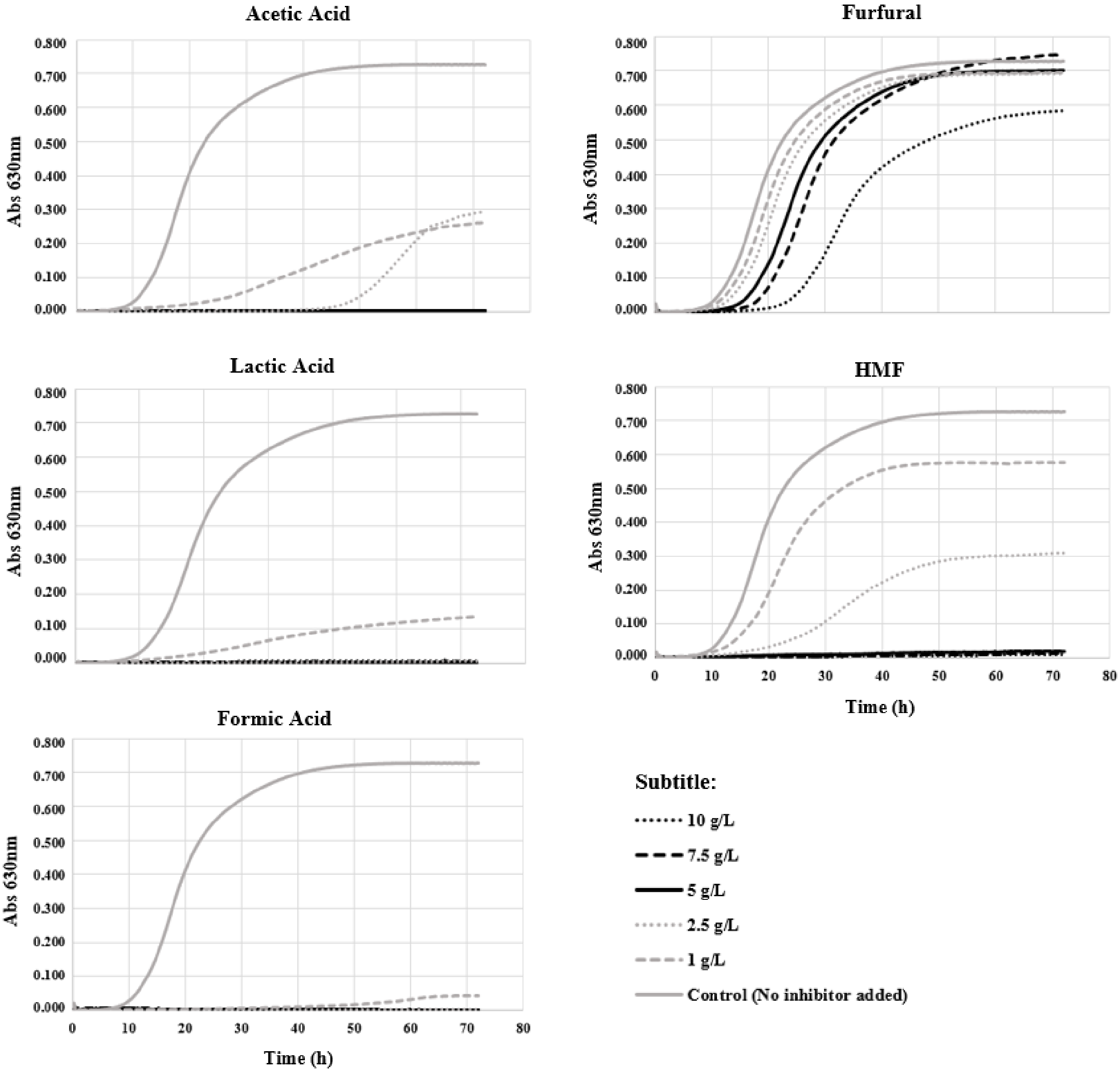
Fig 1. Effect of compounds related to acid hydrolysis (acetic acid, furfural, HMF, and formic acid) and microorganism metabolite (lactic acid) on the growth of *Lactiplantibacillus plantarum* 1462



Growth at 37ºC in MRS media with agitation for 72 h.

Formic acid in all concentrations evaluated inhibited the growth of *Lc. paracasei* 2218 (Fig 2). The same happened for the growth with acetic and lactic acid, which occurred mildly and delayed, compared to the control, only at the lowest concentrations of 2.5 g/L and 1 g/L. The bacteria were able to grow in the presence of furfural, reaching similar growth as the control, in all concentrations tested. On the other hand, the growth in the presence of HMF occurred only at concentrations of 2.5 g/L and 1 g/L, approximately 42% and 79%, respectively, of the control growth.

Fig 2. Effect of compounds related to acid hydrolysis (acetic acid, furfural, HMF, and formic acid) and microorganism metabolite (lactic acid) on the growth of *Lc. paracasei* 2218



Growth at 37ºC at MRS media with agitation for 72 h.

The main mechanisms by which organic acids can influence the growth of microorganisms could be indirectly by reducing medium pH, or directly by penetrating the bacterial cell and altering its physiological homeostasis (LUISE et al., 2020). Acetic, lactic, and formic acids can inhibit the growth of microorganisms by entering the cell, where the pH is 7, in its non-dissociating form, dissociating in the cell and decreasing the cytoplasm pH. Consequently, could provoke inactivation bacterial enzymes, inhibiting intracellular metabolic reactions such as synthesis of macromolecules, leading to slower or non-growth and disruption of internal membranes (LUISE et al., 2020; PARHI; SONG; CHOO, 2022).

Studies showed that the medium pH can influence the growth of *Lactobacillus* in general. *Lactobacillus plantarum* was was able to grow at pH 5, 6 and 7, however, at pH 4 its growth was inhibited. Another probiotic, *Lactobacillus salivarius*, showed lower levels of survival and no cell viability after 6 h exposure to pH 2.6 and the same happened with *Lactobacillus rhamnosus* after 24 h subjected to pH 2(SANHUEZA ET AL., 2015; VERA-PEÑA & RODRIGUEZ, 2020). For *Lactobacillus casei*, the initial pH of culture medium had an impact on growth rates, significantly decreasing growth at pH 4 and 3. Transmission electron microscopy analysis revealed morphological differences between the cells grown at pH 6.5 and 4. At pH 6.5, the analysis showed a typical bacterial cell surface structure, a well-defined cell wall and membrane, however, the cell under acid stress (pH 4) presented alterations in the cell surface structure (NEZHAD; DJ; ML, 2010).

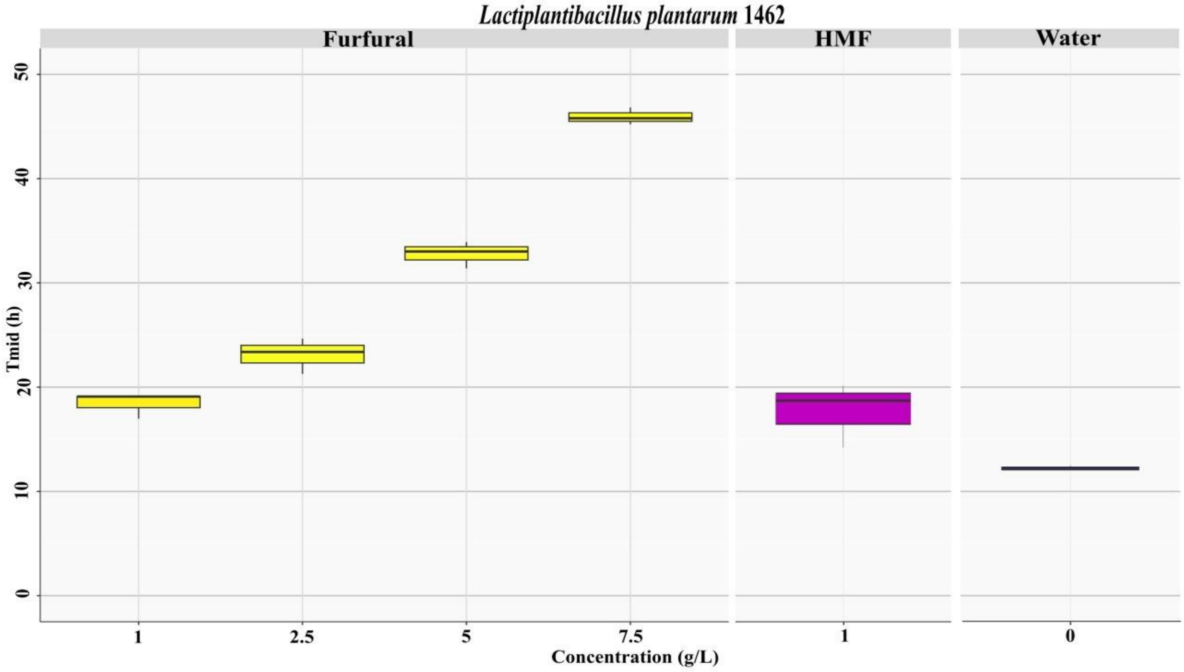
In the present study, the addition of organic acids to the culture media changed the pH. The initial medium pH 6 went down to 4.3, 3.9, and 3.7 for acetic, lactic and formic acids, respectively, with 10 g/L solution concentration. Since the pH of the medium with furfural and HMF was not altered in any of the concentrations, the pH could have an influence on the growth of the microorganisms.

Several studies demonstrate that some *Lactobacillus* strains are able to tolerate certain furfural and HMF concentration. A concentration of 8 g/L of furfural and 6 g/L of HMF did not change the growth of *Lc. plantarum* strain compared to the control, differently from the *Lc. plantarum* 1462, which had its growth reduced in almost all concentrations of furfural and only tolerated HMF at 1 g/L. Strains of *L. brevis* and *L. pentosus* were able to tolerate and grow well on media containing 7 g/L furfural but showed complete inhibition on media containing 10 g/L HMF (BOGUTA et al., 2014;

TU et al., 2019), similar to what occurred with the two Lactobacillus strains used in the present study.

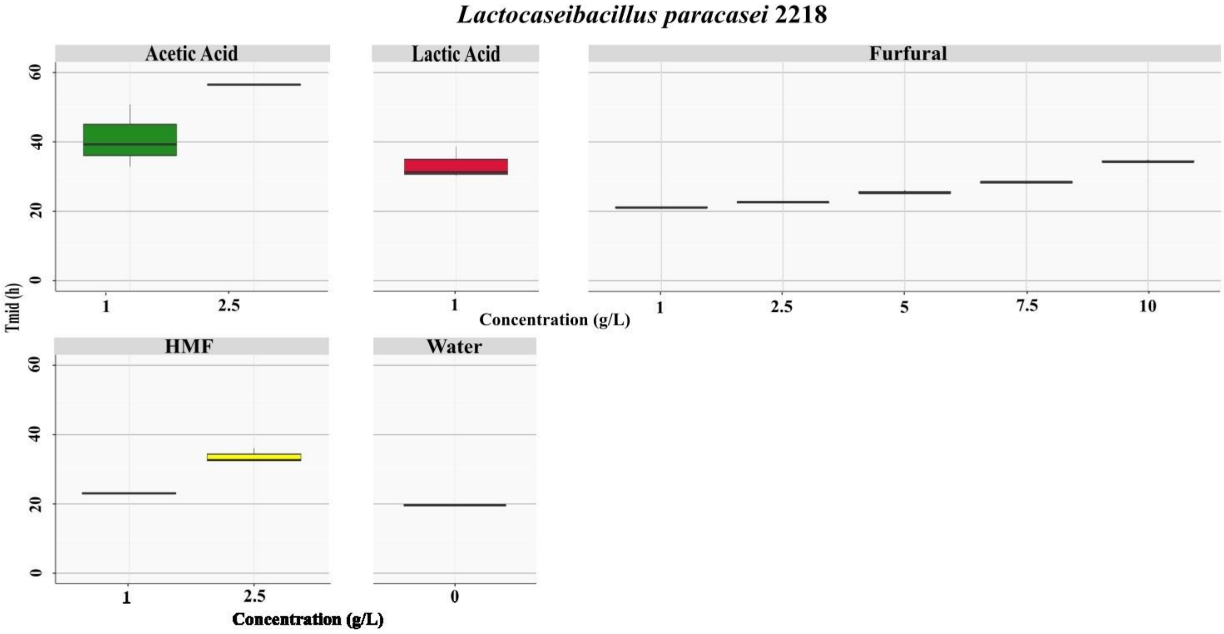
The average time it takes for a microorganism to reach half of the maximum absorbance (Tmid), that is, maximum growth, was evaluated for the conditions where growth occurred. For *Lc. plantarum* 1462 (Fig 3), Tmid of growth without inhibitors (water) was around 12 hours. With the addition of only 1% of furfural and HMF, Tmid increased to 18 h. Regarding furfural, the higher its concentration, the longer it took to reach half of the maximum optical density (OD), which reached over 45 h at a 7.5 g/L compound concentration, despite the similar maximum OD after 72 h. In Fig 4, Tmid of *Lc. paracasei* 2218 was displayed and it can be observed that this probiotic took around 20 h to reach half of its maximum OD in a medium with no inhibitor. Acetic acid was the inhibitor that had the greatest effect on Tmid, as with a 2.5 g/L concentration of the compound, this parameter nearly tripled. Furfural and HMF at concentrations of 1 g/L showed a Tmid similar to that of water. All the compounds used affected the growth of *Lactobacillus*, either completely or partially inhibiting it, or affecting the rate of its growth.

Fig 3. Tmid of *L. plantarum* 1462 growing in media with furfural and HMF



Source: Author

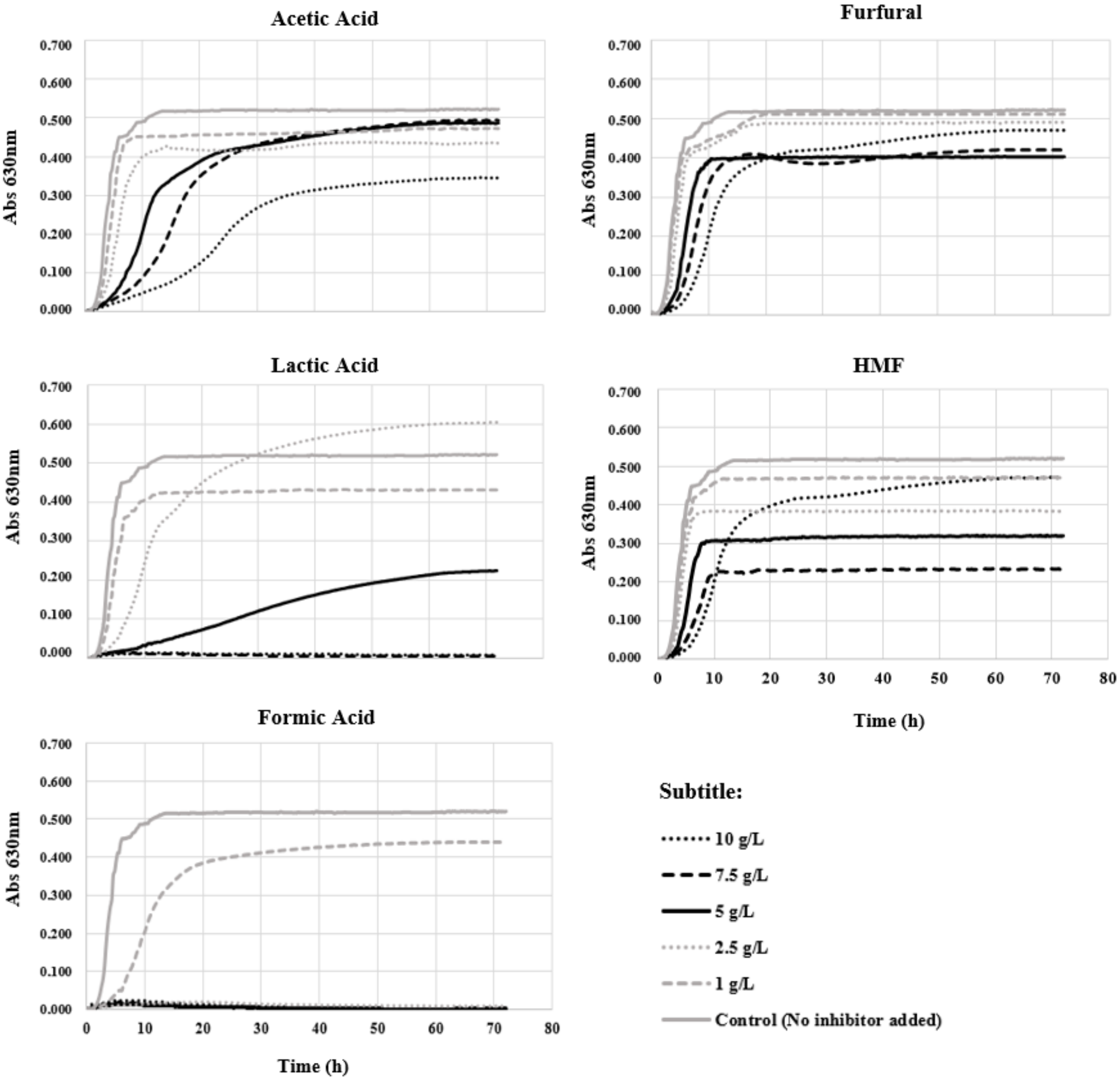
Fig 4. Tmid of *L. paracasei* 2218 growing in media with acetic acid, lactic acid, furfural and HMF.



