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

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES PRODUCTION USING BANANA AND GUAVA WASTE, WITH EVALUATION OF THE PREBIOTIC AND ANTIOXIDANT EFFECT

CAROLINA FROES FORSAN

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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia).
Orientador: Dr. Michel Brienzo.

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AUTORA: CAROLINA FROES FORSAN

ORIENTADOR: MICHEL BRIENZO

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Prof. Dr. MICHEL BRIENZO (Participaçao Virtual)
Laboratorio de Caracterizacao de Biomassa / Instituto de Pesquisa em Bioenergia - IPBEN

Profa. Dra. PATRÍCIA FELIX ÁVILA (Participaçao Virtual)
Faculdade de Engenharia de Alimentos / Universidade Estadual de Campinas - Unicamp

Prof.Dr. PAULO RENATO MATOS LOPES (Participaçao Virtual)
Departamento de Produção Vegetal / Faculdade de Ciências Agrárias e Tecnológicas - UNESP

Prof. Dr. FERNANDO MASARIN (Participaçao Virtual)
Departamento de Engenharia de Bioprocessos e Biotecnologia / Faculdade de Ciencias Farmaceuticas - Unesp

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ABSTRACT

Residues from the processing of fruits such as banana and guava are abundant in Brazil and can be used for the production of cellooligosaccharides (COS) and xylooligosaccharides (XOS). This study aimed to produce COS and XOS with banana pseudostem and leaf and guava seed cake, evaluating antioxidant and prebiotic capabilities. For production it was evaluated pretreatments with sulfuric acid (20% w/w), sodium and potassium hydroxide (20% w/w), and hydrogen peroxide (6% w/v). Grinding in a ball mill and biological pretreatment with endoxylanase from *Aspergillus versicolor* and endoglucanase (celluclast, Novonesis) were also studied. Solutions of COS and XOS (unfiltered and filtered in a Sep-Pak cartridges) and glucose, xylose and commercial cellobiose in concentrations between 0.1 and 16 $g.L^{-1}$ were used in antioxidant activity tests, with the reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH). COS, XOS, xylose, glucose and cellobiose at concentrations of 10 and 20 $g.L^{-1}$ and solution of COS with XOS, both at a concentration of 20 $g.L^{-1}$, in varying proportions, were added to a modified MRS culture medium (containing 25% proteins and 50% mineral salts served) as a carbon source for the growth of probiotics *Bifidobacterium breve* and *Lactiplantibacillus plantarum*. Growth was quantified by optical density (OD), absorbance at 630 nm using the Tecan Sunrise microplate reader. The highest results for converting glucan into COS were obtained using alkali pretreatment. With banana pseudostem the highest conversion of glucan into COS was 53.05% with KOH and the conversion of xylan into XOS was 59.1%. With banana leaf and guava seed cake the highest conversion of glucan into COS was 79.73% and 59.99%, and the conversion of xylan into XOS was 66.87% and 55.14%, respectively, with NaOH. Alkaline pretreatments were generally more efficient, the use of endoxylanase before endoglucanase increased yield in all cases and the use of a ball mill was only positive for materials pretreated with sulfuric acid and sodium hydroxide. Regarding antioxidant activity, only COS and XOS unfiltered in a Sep-Pak cartridges were able to reduce the DPPH radical, XOS were more effective than COS. This activity was probably related to compounds soluble in the solution, such as lignin-fragment, extractives and phenolic compounds, identified and qualified by the method of Folin-Ciocalteu and scanning spectrophotometry. In prebiotic activity assays, *L. plantarum* and *B. breve* were able to grow on all carbon sources used. The OD obtained with COS and XOS (isolated or mixed) in some cases was similar or greater than the OD obtained with glucose (control), and in general the microorganisms were able to consume them almost completely and produce mainly acetic and lactic acid. As conclusion, the use of endoxylanase before endoglucanase, especially in alkaline pretreated materials, increases the COS

yield, by leaving cellulose more accessible to the action of the cellulase, in addition, XOS were also produced. Unpurified COS and XOS solutions showed antioxidant activity and are promising prebiotics, without the need for high purity, which would make the process expensive. The mixture of COS and XOS also presented a prebiotic effect, enabling the simultaneous production of both, without the need for separation.