Source: Author

The growth of *B. breve* in acetic acid decreased at the higher concentration of 10 g/L compared to the control. However, in the other concentrations of the organic acid the growth was not significantly altered but it took longer to reach the exponential phase in higher concentrations (Fig 3). Inhibition of growth occurred in the media with 10 g/L and 7.5 g/L of lactic acid, however, growth in 1 g/L was higher than the control. Formic acid inhibited the growth in all concentrations, except in 1 g/L. Furfural and HMF did not inhibit growth, however, the higher concentrations of these by-products, decreased growth. The only exception was growth at 10 g/L of furfural and HMF, which was similar to the growth at 1 g/L.

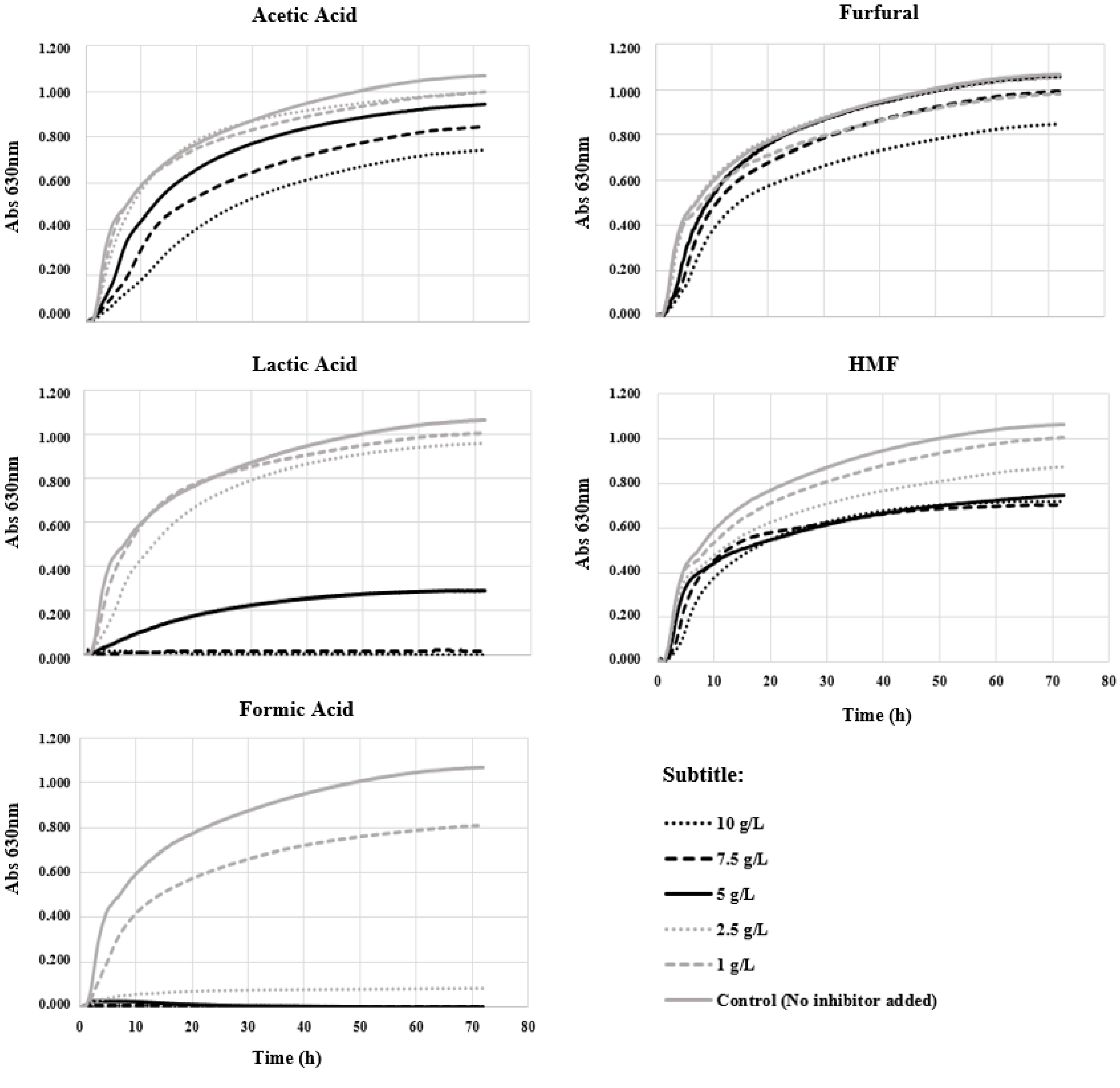
Fig 5. Effect of compounds related to acid hydrolysis (acetic acid, furfural, HMF, and formic acid) and microorganism metabolite (lactic acid) on the growth of *Bifidobacterium breve.*



Growth at 37ºC in MRS media (added with 0.05 L-cysteine) without agitation for 72h.

The interference of the organic acids and sugar degradation by-products on the growth of *B. longum* was similar to the interference on the growth of *B. breve*. The higher the concentration of acetic acid, the higher the decrease in growth compared to the control (Fig 4). The growth was inhibited in the concentration of 10 g/L and 7.5 g/L of lactic acid and in the presence of formic acid growth only occurred at 1 g/L concentration. Furfural and HMF did not inhibit growth, however, lower concentrations of these sugar degradation by-products allowed higher growth.

Fig 6. Effect of compounds related to acid hydrolysis (acetic acid, furfural, HMF, and formic acid) and microorganism metabolite (lactic acid) on the growth of *Bifidobacterium longum*



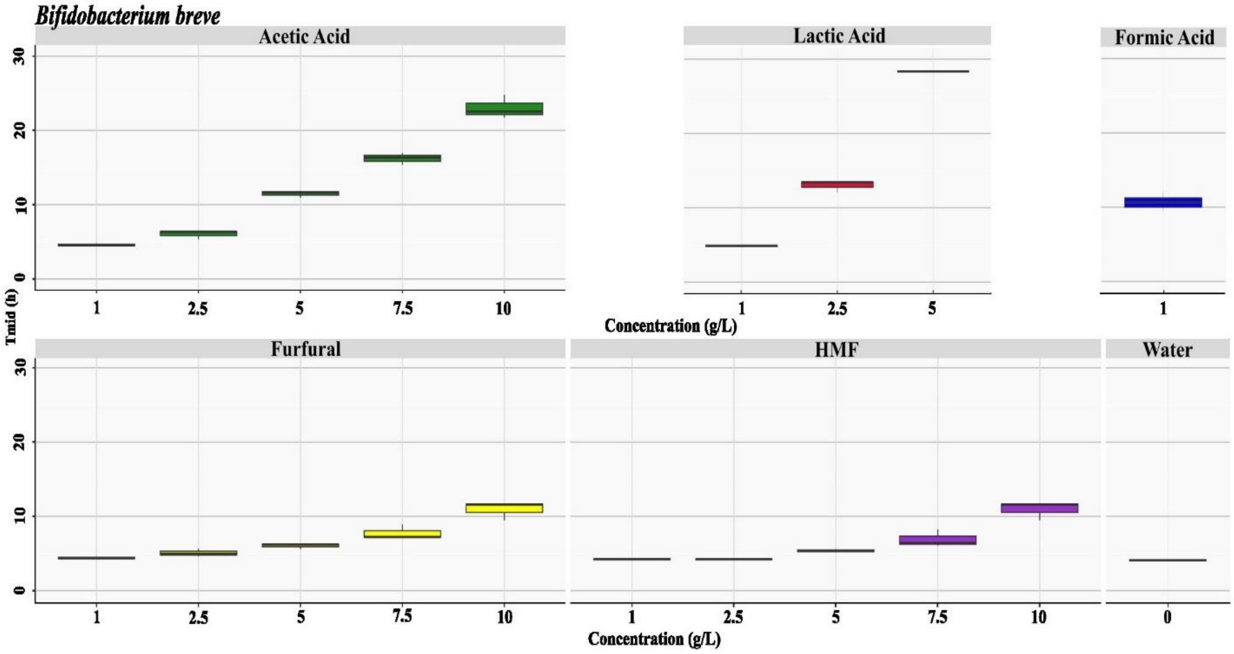
Growth at 37ºC in MRS media (added with 0.05 L-cysteine) without agitation for 72h.

Bifidobacteria grow optimally within pH range 6.5 to 7, however, *B. longum* and *B. breve* have the potential to resist low pH. Strains of both these bacteria showed 50% of cell viability in pH below 4 and presented a small change in growth after the addition of 1 g/L acetic acid in culture media (Parhi et al., 2022; Vlková et al., 2015). Adaptation and survival at low pH can determine the efficacy of *Bifidobacterium* strains as probiotic microorganisms and acid-pH-resistant strains can be originated by exposing them to a medium with acid conditions. Acid-resistant *B. longum* presented viability of 94%, 77%, and 44% when exposed to pH 4, 3.5, and 2.5, respectively (SAARELA et al., 2011; SÁNCHEZ et al., 2007). *Bifidobacterium bifidum* was able to grow in lignocellulosic biomass hydrolysate media containing 2 g/L furfural, 0.8 g/L HMF, 0.7 g/L acetic acid, and 5.3 g/L formic acid (RAJAN et al., 2021).

Depending on where the *Bifidobacterium* strains were isolated from, e.g. if they were isolated from an acid environment, they can present acid and low-pH tolerance. In addition, Bifidobacteria are known to have the ability to produce exopolysaccharides (EPS) under stressful conditions such as acidic pH, providing tolerance and contributing to cell protection and survival. Production of EPS by different strains of *B. longum* and *B. breve* induced by glucose, lactose, and fructose, thus improving tolerance against low pH has been reported (ALP & ASLIM, 2010; PARHI ET AL., 2022).

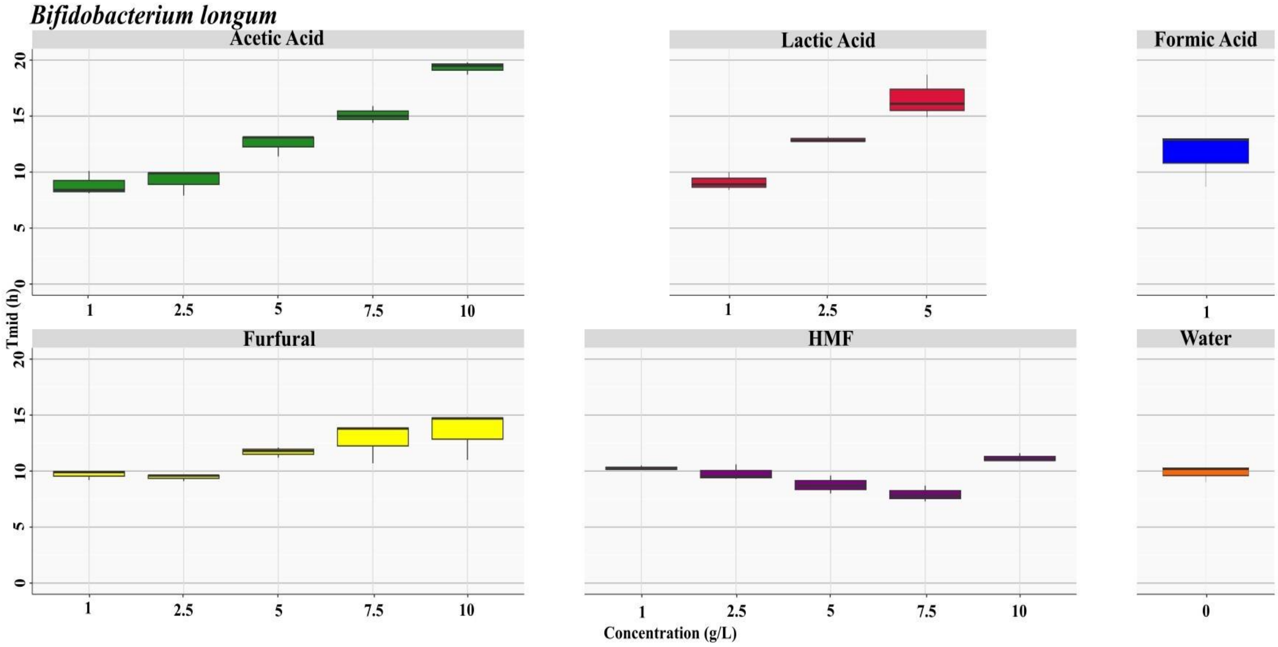
Regarding the time to reach half maximum OD, both *B. breve* and *B. longum* (Figs 7 and 8), at a concentration of 1 g/L in all the compounds, except formic acid, and 2.5 g/L furfural and HMF, obtained a Tmid similar to control experiment, reaching 5 h and 10 h, respectively. The growth of *B. breve* in the highest concentration of furfural and HMF took double the time to reach half the maximum OD. The higher the concentration of the compound, the longer it took to reach half of the maximum OD, except in the growth of *B. longum* in HMF, where at concentrations of 2.5, 5, and 7.5 g/L, this parameter was even lower than that of control experiment. In general, for both bacteria, organic acids showed a greater growth delay compared to furfural and HMF, especially in the higher concentrations.

Fig 7. Tmid of *B. breve* growing in media with acetic acid, lactic acid, formic acid, furfural and HMF.



Source: Author

Fig 8. Tmid of *B. longum* growing in media with acetic acid, lactic acid, formic acid, furfural and HMF.

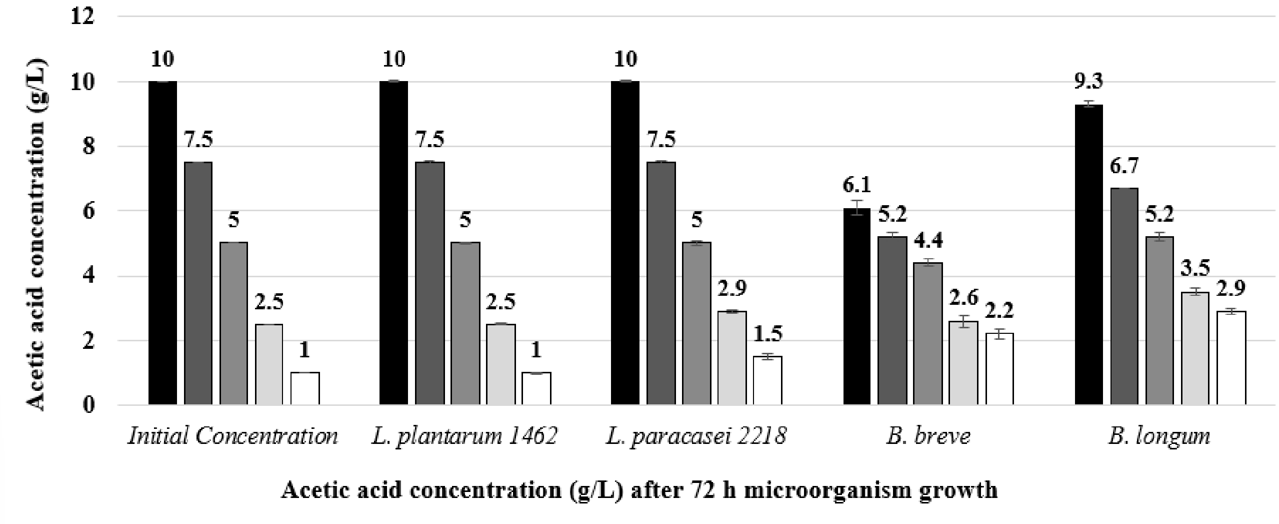


Source: Author

3.2. Concentration of the organic acids and sugar degradation by-products after microorganism growth

The concentration of the organic acids tested was not altered after the growth of both *Lactobacillus* strains (Fig 5, 6, and 7). This may indicate that the presence of the acid itself, lowering pH, was enough to inhibit or highly decrease the growth of these bacteria. However, after 72 h of the *Bifidobacterium* growth, the concentration of the organic acid presented alterations.

Fig 9. Concentration of acetic acid in the medium after 72 h growth of microorganisms



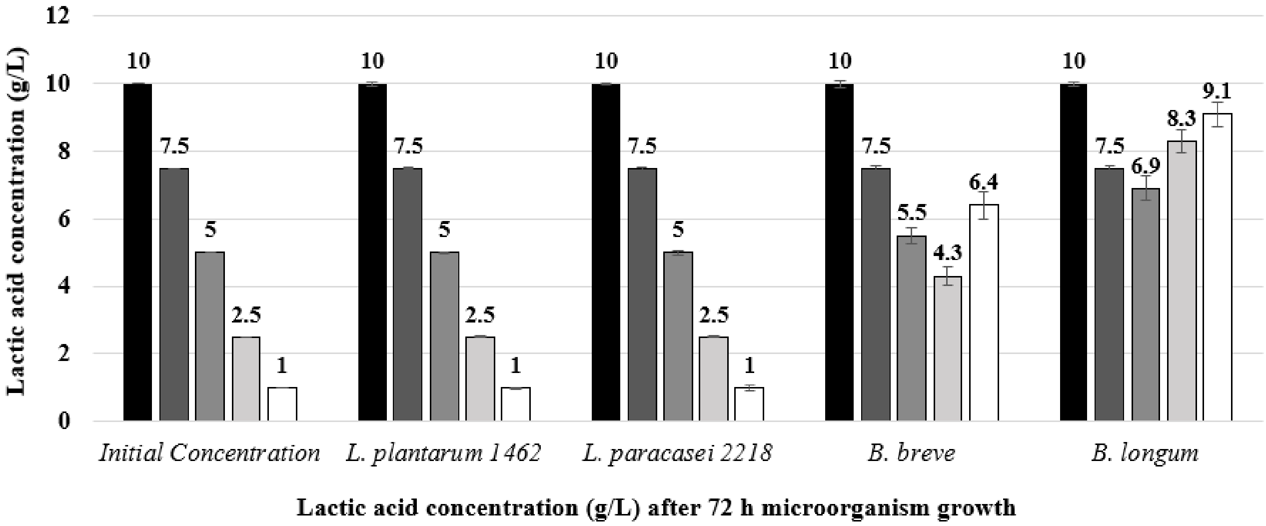
Initial concentration: concentration of acetic acid at time zero; The remaining bars refers to the concentration after 72h. Similar bar colors for comparison with respective initial concentration; The number above the bars represents the exact value.

Source: Author

Acetic acid had its concentrations of 10 g/L, 7.5 g/L and 5 g/L reduced to 6.1 g/L, 5.2 g/L, and 4.4 g/L, respectively, in the medium after *B. breve* growth (Fig 9). A similar occurred after *B. longum* growth, presenting a reduction in these same concentrations. The reduction of the acid concentrations could suggest that the acid penetrated the bacteria cells but not enough to harm it and inhibit growth and/or even the microorganism used it as a carbon source for growth. After the growth of both bifidobacteria at the concentrations of 2.5 g/L and 1 g/L, acetic acid concentration increased, and since they were able to grow well in these concentrations, this can indicate that they produced the exceeding amount.

A different profile was obtained by both bifidobacteria after growth in media with lactic acid. In the concentrations of 10 g/L and 7.5 g/L, in which these bacteria were not able to grow, the amount of lactic acid remained the same (Fig 10), indicating that this inhibition could have happened by the low pH medium. However, in the concentrations that were observed growth, an exceeding amount of lactic acid appeared, indicating the production of it by both *B. breve* and *B. longum*. It is well known that *Bifidobacterium* are able to ferment sugars such as glucose, and produce short-chain fatty acids, acetic and lactic acid among them (KAREENA et al., 2022; OLIVEIRA et al., 2012; PALFRAMAN; GIBSON; RASTALL, 2003a; USTA-GORGUN; YILMAZ-ERSAN, 2020).

Fig 9. Concentration of lactic acid in the medium after 72 h growth of microorganisms

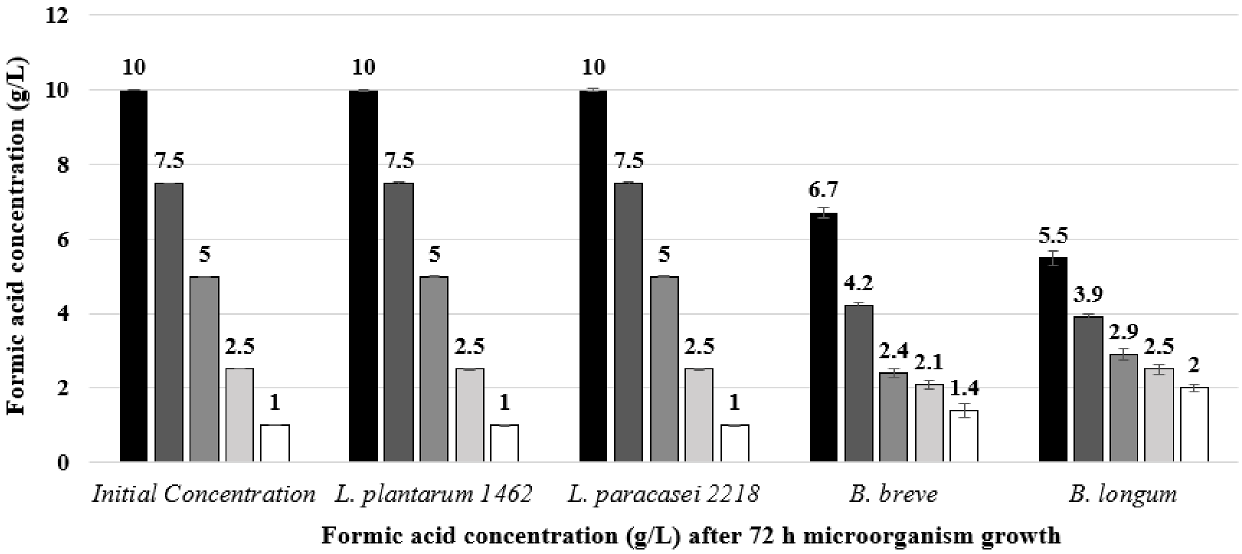


Initial concentration: concentration of acetic acid at time zero; The remaining bars refers to the concentration after 72h. Similar bar colors for comparison with respective initial concentration; The number above the bars represents the exact value.

Source: Author

In the concentrations of formic acid that did not allow the growth of *B. breve* and *B. longum*, after 72 h the amount of acid in the medium decreased (Fig 11), indicating that the formic acid might have penetrated the bacterial cell and inhibited its growth. Despite that, in the concentration of 1 g/L formic acid, where both bifidobacteria were able to grow, the amount of this organic acid in the media increased by 0.4 g/L after *B. breve* growth and 1 g/L after *B. longum* growth. Both of these bacteria have been reported to produce formic acid in small amounts, strains of *B. breve* and *B. longum* were able to produce 1.7 g/L and 0.3 g/L of formic acid, respectively in media with glucose as a carbon source. In addition, usually after the fermentation of carbon sources by Bifidobacteria, more than one organic acid can be produced. Both of these bacteria were able to produce, after fermenting glucose, different amounts of acetic, lactic, and formic acid simultaneously (RIOS-COVIAN et al., 2013). The same occurred in the present study in the concentrations of organic acids which allowed the growth of the bifidobacteria (results not shown).

Fig 10. Concentration of formic acid in the medium after 72 h growth of microorganisms



Initial concentration: concentration of acetic acid at time zero; The remaining bars refers to the concentration after 72h. Similar bar colors for comparison with respective initial concentration; The number above the bars represents the exact value.

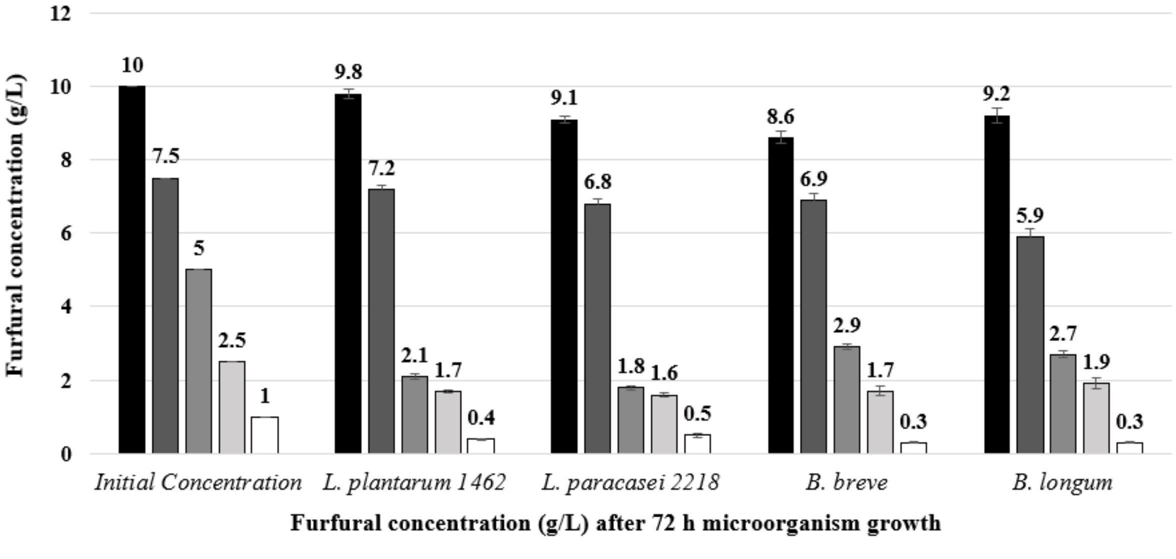
Source: Author

All microorganisms tested were able to grow at high concentrations of furfural, even though some concentrations decreased their growth compared to the control. After

72 h, the concentration of furfural decreases in all media (Fig 12), and this can indicate that these microorganisms not only tolerate it at high concentrations but are even able to use it. The same occurred with the HMF in the media where there was growth. This compound was reduced in the medium (Fig 13), indicating consumption by the bacteria.

A strain of *Lc. plantarum* was able to reduce 65% of furfural and 80% of HMF in a media, which initial concentration was 0.53 g/L and 0.425 g/L, respectively, after 60 h (ALVES DE OLIVEIRA et al., 2018). Strains of *Limosilactobacillus fermentum* and *Lacticaseibacillus paracasei* were also able to grow in media containing up to 2.5 g/L furfural and 4 g/L HMF, consuming these compounds, in some cases even completely (GIACON et al., 2022). The results present in this study showed that the highest reduction of furfural was 58% for *Lc. plantarum* 1492 at 5 g/L concentration and 64% for *Lc. paracasei* 2218 at the same concentration. Despite that, the highest reduction of HMF was performed by the bifidobacteria, which were able to reduce 90% and 98%, in the media with 1 g/L, by *B. breve* and *B.longum*, respectively.

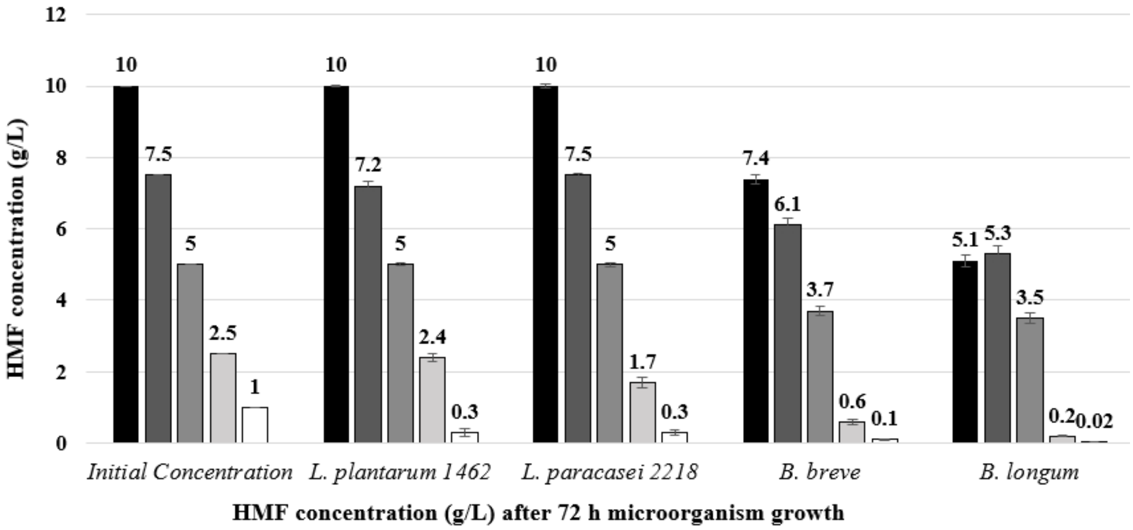
Fig 11. Concentration of furfural in the medium after 72 h growth of microorganisms



Initial concentration: concentration of acetic acid at time zero; The remaining bars refers to the concentration after 72h. Similar bar colors for comparison with respective initial concentration; The number above the bars represents the exact value.