Keywords: cellulose; chemical pretreatment; hemicellulose; enzymatic pretreatment; physical pretreatment.

RESUMO

Resíduos do processamento de frutas como banana e goiaba são abundantes no Brasil e podem ser utilizados para produção de celo-oligossacarídeos (COS) e xilo-oligossacarídeos (XOS). Este estudo teve como objetivo produzir COS e XOS com pseudocaule e folha de bananeira e torta de semente de goiaba e avaliar a capacidade antioxidante e prebiótica. Para a produção foram avaliados pré-tratamentos químicos com ácido sulfúrico (20% m/m), hidróxido de sódio e potássio (20% m/m), e peróxido de hidrogênio (6% m/v). Moagem em moinho de bolas e pré-tratamento biológico com endoxilanase de *Aspergillus versicolor* e endoglucanase (celluclast, Novonesis) também foram estudados. As soluções de COS e XOS (não filtrados e filtrados em cartucho Sep-Pak) e glicose, xilose e celobiose comerciais em concentrações entre 0.1 e 16 $g.L^{-1}$ foram usadas em testes de atividade antioxidante usando o reagente 2,2-difenil-1-picrilhidrazil (DPPH). Soluções de COS, XOS, xilose, glicose e celobiose nas concentrações de 10 e 20 $g.L^{-1}$ e soluções de COS misturado com XOS, ambos na concentração de 20 $g.L^{-1}$, em proporções variadas, foram adicionados a um meio de cultura MRS modificado, (contendo 25% de proteínas e 50% de sais minerais) e serviram como fonte de carbono para o crescimento dos probióticos *Bifidobacterium breve* and *Lactiplantibacillus plantarum*. O crescimento foi quantificado por densidade óptica (DO), absorbância de 630 nm usando o leitor de microplaca Tecan Sunrise. Os maioress resultados para conversão de glucano em COS foram obtidos utilizando pré-tratamentos alcalinos. Com pseudocaule de bananeira a maior conversão de glucano em COS foi de 53,05% com KOH e a conversão de xilana em XOS foi de 59,1%. Com folha de bananeira e torta de semente de goiaba a maior conversão de glucano em COS foi de 79,73% e 59,99%, e a conversão de xilana em XOS foi de 66,87% e 55,14%, respectivamente, com NaOH. Os pré-tratamentos alcalinos foram geralmente mais eficientes, o uso de endoxilanase antes da endoglucanase aumentou o rendimento em todos os casos e o uso de moinho de bolas só foi positivo para materiais pré-tratados com ácido sulfúrico e hidróxido de sódio. Em relação a atividade antioxidante, apenas COS e XOS não filtrados em cartucho Sep-Pak foram capazes de reduzir o radical DPPH, XOS foram mais eficazes do que COS. Essa atividade provavelmente foi relacionada a compostos solubilizados na solução, como fragmentos de lignina, extrativos e compostos fenólicos, identificados e qualificados pelo método de Folin-Ciocalteu e espectrofotometria de varredura. Nos ensaios de atividade prebiotica, *L. plantarum* e *B. breve* foram capazes de crescer em todas as fontes de carbono usadas. A DO obtida com COS e XOS (isolados ou misturados) em alguns casos foi similar ou maior do que a

DO obtida com a glicose (controle), e em geral os micro-organismos foram capazes de consumi-los quase totalmente e produzir como metabólito principalmente ácido acético e ácido láctico. Como conclusão o uso da endoxilanase antes da endoglucanase, principalmente em materiais pré-tratados alcalinamente, aumenta o rendimento de COS, por deixar a celulose mais acessivel à ação da celulase, além disso, XOS, também foram produzidos. Soluções de COS e XOS não purificados mostraram atividade antioxidante, e são prebióticos promissores, sem que haja necessidade de alta pureza, o que tornaria o processo caro. A mistura de COS e XOS também apresentou efeito prebiótico, possibilitando a produção simultânea de ambos, sem necessidade de separação.