Source: Author

Fig 12. Concentration of HMF in the medium after 72 h growth of microorganisms



Initial concentration: concentration of acetic acid at time zero; The remaining bars refers to the concentration after 72h. Similar bar colors for comparison with respective initial concentration; The number above the bars represents the exact value.

Source: Author

A *Lactobacillus reuteri*, on media with glucose as a carbon source, was able to use furfural (1 g/L), which was completely converted to furfuryl alcohol. Different bacteria of the bacillus genus were able to produce 2-furoic acid and 5-hydroxymethyl-2-furancarboxylic acid as the main degradation products of furfural and HMF, respectively (BECERRA et al., 2022; VAN NIEL et al., 2012).

The possibility that some of these microorganisms are able to grow in the presence of furfural and HMF, even in high concentrations, and also have a metabolic pathway to use and remove them from the medium is interesting for the production of XOS by acid hydrolysis of biomass. Since there would be the formation of these sugar-degradation by-products in the process and, if the microorganisms are not inhibited by them, depending on the concentration, a step to remove them would not be necessary. In Brazil, according to the Ministry of Agriculture, Ordinance No. 6, of July 25, 1985, the amount of HMF allowed in honey is 60 mg/kg. In addition, the *European Food Safety Authority* (*EFSA*) established that a safe amount of furfural in cosmetic products is up to 0.5 mg/kg. Perhaps, a certain concentration of furfural and HMF could be tolerable for a final product for human or animal consumption.

3.3. XOS and sugar degradation by-products production by diluted acid hydrolysis

Banana pseudostem (BPS) biomass was submitted to dilute acid hydrolysis for XOS production. The amount of XOS produced (Table 1) was similar using the biomass with and without extractives. Xylose was produced in small amounts, which is important since the goal is to produce XOS. The concentration of XOS obtained in the present study was lower than the one obtained using the same treatment with sugarcane bagasse, which was 24.5 g/L. However, the conversion was around 90%, similar to the one presented in Table 1 (FORSAN et al., 2021).

Table 1. Production of XOS and by-products from sugar degradation by dilute acid hydrolysis using 2% sulfuric acid

Biomass	Xylose (g/L)	XOS (g/L)	Yield (%)	Furfural (g/L)	HMF (g/L)
BPS with extractives	0.34 ± 0.01	14.72 ± 0.38	88.59 ± 2.22	0.027 ± 0.002	0.002 ± 0.001
BPS without extractives	0.19 ± 0.01	14.80 ± 0.52	88.17 ± 3.04	0.075 ± 0.004	0.007 ± 0.001

BPS: Banana pseudostem

The concentration of furfural and HMF produced were low for the condition applied, however using other treatment conditions these results can vary (FORSAN et al., 2021). A sugarcane bagasse hydrolysate that suffered an acid pretreatment using 0.5% (v/v) sulfuric acid, at 140 ºC for 15 min had up to 0.92 g/L furfural and 2.37 g/L HMF (ALVES DE OLIVEIRA et al., 2018). Taking into consideration the growth results of the microorganisms in the presence of furfural and HMF, they would be able to grow in the hydrolysate produced in this study.

3.4. Growth of probiotics using XOS produced from acid hydrolysis as carbon source

Xylooligosaccharides (XOS) were produced using both banana pseudostem with and without extractives. Additionally, the produced XOS underwent both filtered and unfiltered processes for phenolic compounds removal (furfual and HMF). The XOS produced by diluted acid hydrolysis were added to a growth medium as carbon source for the probiotics. Their growth was monitored for a period of 96 h, compared with glucose and xylose as carbon source.

Growth of *B. longum* (Fig 14) was not significantly influenced by either purification or removal of the extractives, although the highest growth occurred in the medium with filtered XOS (phenolic-free) without extractives. In all tested XOS conditions, the exponential growth phase extended until approximately 18 h, which was slightly shorter in the growth media with XOS containing extractives, both filtered and unfiltered. On the other hand, the growth of *B. breve* (Fig 15) was more influenced by the removal of extractives than by filtration, as the growth in both filtered and unfiltered XOS containing medium showed no significant difference. The exponential growth phase was longer, and the growth was greater in the medium containing XOS without extractives. For both microorganisms, the growth was significantly lower when compared to the glucose and xylose control groups. Growth of *B. longum* and *B. breve* using XOS as carbon source represented 26% and 40%, respectively, of the growth using xylose as carbon source.

Fig 13. Growth of *B. longum* on media containing XOS produced by dilute acid hydrolysis as carbon source

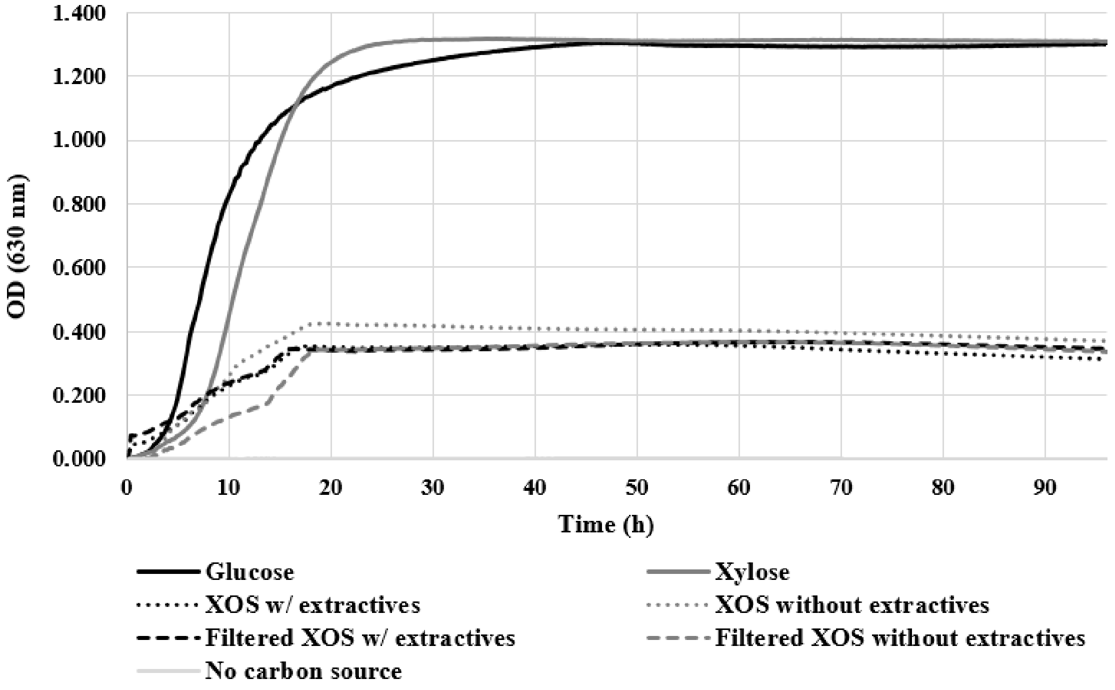
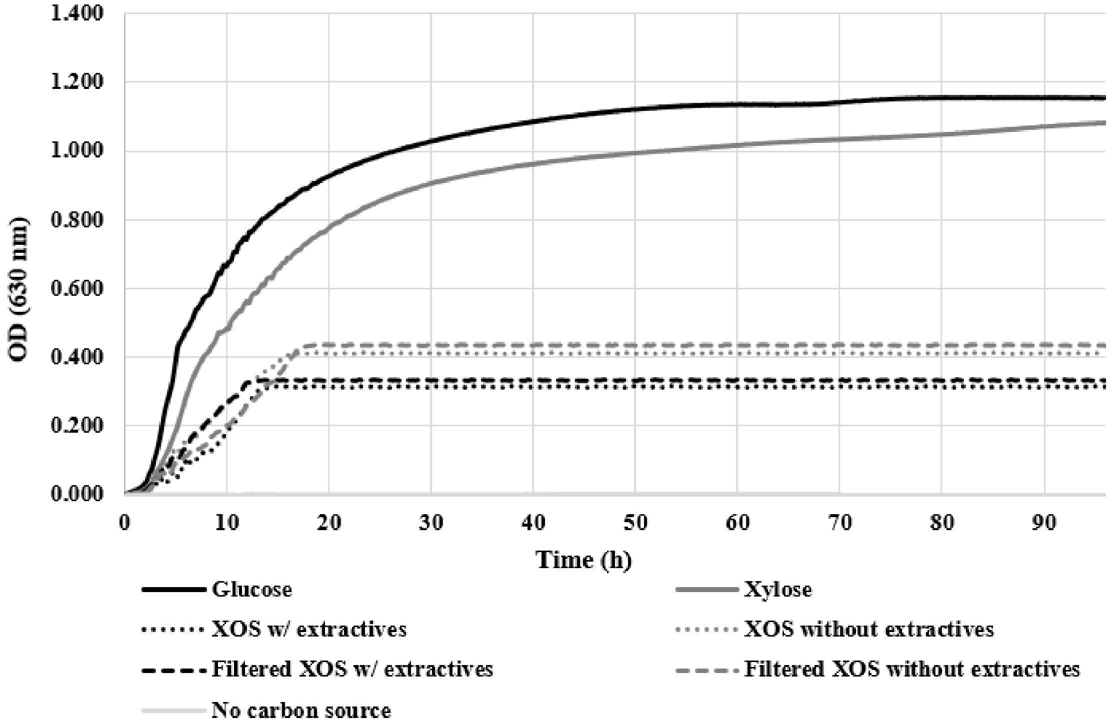


Fig 14. Growth of *B. breve* on media containing XOS produced by dilute acid hydrolysis as carbon source



XOS produced by acid hydrolysis using malic and citric acid were tested as carbon source for the growth of *Bifidobacteria adolescentis*. After 48 h growth in media with 2 g/L of XOS, optical density (600 nm) reached around 0.48 and 0.60, for malic and citric acid XOS, respectively (YAN et al., 2022b). The growth of the same bacteria, in media with commercial XOS as carbon source were much lower, approximately 0.15 (optical density at 650 nm). In addition, the growth of *B. lactis* and *B. infantis*, approximately 0.12 and 0.25, respectively (FIORELI, 2023), was also lower than growth of *B. breve* and *B. longum* in the present study.

Filtration of XOS had no effect on the growth of *L. plantarum* 1462 (Fig 16), however the removal of extractives improved 27.5% and 22% of growth, compared with filtered and unfiltered XOS, respectively. Even though it took longer to reach the exponential phase (approximately 15 h), the highest growth was on the media with unfiltered XOS without extractives, reaching OD (630nm) of 0.515, which represents 52% of growth using xylose as carbon source. Growth of *L. paracasei* 2218 followed a similar pattern since it also was lower on the media with filtered XOS. The highest growth was using unfiltered XOS without extractives as carbon source and after 96 h, the microorganism growth curve was still in the exponential phase. It represented 79% of the growth with media containing xylose as carbon source.

Fig 15. Growth of *L. plantarum* 1462 on media containing XOS produced by dilute acid hydrolysis as carbon source

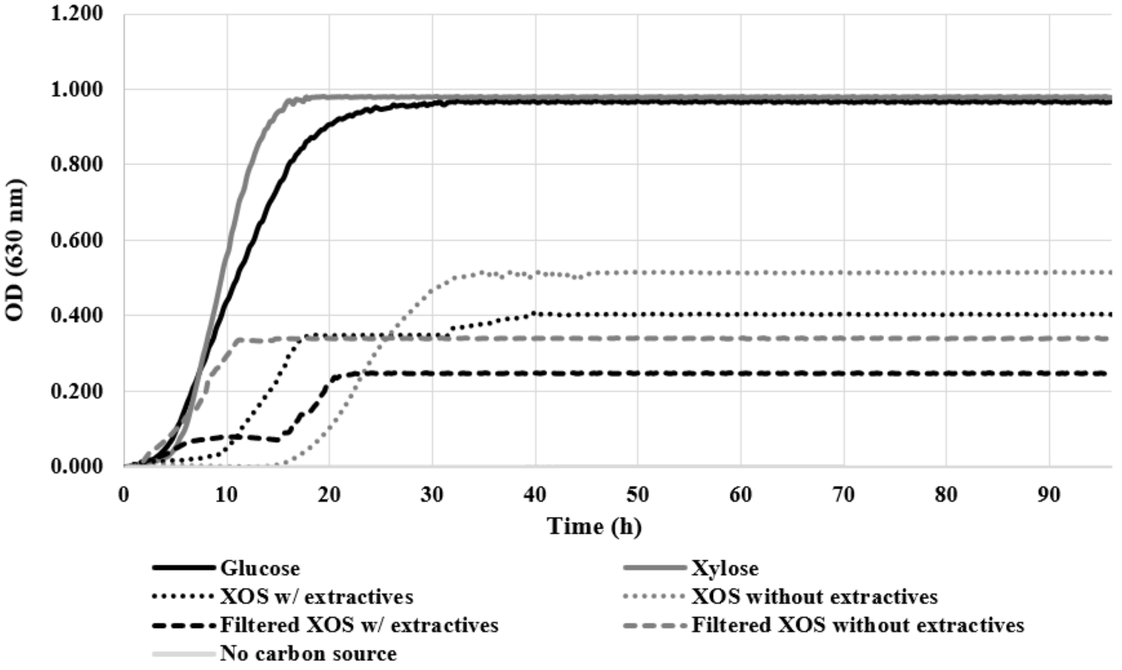
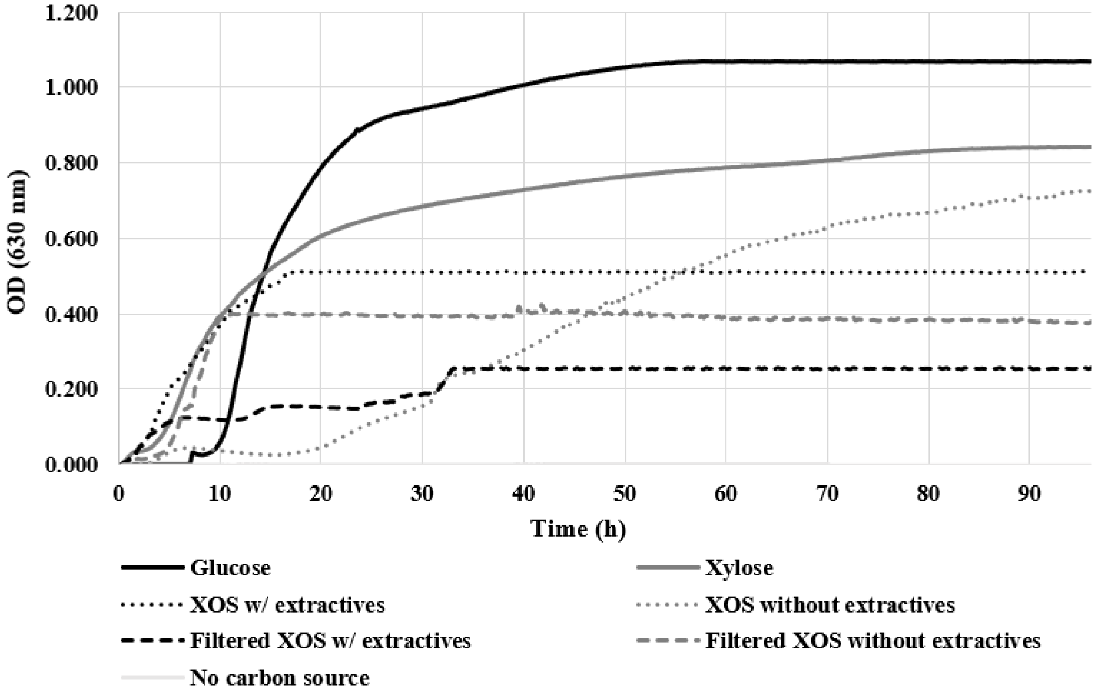


Fig 16. Growth of *L. paracasei* 2218 on media containing XOS produced by dilute acid hydrolysis as carbon source



Xylooligosaccharides generated through acid hydrolysis employing malic and citric acid were introduced into a growth medium for *L. acidophilus* cultivation. Following a 48 h incubation period, the optical density (600 nm) at a concentration of 2 g/L for malic acid-derived XOS reached 0.51, while citric acid-derived XOS exhibited an optical density of 0.4 at the same concentration (YAN et al, 2022). Furthermore, *Levilactobacillus brevis* demonstrated a capacity for growth with an optical density of 0.416 when cultivated in a medium fueled by corn fiber-derived XOS through autohydrolysis as the carbon source. Notably, when xylose was employed as the carbon source, the growth rate during the same timeframe was twice as high (SAMALA et al., 2015). The outcomes obtained from this study exhibited similarities, both in terms of growth capacity using XOS as carbon source and when compared to growth observed on xylose.