Palavras-chave: celulose; hemicelulose; pré-tratamento químico; pré-tratamento físico; pré-tratamento enzimático.

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CHAPTER I: INTRODUCTION AND OBJECTIVES

1.1 Introduction

Brazil is the third largest fruit producer in the world, with a production of approximately 41 million tons in 2022 (ABRAFRUTAS, 2024). Tons of fruit waste are generated annually, which include peels, seeds, pomace, and leaves, originated from harvesting and industrial processing and fruits rejected by consumers due to imperfections often caused by improper handling (FAVARETTO et al., 2023). When these wastes are improperly disposed of, they can cause negative impacts on the environment such as soil and groundwater contamination, attraction of animals and insects, and unpleasant odors (PEREIRA et al., 2022a). In addition, there is also economic loss impacting the price of produced fruit to the final consumer. Banana and guava are among the most produced fruits in Brazil; therefore, they were chosen as raw materials in the present study (SÁ et al., 2019; VITTI; LIMA; MARTINES FILHO, 2020). During the handling and processing of bananas, around 20% of waste is generated, and in relation to guava, this corresponds to 4 to 12% of the total fruit mass. These biomasses can be used to generate biofuels, bioplastics, organic acids, hydrogels, resins, and oligosaccharides such as cellooligosaccharides (COS) and xylooligosaccharides (XOS) through physicochemical and biological treatments, which aim to reduce biomass recalcitrance (PEREIRA et al., 2022b; FAVARETTO et al., 2023; RAMADAN et al., 2023; NARGOTRA et al., 2022).

In the case of the physical method, there is a reduction in particle size and an increase in surface area by mechanical methods, facilitating the accessibility of enzymes and accelerating enzymatic hydrolysis (WU et al., 2023). Chemical methods use reagents such as alkali, acid, oxidizing compounds, and solvents, among others, to disestruturate the components of lignocellulosic biomass (SAI BHARADWAJ et al., 2023). They are widely used in industry because of their simplicity and high efficiency. However, degradation products and inhibitors are generally produced and can cause environmental pollution (LI et al., 2022). Enzymatic hydrolysis is highly promising compared to chemical methods, as undesired by-products are not formed during the process, and the final product has high purity. However, the yield is often low when applied to untreated material, and the process can be expensive and slow, requiring a combination of a physical-chemical and enzymatic process to achieve a high yield (FORSAN et al., 2021a).

COS and XOS are oligomers of glucose and xylose, respectively, with a degree of polymerization between 2 and 7 units (GONÇALVES et al., 2023). The interest in these

oligosaccharides is growing due to their numerous possibilities of use: in agriculture, they can increase the resistance of plants to diseases due to the stimulation of the immune response, and they act as fertilizers, increasing the number of microorganisms in the soil and helping to plant growth (MA et al., 2022). They can act as prebiotics, a non-digestible food component, which is fermented by some beneficial microorganisms in the intestinal microbiota, providing numerous benefits to the host's health such as stimulating the immune system, protection against pathogens, and production of vitamins (HOU et al., 2022). Antioxidant activity has been reported to XOS and COS, which are substances that can inhibit oxidation caused by a free radical (VALLS et al., 2018). Antioxidants can prevent or postpone various diseases such as cancer, rheumatic arthritis, atherosclerosis, muscular hypertrophy, and neurodegenerative diseases (OLIVEIRA, 2015).

In this scenario, the objective of this study was to produce COS by comparing chemical and physical pretreatments, and the use of the commercial endoglucanase (Celluclast cocktail – Novonesis), varying several analysis parameters. In some stages, the endoxylanase from *Aspergillus versicolor* was used, in an attempt to remove xylan, and ball mill was applied to reduce the crystallinity and recalcitrance of the biomass. The process combination offered the advantage of the secondary production of XOS, which are already extensively studied and commercialized throughout the world, contributing to a billion-dollar global market.