3.5. Estimative of production costs of XOS through diluted acid hydrolysis

The production cost of XOS through acid hydrolysis can be estimated by summing up the cost ratio of the necessary components for its production (Table 4).

Table 4. Price of necessary items for XOS production by diluted acid hydrolysis

Items	**Quantity**	**Price (R$)**
Sulfuric acid	100 mL	70.00
Biomass (banana pseudostem)	5 g	no cost
Power (water bath)	0.55 kW	0.85 kWh

The amount of sulfuric acid required for 50 mL of a 2% (w/v) solution is 0.54 mL, totaling R$0.38. The energy consumed in the water bath for the reaction to occur over a period of 35 minutes is approximately 0.3211 kWh, resulting in a cost of R$0.28. Taking this into account, the production of 0.7381 g of XOS, or approximately 14.8 g/L, incurred a cost of R$0.66.

4. Conclusion

The growth of probiotic bacteria is influenced by various factors, including organic acids, pH levels, and by-products of sugar degradation such as furfural and HMF. In our study, it was observed that bifidobacteria exhibited greater resistance to these compounds and acidic pH compared to the Lactobacillus strains used. Interestingly, as the concentration of these compounds increased, the growth of *Lactobacillus* decreased. Additionally, in some cases, the concentrations of acetic, lactic, and formic acid increased after the growth of bifidobacteria, suggesting that they also produced these organic acids.

Furthermore, both furfural and HMF inhibited the growth of certain microorganisms at specific concentrations. However, when these microorganisms were able to grow in the presence of these compounds, they also demonstrated the ability to metabolize and consume them. Notably, the microorganisms were able to thrive even in the presence of furfural and HMF produced during the acid hydrolysis process. In fact, when XOS produced through acid hydrolysis were used as the carbon source in the culture medium, all tested microorganisms exhibited growth.

While most microorganisms were not significantly affected by the filtration of XOS, the removal of extractives from the XOS resulted in a substantial improvement in their growth rates. Particularly, *Lc. paracasei* 2218 showed the highest growth in the medium containing purified XOS without extractives as the carbon source, achieving growth equivalent to 79% of that observed when xylose was used as the carbon source. In light of these findings, XOS generated through acid hydrolysis hold promise as effective prebiotics capable of promoting the growth of probiotic microorganisms.

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CHAPTER 6: SHORT-CHAIN FATTY ACID PRODUCTION FROM THE FERMENTATION OF XYLOOLIGOSACCHARIDES BY PROBIOTIC BACTERIA

ABSTRACT

XOS possess unique attributes such as resistance to digestion by human enzymes and gastric juice, making them promising prebiotics. Prebiotics selectively promote beneficial gut microorganisms, leading to the production of short-chain fatty acids (SCFAs) with diverse health benefits. This study aimed to generate XOS through enzymatic hydrolysis and explore the metabolites produced by probiotics during XOS fermentation. XOS were produced by enzymatic hydrolysis and were used as carbon source on media in different concentrations (5 – 30 g/L) to evaluate the growth of four probiotic microorganisms (*B. breve*, *B. longum*, *Lc. plantarum* 1462 and *Lc. paracasei* 2218). Their growth was evaluated in the presence of glucose, xylose and fructooligosaccharides as carbon sources. After 96 h, the production of metabolites (acetate, lactate, propionate and bytirate) was evaluated. Generally, growth of all microorganisms in the presence of xylose was directly proportional to the concentration. Bifidobacteria took longer to assimilate fructooligosaccharides since the exponential growth phase took longer to be reached. However, the concentration of this carbon source significantly affected growth only at the lowest concentration, which was also observed for *Lactobacillus*. For most of the microorganisms, XOS concentration that yielded the highest growth was 20 g/L. *B. breve* produced the highest amount of lactate during xylose fermentation, while acetic acid was most abundant during FOS fermentation. XOS fermentation resulted in significant butyric acid production. *B. longum* produced lactic acid predominantly with glucose, xylose, and FOS, but acetic acid was most prominent during XOS fermentation. Both *Lactobacillus* species produced lactic acid abundantly with glucose and FOS, while acetic acid was most abundant during xylose and XOS fermentation. Butyric acid production varied between the *Lactobacillus* species and carbon sources, and propionic acid was detected primarily after xylose fermentation. In conclusion, this study successfully demonstrated the potential of XOS as an effective carbon source for the growth of beneficial gut microorganisms, with distinct impacts on the production of key metabolites.

Keywords: xylooligosaccharides, prebiotics, probiotics, fermentation metabolites

1. Introduction

The biorefinery concept involves transforming biomass into valuable products. Hemicellulose, a crucial component of plant materials, can be readily hydrolyzed into safe substances like oligosaccharides and monosaccharides through biorefinery processes. In recent decades, non digestible oligosaccharides (NDO) have gained increasing commercial attention and interest. Within the various categories of NDOs, xylan oligosaccharides (XOS) stand out as an emergent food ingredient derived from feasible and sustainable sources like agro-industrial residues (MENSAH et al., 2023).

Xylooligosaccharides are oligomers characterized by a 1,4-linked xylose backbone, along with substitutions like uronic acids, acetyl groups, and arabinose units. They display remarkable variability in their degree of polymerization (DP), and types of linkages (NARISETTY et al., 2022). In the bioconversion of organic wastes, including agro-industrial lignocellulose residues, hydrolytic enzymes known as hydrolases are commonly employed. The enzymatic conversion occurs under gentle reaction conditions, devoid of the need for harmful chemicals, making it well-suited for a sustainable process in XOS production. Moreover, the application of xylanolytic enzymes offers notable precision and efficacy, enabling enhanced control over the degree of polymerization and reducing the presence of unwanted side products such as xylose and sugar degradation by-products (DONG et al., 2023).

The physical and biological attributes of XOS are contingent on the specific types of substitutions, their arrangement along the backbone, and the distribution of molecular weights across the xylan structure (NARISETTY et al., 2022). XOS exhibits resistance to both acidity and heat. They function as low-calorie sweeteners (1.5kcal/g), and possess a solid and colorless nature, although this aspect can vary based on the source of xylan and the purification process employed (YAN et al., 2022d). XOS cannot be broken down by the human digestive system due to the absence of enzymes needed to break the β-linkages found in these compounds. This characteristic makes these oligosaccharides promising candidates as prebiotics, as they remain intact as they reach the gastrointestinal tract and are accessible for the intestinal flora without undergoing degradation (BHATIA et al., 2020).

The definition of prebiotic according to the International Scientific Association for Probiotics and Prebiotics (ISAPP) is ″a substrate that is selectively utilized by host microorganisms conferring a health benefit” (GIBSON et al., 2017c). These substances are typically carbohydrates that undergo fermentation in the colon, thereby influencing the levels of lactobacilli and bifidobacteria present there. Among the significant health-enhancing advantages of such microbial groups is their capacity to generate short-chain fatty acids (SCFAs). These SCFAs play a role in regulating various functions of both the intestinal and extraintestinal systems, including the function of the gut's epithelial and mucus barriers, immunity, inflammation, as well as processes like glucose and lipid metabolism, energy expenditure, and sensation of satiety (CORIM MARIM; GABARDO, 2021b; CUNNINGHAM et al., 2021).

Short-chain fatty acids, including butyrate, propionate, acetate, and lactate, are essential products of dietary fiber fermentation by gut bacteria. Butyrate, a four-carbon SCFA, serves as a primary energy source for colonocytes, supports colon health, and exhibits anti-inflammatory properties (HAMER et al., 2008). Propionate, a three-carbon SCFA, influences metabolism by participating in gluconeogenesis, aiding in blood sugar regulation, and potentially impacting appetite and weight management (DEN BESTEN et al., 2013). Acetate, a two-carbon SCFA, contributes to cholesterol and fatty acid synthesis, as well as playing a role in regulating appetite and energy balance (BYRNE et al., 2015). While not a classical SCFA, lactate, derived from carbohydrate fermentation, contributes to gut microbiome health by providing energy for beneficial bacteria and maintaining microbial balance (MACFARLANE; MACFARLANE, 2012).

Collectively, SCFAs play a vital role in gut health, immune modulation, and metabolic regulation. They offer multifaceted benefits, including supporting the integrity of the colon lining, dampening inflammation, impacting metabolism, and influencing appetite. Ongoing research continues to unveil the intricate interplay between SCFAs, the gut microbiome, and overall well-being, making them key players in maintaining a healthy digestive system and promoting overall health (KOH et al., 2016b).

Driven by the growing enthusiasm for sustainable XOS production and their incorporation into functional foods, this research sought to produce XOS through enzymatic hydrolysis and investigate the metabolites generated by probiotics during the fermentation of this oligosaccharide. The source of XOS, xylan from banana pseudostem, is a waste little explored and largely produced. The production of metabolites know as short chain fatty acids such as acetate, butyrate and propionate was evaluated because their production by probiotics after an oligomer fermentation is one indication of prebiotic property. In addition, other carbon sources (xylose and fructooligosaccharides) were evaluated for comparison.

2. Material and Methods

2.1 Production of xylooligosaccharides by enzymatic hydrolysis

Solubilized xylan extracted from banana pseudostem underwent enzymatic hydrolysis. This process took place within flasks, each containing 25 mL of 0.05 mol/L sodium phosphate buffer at a pH of 6. These flasks were placed in a shaker at 55 ºC, and agitation was maintained at 120 rpm. The enzymatic reaction featured the use of a previously purified *Aspergillus versicolor* endoxylanase (CANO CARMONA et al., 1998). An enzyme load of 50 IU/g was applied, and the reaction took 24 h, based on lieterature study (DE FREITAS et al., 2021). To halt the enzyme activity, the flasks were subjected to a 5-min boil in water. Before conducting XOS analysis, the hydrolysate was filtered using a 0.22 µm syringe filter.

2.2 Quantification of xylooligosaccharides

To quantify the concentration of xylooligosaccharides (XOS), the samples from enzymatic hydrolysis and after microorganisms growth underwent analysis using high-performance liquid chromatography (HPLC) on WATERS equipment. The procedure employed an Aminex HPX-87C BIO-RAD column (300 x 7.8 mm) maintained at a temperature of 80 °C. Ultrapure water with a flow rate of 0.6 mL/min was utilized as the eluent. A sample volume of 20 µL was injected into the system, and a refractive index detector (Waters 2414) was employed. For calibration, varying concentration solutions of xylose (Sigma), as well as xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) from Megazyme, were used as standards.

2.3. Growth of probiotic bacteria on media with different carbon sources

2.3.1 Microorganisms

Lactiplantibacillus plantarum 1462 and *Lacticaseibacillus paracasei* 2218 were sourced from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), affiliated with the State University of Campinas, Brazil. These microorganisms were upheld as stock cultures on solid MRS (de Man, Rogosa, and Sharpe) medium at a temperature of 4 ºC.

Bifidobacterium longum BB-05 Danisco and *Bifidobacterium breve* BB-03 Danisco were acquired from the Laboratory of Industrial Biotechnology at São Paulo State University, located in Assis, São Paulo, Brazil. These microorganisms were also sustained as stock cultures on solid MRS medium, enriched with 0.05% cysteine, and stored at a temperature of 4 ºC.

2.3.2. Growth media and method

A formulated MRS (De Man et al., 1960) medium with 25% of proteins and 50% of mineral salts was prepared and sterilized via autoclaving for 20 min at a temperature of 121 ºC. For *Bifidobacteria* cultures, cysteine at a concentration of 0.05% was introduced into the medium. Distinct carbon sources, namely glucose, xylose, fructooligosaccharides (FOS), and xylooligosaccharides (XOS), were incorporated into the medium at varying concentrations (30, 25, 20, 15, 10, and 5 g/L). Subsequently, the strains were cultivated at a temperature of 37 ºC for a duration of 72 h within MRS media. These cultures were established in a 96-well plate, with agitation applied for *Lactobacilli* and without agitation for *Bifidobacteria*. Each concentration was subjected to quadruplicate cultivation.

To monitor growth, the optical density (OD) at a wavelength of 630 nm was measured every 15 min using the Tecan Sunrise™ absorbance microplate reader. This systematic approach facilitated the assessment of microbial growth dynamics under various carbon source concentrations for each strain.

2.4 Production of short-chain fatty acids by probiotic bacteria

Following the cultivation phase, the samples underwent filtration using a 0.22 µm syringe filter. For assessing the concentration of lactic, acetic, butyric, and propionic acids within the medium subsequent to microbial growth, High Performance Liquid Chromatography (HPLC) was conducted using a BIO-RAD Aminex HPX-87H column on Waters Equipment. The analysis was performed under the following conditions: a temperature of 50 ºC, a flow rate of 0.6 mL/min using a sulfuric acid solution of 0.05 mol/L as the mobile phase, and detection by a refractive index detector.

3. Results and discussion

3.1. Probiotic growth on media with different carbon sources

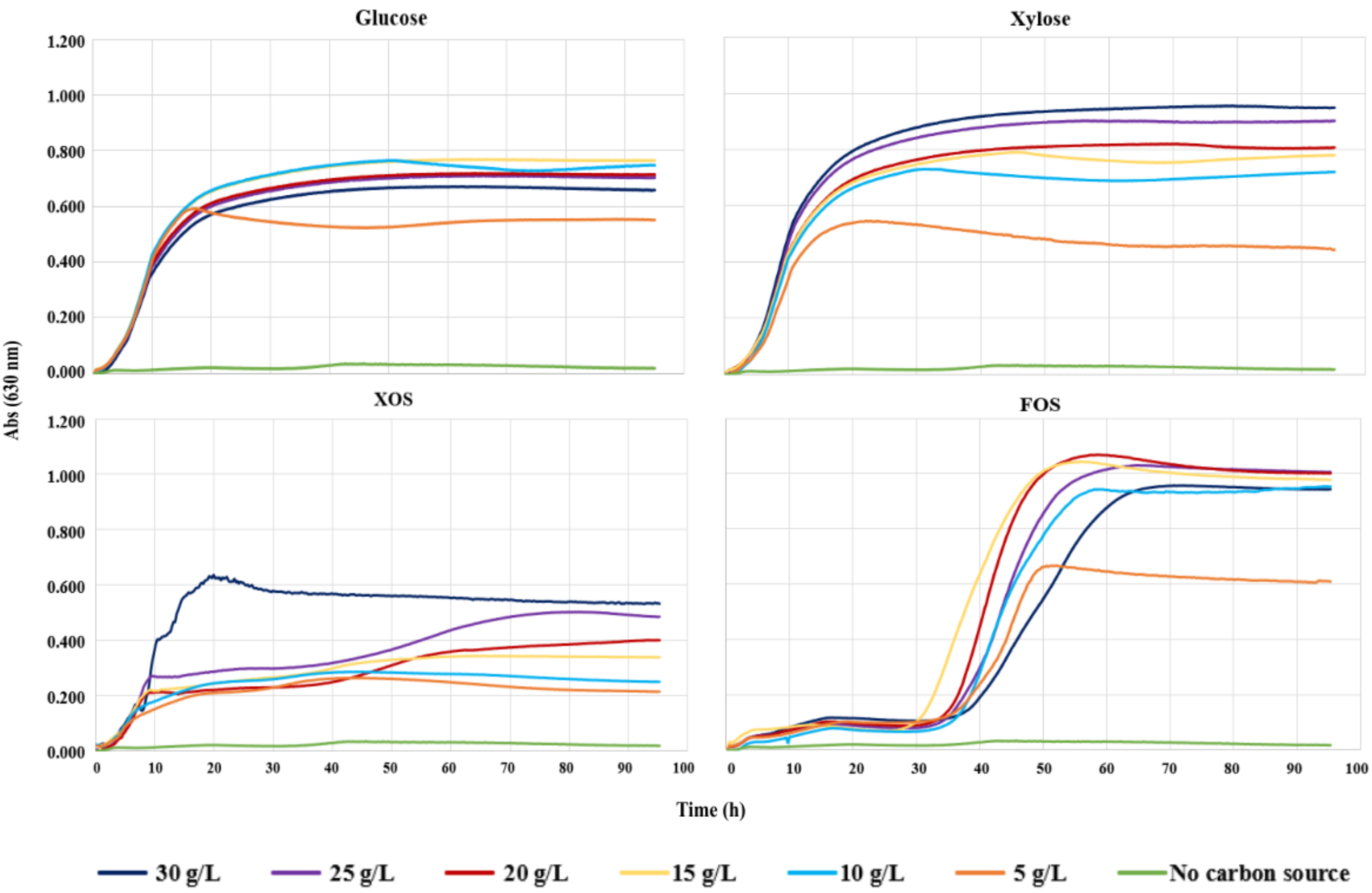
Probiotic microorganisms were subjected to growth in a culture medium containing different carbon sources (glucose, xylose, fructooligosaccharides, and xylooligosaccharides) at different concentrations (30, 25, 20, 15, 10, and 5 g/L) for 96 h. Growth was also evaluated at media with no carbon source in order to verify if another media component could be helping bacteria growth.

Bifidobacteria exhibit a remarkable enzymatic capacity to metabolize various complex carbohydrates, including xylooligosaccharides, fructooligosaccharides, xylan, and xylose. This metabolic prowess is attributed to the production of specific enzymes by these bacteria. Xylanases breakdown xylan and xylooligosaccharides into xylose. Fructofuranosidases hydrolyze glycosidic bonds in fructooligosaccharides, yielding fructose and shorter oligosaccharides. Moreover, Bifidobacteria possess xylose isomerase, enabling them to convert xylose into xylulose, thus utilizing it as a carbon source. These enzymatic capabilities provide Bifidobacteria with a competitive edge in efficiently metabolizing complex carbohydrates, contributing to their role as beneficial gut microbes (POKUSAEVA; FITZGERALD; VAN SINDEREN, 2011).

For the growth of *B. breve* in the medium containing glucose, the concentration of the carbon source did not significantly affected the growth, except for a reduction observed with the lowest concentration of 5 g/L (Fig 1). Among the carbon sources used, glucose is the one that is most readily assimilated y the microbial cell. On the other hand, the concentration of xylose in the medium affected the growth of the microorganism, as higher concentrations led to greater growth.

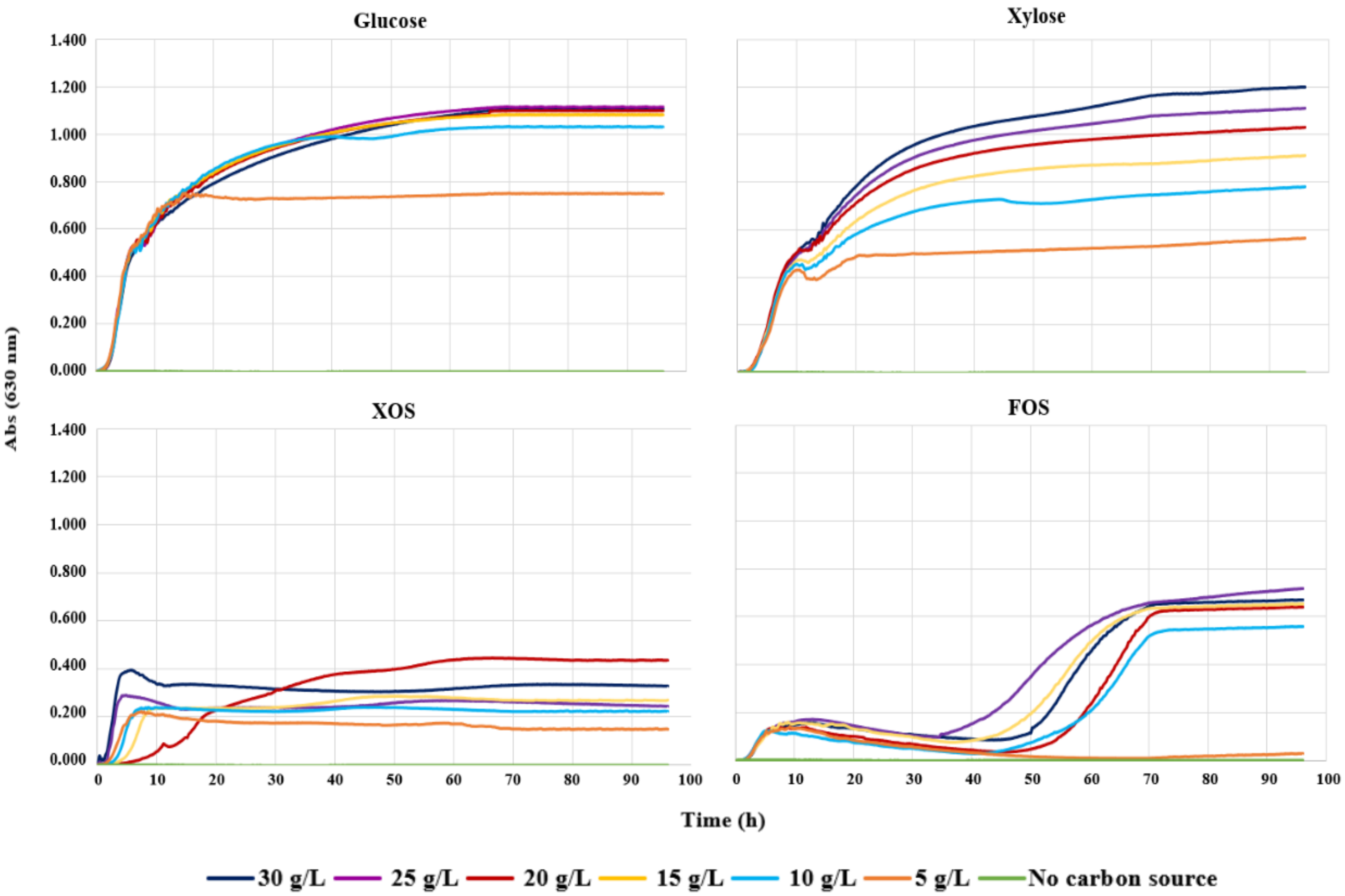
The growth in the medium containing XOS as a carbon source followed the same pattern, with greater growth observed at higher concentrations. However, the growth in the medium with 30 g/L of XOS was only about 57% of the growth in the medium containing xylose at the same concentration. Fructooligosaccharide (FOS) was the carbon source that took the longest time to be assimilated by the microorganism, as the exponential phase occurred only after approximately 35 h of cultivation. Growth was similar among the different concentrations, reaching absorbance levels close to those of glucose and xylose, with a significantly lower growth observed only at the lowest concentration of 5 g/L.

Fig 17. Growth of *B. breve* in media with different types and concentration of carbon source



The growth of *B. longum* was quite similar (Fig. 2), as in the medium containing glucose as the carbon source, growth was not significantly affected by the concentration except for the lowest one. The concentration of xylose as a carbon source influenced the growth, with lower growth observed at lower concentrations. The concentration of XOS that resulted in the highest growth was 20 g/L, but it represented only approximately 42% of the growth in the medium containing xylose at the same concentration. The exponential phase began around 40 h of cultivation in the medium containing FOS as a carbon source, and growth was drastically reduced at a concentration of 5 g/L.

Fig 18. Growth of *B. longum* in media with different types and concentration of carbon source



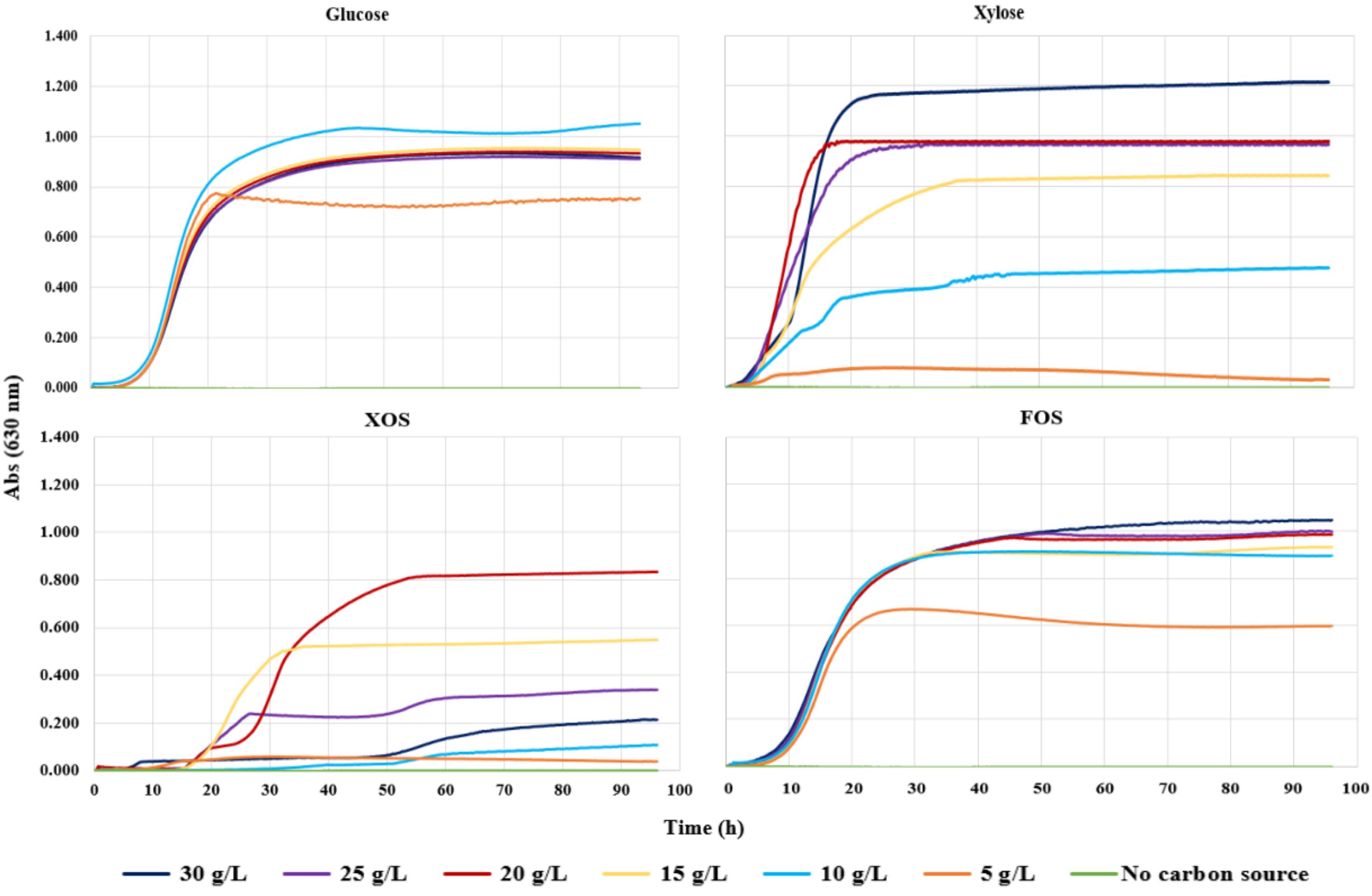
In a research study examining the carbohydrate preferences of nine distinct *Bifidobacterium* species isolated from the human gut, it was observed that these bifidobacteria exhibited diverse fermentation profiles when exposed to various carbohydrates (glucose, xylose, xylan, XOS and FOS). While the majority of species showed a preference for glucose as their primary carbon source, *B. longum* displayed a preference for xylose and fructooligosaccharides (FOS) as carbon sources, whereas *B. breve* exhibited a preference for glucose and FOS as its favored carbon sources (PALFRAMAN; GIBSON; RASTALL, 2003b). In our current investigation, *B. longum* demonstrated a distinct inclination towards xylose as a carbon source, although its growth on FOS was comparatively lower. On the other hand, *B. breve* exhibited greater growth on xylose compared to glucose, aligning with the findings in the referenced study where FOS was the preferred carbon source for growth.

In a four-week study, mice were administered fructooligosaccharides (FOS) at varying doses. The two distinct FOS dosages used included a low-dose group containing 5% FOS (w/w) and a high-dose group containing 25% FOS (w/w). The impact of these FOS doses on the proliferation of bifidobacteria was investigated, revealing a significant stimulation of bifidobacterial growth, particularly within the high-dose group. Following FOS treatment, there was a notable increase in the relative abundance of bifidobacteria, specifically *Bifidobacterium pseudolongum*, constituting over 95% of the total bifidobacterial population (MAO et al., 2018). In the current study, it was observed that higher dosages of FOS further facilitated the proliferation of Bifidobacteria.

Both commercial and autohydrolysis-produced xylooligosaccharides were introduced into the growth media for *B. adolescentis* and *B. longum*. When measuring growth at an optical density of 600 nm, *B. adolescentis* exhibited growth levels of 0.600 and 0.700 in the presence of commercial and autohydrolysis XOS, respectively. In contrast, *B. longum* displayed growth values of 0.200 on commercial XOS and 0.150 on autohydrolysis XOS, which is consistent with the results observed in the current study (MOURA et al., 2007).