1.2 Objectives

The objective of this study was to compare physical and chemical pretreatments and enzymatic hydrolysis for cellooligosaccharides and xylooligosaccharides production, using banana pseudostem and leaves and guava seed cake. The antioxidant and prebiotic activity of COS and XOS produced were also evaluated.

1.2.1 Specific Objectives

- Evaluate the influence of grinding with a ball mill in combination with chemical pretreatment: sulfuric acid, sodium and potassium hydroxide, and hydrogen peroxide with enzymatic hydrolysis for cellooligosaccharides and xylooligosaccharides production;
- Produce cellooligosaccharides through enzymatic hydrolysis with variable enzymatic loads of endoglucanase; (produzir COS com carga ideal de endoglucanase)
- Evaluate the use of endoxylanase from *Aspergillus versicolor* before endoglucanase for

cellooligosaccharides production;

- Evaluate the antioxidant activity of cellooligosaccharides and xylooligosaccharides solution unpurified and purified employing Sep-pak C_{18} cartridges, using DPPH as a reagent;
- Evaluate the growth of probiotics: *Lactiplantibacillus plantarum*, and *Bifidobacterium breve*, using cellooligosaccharides and xylooligosaccharides isolated or mixed as carbon sources;
- Quantify lactic acid and short-chain fatty acids (acetic, formic, butyric and propionic acid), produced by bacteria after fermentation of cellooligosaccharides, xylooligosaccharides and mixture.

1.3 Thesis format and organization

This thesis was organized into chapters, which were designed considering independent publications such as book chapters and papers, respecting the structure of the scientific journal.

The organization was like Chapter 1: General introduction and general and specific objectives - Chapter 2: Bibliographic review - Cellooligosaccharides and xylooligosaccharides production processes, potential prebiotics, and routes of metabolism by *Lactobacillus* and *Bifidobacterium* - Chapter 3: Correspond to scientific paper published in BioEnergy Research journal and patented by National Institute of Industrial Property - Cellooligosaccharides and xylooligosaccharides production by a combination of mechanical, chemical, and enzymatic treatments of banana pseudostem, leaves and guava seed cake – Chapter 4: Antioxidant activity of cellooligosaccharides and xylooligosaccharides from banana leaves and pseudostem and guava seed cake by DPPH method – Chapter 5: Potential prebiotic activity of cellooligosacharides and xylooligosaccharides from banana pseudostem and leaf and guava seed cake in *Lactiplantibacillus plantarum* and *Bifidobacterium breve* - Chapter 6: General conclusion.

1.3.1 Qualification rational research line

The Chapter 1 presented general introduction and objectives of the thesis.

The Chapter 2 presented a literature review about structure of cellooligosaccharides and xylooligosaccharides, health benefits of their consumption such as prebiotic effect, glucose and xylose metabolism by probiotic microorganisms, the importance of using biomass, data on the biomass used in the study (banana and guava), the structure of lignocellulosic materials.

Additionally, the chapter presents data from the literature on the production processes for cellooligosaccharides and xylooligosaccharides, emerging technologies and challenges and future perspectives in the production of oligosaccharides.

The Chapter 3 consists of a paper on the production of cellooligosaccharides by enzymatic hydrolysis from banana leaves and pseudostem and guava seed cake. The study conditions were: (I) untreated and pretreated biomasses with sulfuric acid, sodium and potassium hydroxide and hydrogen peroxide, (II) a part of the material was not ground and a part was ground in a ball mill for 30 min, (III) ground and unground material hydrolyzed with commercial cellulase Celluclast, and (IV) ground and unground material hydrolyzed with endoxylanase *Aspergillus versicolor* and commercial cellulase Celluclast. According conversion cellulose into COS, it was possible to evaluate the influence of chemical, physical and biological pretreatments on cellulose crystallinity.

The Chapter 4 is an article about antioxidant activity of COS and XOS. The capacity of the produced oligosaccharides and their monomers, glucose and xylose (commercial) and commercial cellobiose, to reduce the DPPH radical was evaluated. COS and XOS solutions filtered in a Sep-Pak filter were also tested, with the aim of evaluating whether pure oligomers reduce the radical or this activity is related to compounds present in the solution obtained after enzymatic hydrolysis.