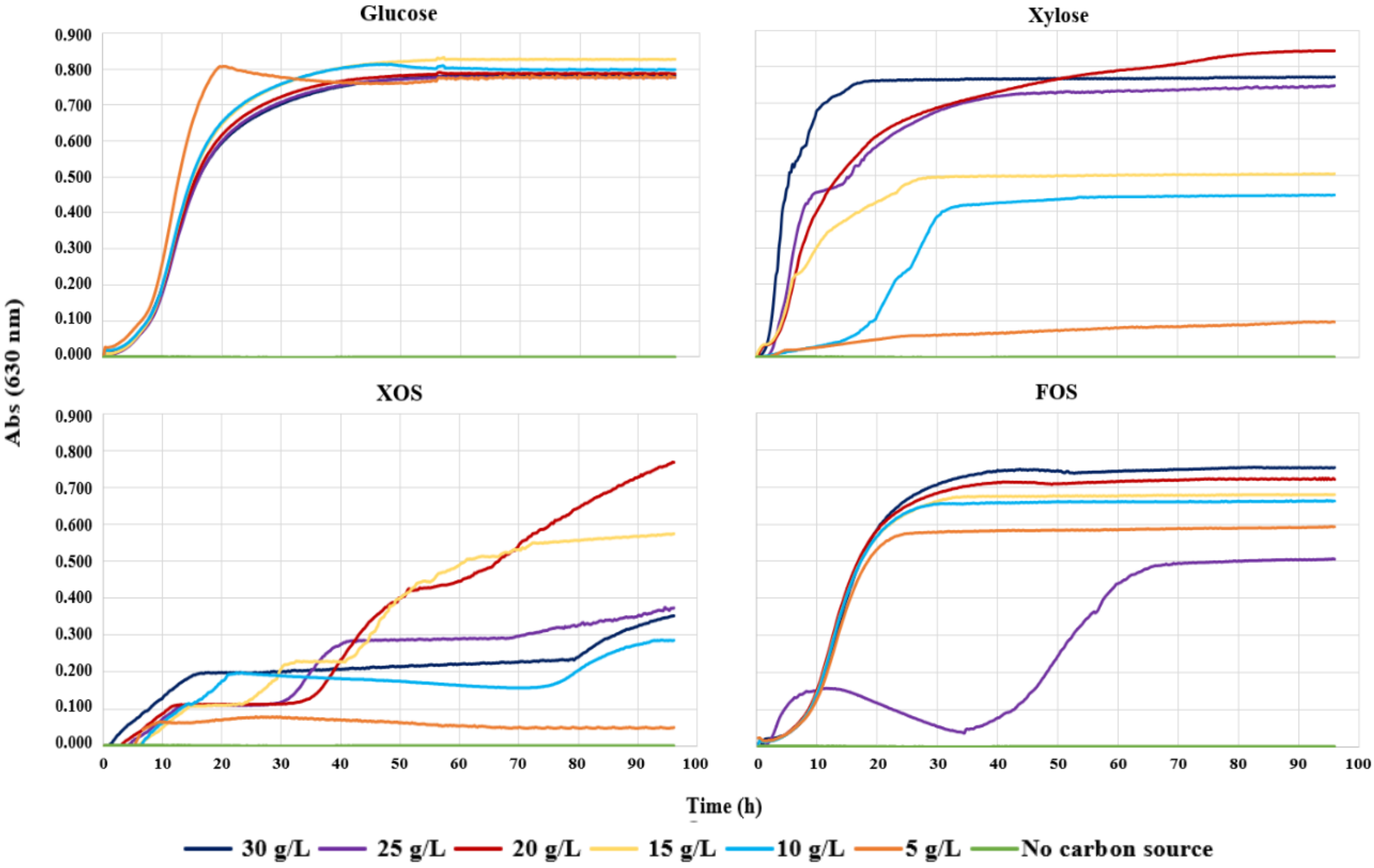
The growth of both *Lc. plantarum* 1462 and *Lc. paracasei* 2218 (Figs 3 and 4) remained relatively unaffected by variations in glucose concentration, different to their growth in media containing xylose, which showed an increase in growth at higher concentrations. The growth pattern on FOS was consistent for both microorganisms, as higher concentrations generally resulted in higher growth, except for the 25 g/L concentration, where the growth of *Lc. paracasei* 2218 was notably lower compared to other concentrations. Concerning the media containing XOS, it was observed that both microorganisms exhibited their optimal growth at a concentration of 20 g/L, with the second-best growth seen at 15 g/L. Higher concentrations of XOS might have had a partially inhibitory effect on their growth. Furthermore, it's worth noting that even after 96 h, the growth of *Lc. paracasei* 2218 at a concentration of 20 g/L XOS was still in the exponential phase.

Fig 19. Growth of *Lc. plantarum* 1462 in media with different types and concentration of carbon source



The growth of both *L. paracasei* and *L. paraplantarum* strains was investigated in a literature study at 37°C in the presence of 1% FOS. Under anaerobic conditions, *L. paracasei* B4564 demonstrated growth on FOS, reaching an optical density of approximately 0.5 at 600 nm after 24 h. However, when exposed to FOS in the presence of oxygen, it experienced a prolonged lag phase before entering a significant growth phase. Similarly, *L. paraplantarum* B23115 displayed growth on FOS under anaerobic conditions, achieving an optical density of approximately 1.5 at 600 nm after 24 h (RENYE; WHITE; HOTCHKISS, 2021). Comparing these findings with the results obtained in the present study, it was observed that the growth of *L. paracasei* 2218 in the presence of 10 g/L FOS, which is equivalent to 1%, reached an optical density (630 nm) of 0.663. In contrast, the growth of *L. plantarum* 1462 in the same conditions reached a higher optical density of 0.931.

Fig 20. Growth of *Lc. paracasei 2218* in media with different types and concentration of carbon source



In the research conducted by (RATNADEWI et al., 2020), they observed the proliferation of *Lactobacillus casei* on media containing XOS sourced from coffee peel. During the initial 12-h incubation period, there was a noticeable surge in bacterial growth, followed by a gradual decline over the subsequent 48 h. Notably, when 20% XOS extracted from coffee peel was introduced, it led to the most substantial increase in growth compared to other XOS concentrations. In the present study, the optimal concentration for promoting the growth of Lactobacillus was found to be 20 g/L, which is equivalent to 2%.

The functional potential of XOS as prebiotics was assessed by examining their capacity to promote the growth of the probiotic bacterium *Lactobacillus plantaru*m M-13 (LPM-13). The results clearly demonstrated that XOS, when used as the sole carbon source, provided excellent support for the growth of LPM-13, resulting in a substantial population of 18.77 log CFU/mL (GUPTA et al., 2022b). Remarkably, this level of growth was on par with or even superior to that observed when the standard MRS medium, which contained glucose as the carbon source, was used. In the current investigation, the growth of *L. plantarum* in media containing XOS was found to be comparable to that achieved in the standard MRS medium with glucose, although it's worth noting that the growth with glucose as the carbon source was still slightly higher.

3.2. Organic acid production by probiotic bacteria

Short-chain fatty acids (SCFAs), including acetic acid, propionic acid, and butyric acid are vital organic compounds produced via the gut microbiota's fermentation of dietary fibers and non-digestible carbohydrates in the colon. They play a central role in preserving both intestinal and immune equilibrium within the human body (MARKOWIAK-KOPEĆ; ŚLIŻEWSKA, 2020). SCFAs serve multiple crucial functions: they act as an energy source, providing up to 10% of the daily caloric requirements, particularly supporting the intestinal lining's integrity via butyric acid, and they also exhibit immunomodulatory effects, promoting the production of regulatory T cells to foster immune tolerance and reduce inflammation. Furthermore, SCFAs possess anti-inflammatory properties, inhibiting pro-inflammatory cytokines while encouraging anti-inflammatory cytokine production. Additionally, they maintain a balanced gut microbiota by stimulating beneficial bacteria growth and inhibiting harmful ones through the creation of an acidic colon environment, unfavorable to pathogenic bacteria (RATAJCZAK et al., 2019).

Diet exerts a profound influence on the balance of the intestinal microbiome, with its composition impacting the diversity and abundance of microbial species in the gut. Certain dietary elements, like FOS and XOS, function as prebiotics, nourishing beneficial gut bacteria. This nourishment promotes their growth and activity, fostering a more diverse and harmonious microbiome (MARKOWIAK; ŚLIZEWSKA, 2017). Taking this into consideration, in the present study, we assessed the production of short-chain fatty acids generated by probiotic microorganisms following the fermentation of four different carbon sources, including two prebiotics (XOS and FOS).

Fermentation of glucose, xylose, and XOS by *B. breve* (Fig 5) resulted in significantly higher lactate production compared to other organic acids, with the peak yield reaching 6.7 g/L during 30 g/L xylose fermentation. Interestingly, the fermentation of FOS led to the highest production of acetic acid, reaching 1.3 g/L in a medium containing 15 g/L of FOS. Moreover, XOS fermentation stood out as the primary source of butyric acid production by this probiotic among the tested carbon sources, yielding 0.94 g/L. Regarding the production of short-chain fatty acids by *B. longum* (Fig 6), lactic acid was the most abundantly produced in culture media containing glucose, xylose, and FOS as carbon sources. However, after XOS fermentation, acetic acid stood out as the most prominently generated compound, reaching approximately 3 g/L in a medium with 20 g/L of XOS. Additionally, propionic acid was detected, albeit at lower concentrations, only following the fermentation of xylose and XOS.

Fig 21. Organic acid production by *B. breve* after 96 h fermentation of different carbon sources.

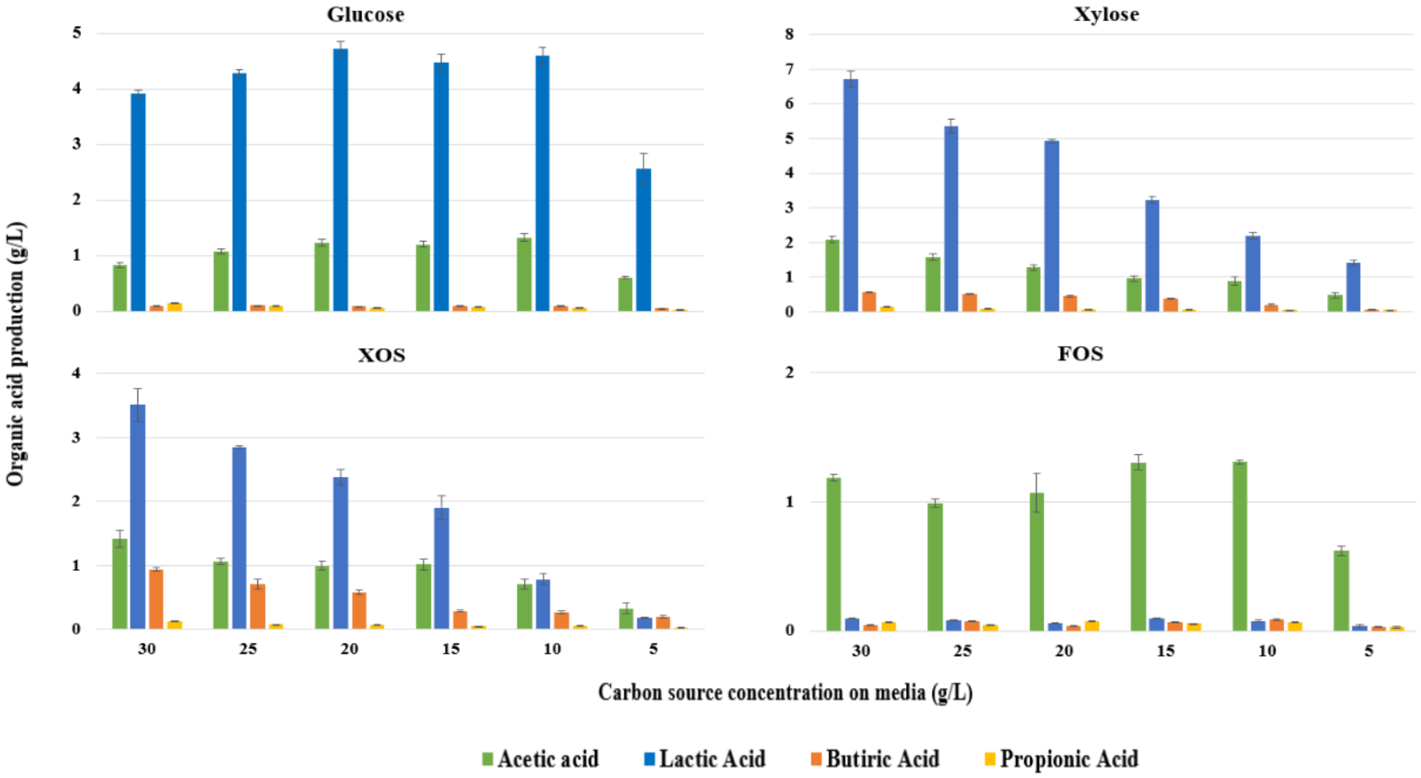
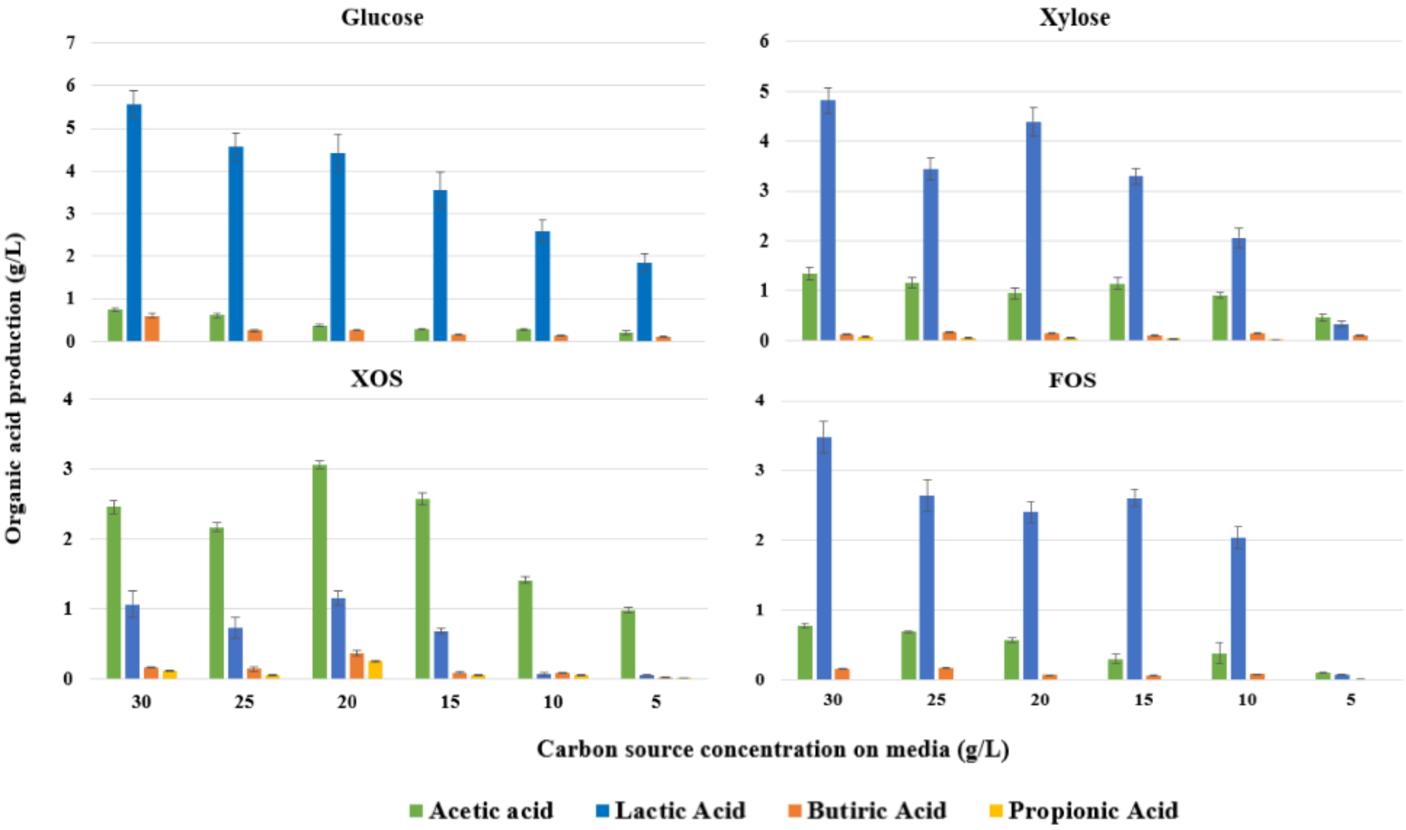


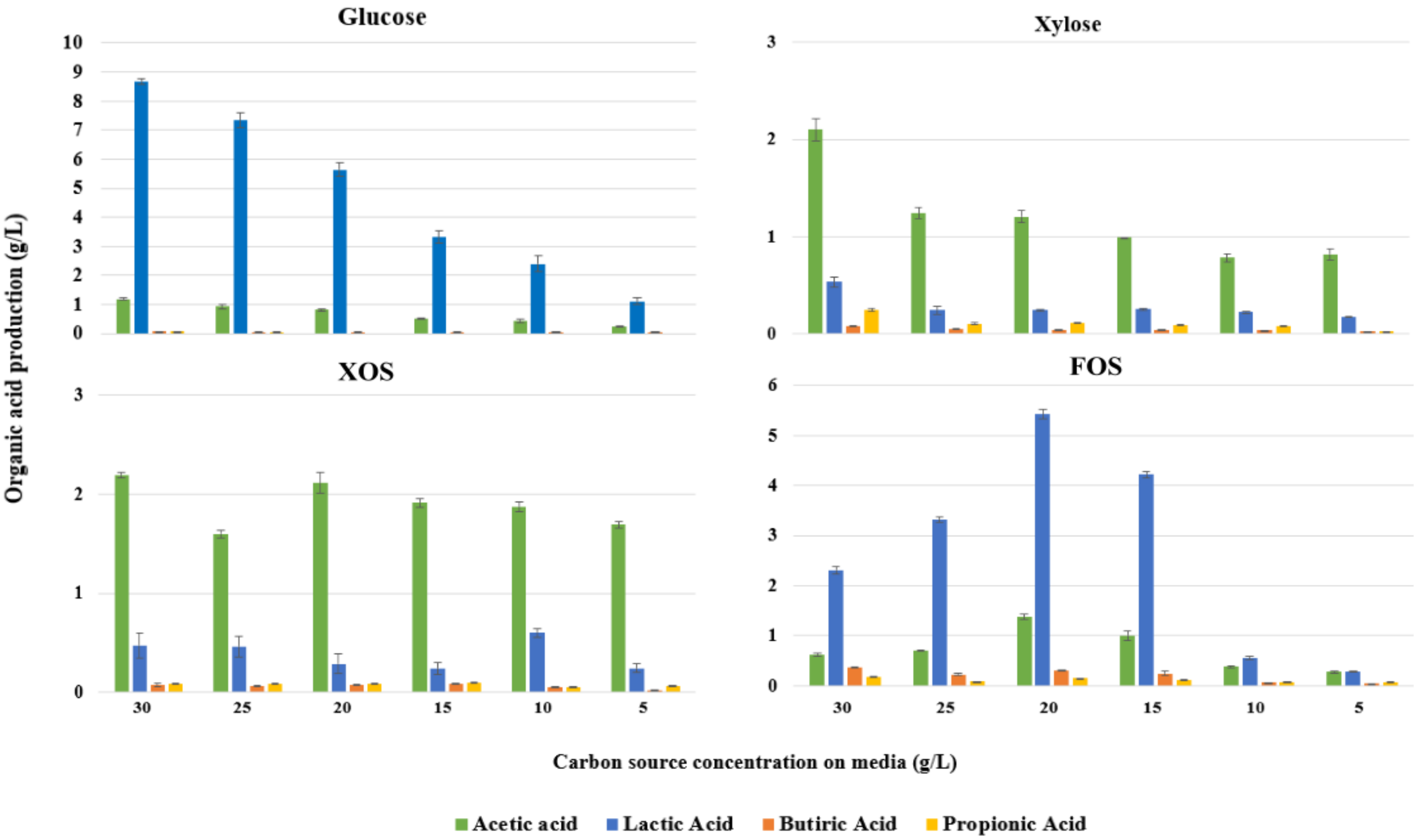
Fig 22. Organic acid production by *B. longum* after 96 h fermentation of different carbon sources.



Human colonic microbes were subjected to a 48-h fermentation process involving XOS and FOS, along with various *Bifidobacterium* and *Lactobacillus* species. The outcomes indicated that both XOS and FOS fermentation promoted the proliferation of *Bifidobacterium*, particularly *Bifidobacterium lactis*. Furthermore, the fermentation of these two prebiotics primarily resulted in the production of acetate and butyrate. In the case of FOS fermentation, approximately 100 mmol/g of acetate and 40 mmol/g of butyrate were generated, equivalent to 6 g/L and 3.5 g/L, respectively. On the other hand, XOS fermentation yielded 80 mmol/g of acetate and 40 mmol/g of butyrate, corresponding to 4.8 g/L and 3.5 g/L, respectively (MÄKELÄINEN et al., 2010). In this study, *B. longum* and *B. breve* exhibited variations in the production of organic acids based on the carbon source, with lactic and acetic acids being the predominant products.

The two *Lactobacillus* species utilized in this study also exhibited variations in the predominant organic acid produced depending on the fermented carbon source (Fig 7 and 8). Lactic acid emerged as the most abundantly produced organic acid in the medium containing 30 g/L of glucose, reaching approximately 9 g/L for both *Lactobacillus* species. This same trend was similar for the medium containing FOS, where lactic acid was the predominant organic acid generated by both *Lactobacillus* species. Acetic acid was the most abundantly produced short-chain fatty acid following the fermentation of xylose and XOS by both *Lactobacillus* species. However, butyric acid production was higher after the fermentation of FOS by *Lc. paracasei* 2218, whereas it was more pronounced after the fermentation of XOS by *Lc. plantarum* 1462. Additionally, the production of propionic acid was greater following the fermentation of xylose by both Lactobacillus species.

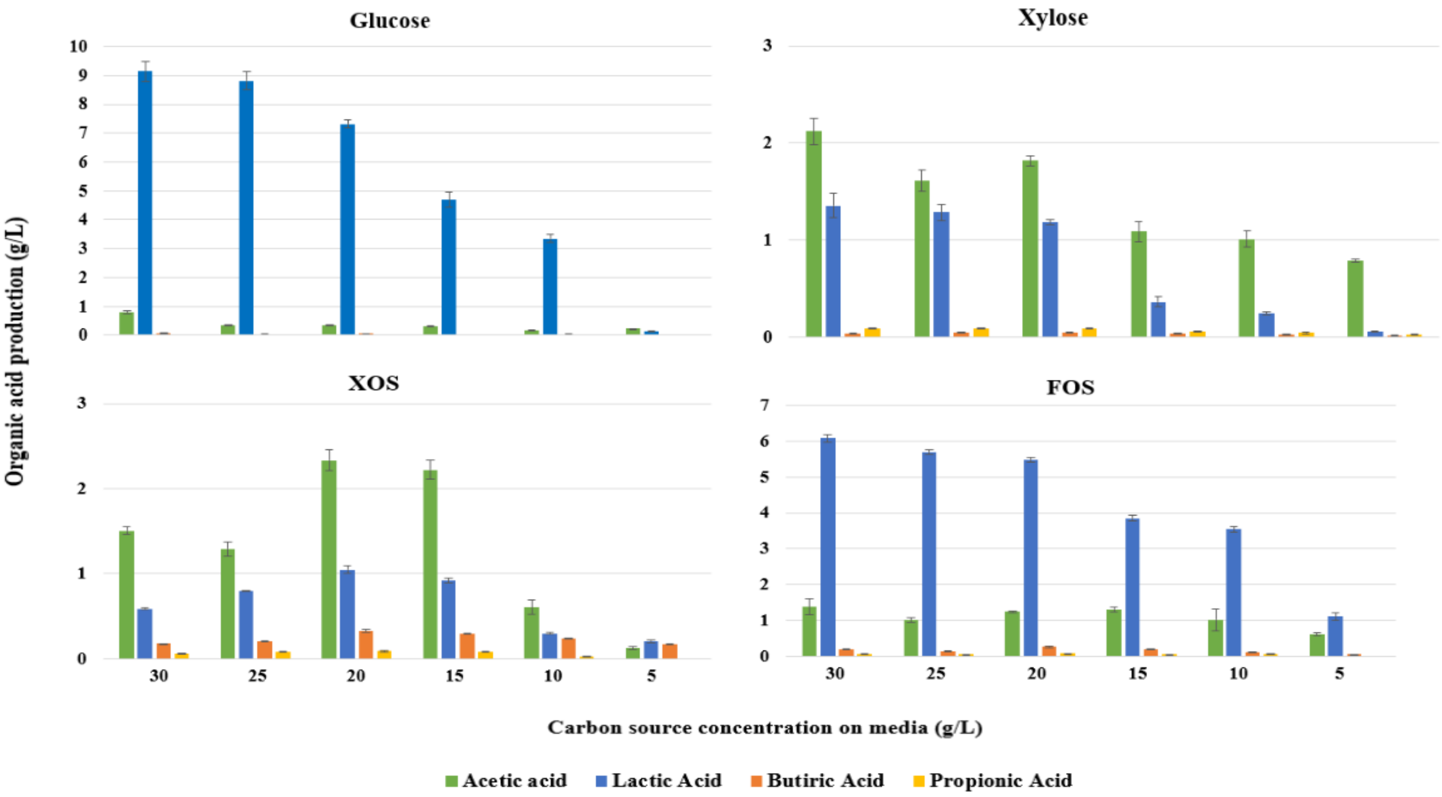
Fig 23. Organic acid production by *Lc. paracasei* 2218 after 96 h fermentation of different carbon sources.



After a 48-h incubation period of *L. plantarum* and *L. brevis* in media containing 2% glucose, xylose, or XOS, the production of lactic and acetic acids was assessed. Glucose fermentation by *L. plantarum* and *L. brevis* yielded 3.25 g/L and 7.76 g/L of lactic acid, respectively, accompanied by a minimal production of acetate, approximately 0.20 g/L for both bacteria (ILIEV et al., 2020). Furthermore, when glucose at the same concentration was fermented by *Lc. plantarum* 1462 and *Lc. paracasei* 2218 in the current study, it resulted in higher lactate production, around 7 g/L and 5.5 g/L, respectively, with acetate production being significantly lower, approximately 0.350 g/L for *Lc. plantarum* and 0.752 g/L for *Lc. paracasei*.

Lactobacillus plantarum, in media containing 2% xylose, produced 1.26 g/L of acetic acid and 0.94 g/L of lactic acid, while *Lactobacillus brevis* generated 1.74 g/L of acetic acid and 1.32 g/L of lactic acid (ILIEV et al., 2020). In the current study, both *Lactobacillus* also produced more acetic acid than lactic acid after xylose fermentation and the concentration produced was similar. Regarding the XOS fermentation, the result obtained in this study was similar to that of the cited article, as acetate production exceeded lactate production. However, it's worth noting that the concentrations achieved by the microorganisms used in the cited article were higher.

Fig 24. Organic acid production by *Lc. plantarum* 1462 after 96 h fermentation of different carbon sources.



4. Conclusion

This study highlights the promising potential of xylooligosaccharides (XOS) in the prebiotic market and underscores the need for further research on diverse production techniques and their application in human consumption. The research demonstrated that enzymatic hydrolysis of banana pseudostem biomass, particularly through partial delignification, milling, and the use of endoxylanase, can effectively enhance XOS production. However, the concentration of XOS was found to be higher when hydrolysis was performed on previously solubilized xylan.

Additionally, the study explored the viability of diluted acid hydrolysis of banana pseudostem biomass as an alternative method for XOS production. This approach showed good conversion rates with a lower production of xylose under optimized conditions. The probiotic microorganisms used in the study could grow in mediums containing by-products of this reaction, like furfural and HMF, although the presence of organic acids was inhibitory to Lactobacillus. Notably, the concentration of XOS produced through acid hydrolysis was lower compared to enzymatic hydrolysis.

The study also found that the growth and metabolic activities of microorganisms were significantly influenced by the type and concentration of carbon sources in their environment. Glucose was consistently the preferred and most easily assimilated carbon source for all studied microorganisms. However, their responses to other carbon sources, such as xylose, XOS, and FOS, varied, indicating specific preferences or efficiencies in metabolizing these alternatives. Some carbon sources led to delayed growth phases or exhibited inhibitory effects at higher concentrations. Notably, the fermentation of XOS and xylose resulted in a higher production of beneficial short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, compared to glucose fermentation, which mainly produced lactate. This finding suggests that XOS possesses favorable prebiotic properties, promoting the production of beneficial SCFAs by the microorganisms.

Overall, this research contributes valuable insights into the production and application of XOS as prebiotics, emphasizing their role in supporting the growth and metabolic activity of beneficial gut microorganisms. The findings of this study pave the way for further exploration into the effective utilization of XOS in promoting gut health and well-being.

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CHAPTER 7: CONCLUSION

In order for the production of xylooligosaccharides to meet the high and growing demand in the prebiotic market, more studies need to be conducted on different production techniques. Furthermore, the use of these prebiotics, produced by different techniques, by probiotic microorganisms should also be studied in order to evaluate the best way for them to be introduced for human consumption.

XOS production by enzymatic hydrolysis of banana pseudostem biomass is improved by partial delignification, milling and using only endoxylanase. However, the concentration of XOS produced is higher if the hydrolysis is performed in previous solubilized xylan. The increase in xylan concentration led to a decrease in yield but an increase in XOS concentration. Interestingly, a higher reaction volume improved the conversion of xylan into XOS. A cycle of a second hydrolysis adding a new enzyme load to the remaining substrate has a low increment in XOS yield. The molecular weight and polydispersity of remaining xylan increased after the second hydrolysis compared to the first one, making enzyme action more difficult.

The production of XOS through diluted acid hydrolysis of banana pseudostem biomass can be an alternative since there is a good conversion and low production of xylose for optimized conditions. Furthermore, the probiotic microorganisms used were able to grow in the medium containing different concentrations of the by-products generated in this reaction (furfural and HMF) but for the *Lactobacillus*, the presence of the organic acids was inhibitory. However, the concentration of XOS produced via acid hydrolysis is also lower compared to the enzymatic hydrolysis of xylan. The study demonstrated that all evaluated microorganisms could thrive when XOS produced through acid hydrolysis was used as the carbon source in the culture medium. While most microorganisms were unaffected by the purification of XOS, the removal of extractives from biomass significantly enhanced their growth. This suggests that XOS produced through acid hydrolysis has the potential to be utilized as prebiotics to stimulate the growth of probiotic microorganisms.

Additionally, the growth and metabolic activities of microorganisms were significantly influenced by the type and concentration of carbon sources present in their environment. Glucose consistently emerged as the preferred and easily assimilated carbon source among all the microorganisms studied. However, their responses to other carbon sources such as xylose, XOS (xylooligosaccharides), and FOS (fructooligosaccharides) varied, suggesting specific preferences or efficiencies in metabolizing these alternative sources. Notably, some carbon sources resulted in delayed growth phases, while others exhibited potential inhibitory effects, particularly at higher concentrations.

For most of the microorganisms examined, the fermentation of XOS and xylose led to a higher production of short-chain fatty acids (including acetate, propionate, and butyrate) compared to glucose fermentation, which primarily resulted in higher production of lactate. This observation suggests that the XOS produced in this study possesses favorable prebiotic properties, as it promotes the production of beneficial short-chain fatty acids by the microorganisms.