The Chapter 5 is about prebiotic effect of COS and XOS, on *L. plantarum* and *B. Breve*, probiotic bacteria, present in human and animal microbiota. The consumption of carbon sources and the concentration of lactic acid and short-chain fatty acids, formed during the fermentation of COS and XOS, were also evaluated.

The Chapter 6 presented general conclusion of the thesis

CHAPTER II: CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES: PRODUCTION PROCESSES, POTENTIAL PREBIOTICS, AND METABOLISM ROUTES BY *LACTOBACILLUS* AND *BIFIDOBACTERIUM*

Abstract

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) are promising as prebiotics, helping the intestinal microbiota. As antioxidants, they reduce the harmful effects of free radicals generated during metabolic processes. They can be produced from biomass waste, a cheap and abundant material, but the most significant challenge is to separate the components of the cell wall so that the polymers can be converted into oligomers and other high-value products. This study reviews the structure of lignocellulosic materials and the main physicochemical pretreatments as emerging technologies used to decrease cellulose crystallinity, solubilize hemicellulose, and remove lignin to promote feasibility in enzymatic treatment for COS and XOS production. The metabolic routes of Lactobacillus and Bifidobacterium in the fermentation of COS and XOS were explored, focusing on short-chain fatty acids and their effects on human health.

Keywords: antioxidant; gut microbiota; oligosaccharides; probiotic; short chain fatty acids.

2.1 Introduction

There is growing concern among people about maintaining a healthy lifestyle and seeking beneficial foods. In this context, cellooligosaccharides (COS) and xylooligosaccharides (XOS) (glucose and xylose oligomers, respectively) are emerging, due to their possible positive effects, such as prebiotic and antioxidant effects (FAN et al., 2024; FORSAN; DE FREITAS; BRIENZO, 2023; PALANIAPPAN; MAPENGO; EMMAMBUX, 2023).

Prebiotic compounds are not digested by the host and act as a substrate for the growth of some microorganisms in the intestinal microbiota, such as species of *Lactobacillus, Bifidobacterium, Lactococcus* and *Streptococcus* (KIM et al., 2022). These microorganisms metabolize oligosaccharides, producing substances such as short-chain fatty acids, which provide health benefits such as prevention of cardiovascular diseases, obesity, osteoporosis, diabetes,

hypercholesterolemia, gastrointestinal infections and colon cancer (RIBEIRO et al., 2022; MACHADO; SENCIO; TROTTEIN, 2021; FUSCO et al., 2023).

Oligosaccharides also have the potential to be used as antioxidants—substances that eliminate free radicals, unstable molecules generated during the normal physiological metabolism of living beings. These free radicals react with other molecules or atoms to achieve stability, leading to oxidative stress, which damages cells, breaks the DNA chain, and induces gene mutations. If the damage caused by the stress oxidative is not repaired, cardiovascular diseases, neurological and metabolic disorders and tumor formation may occur (MARTEMUCCI et al., 2022; ZARIC; MACVANIN; ISENOVIC, 2023; XIONG et al., 2021). Despite the numerous benefits, excessive intake of oligosaccharides can result in a laxative effect, abdominal pain, and distension due to excessive gas production during the fermentation of these carbohydrates (NORDIN et al., 2022; JUHÁSZ; PENKSZA; SIPOS, 2020).

By-products obtained from lignocellulosic materials are a promising source for oligosaccharide production, as they are readily available and commercially cheap. Oligosaccharide can be obtained from agricultural by-products such as guava and sugarcane bagasse, banana leaves and pseudostems, corn cobs, wheat and rice straw, forestry and industrial residues, among others, which are generally burned, causing environmental damage (FORSAN et al., 2021a; FORSAN et al., 2021b; PEREIRA et al., 2022). To convert biomass into value-added products, it must undergo to reduce its recalcitrance to be hydrolized by enzymatic treatments (LORENCI WOICIECHOWSKI et al., 2020).

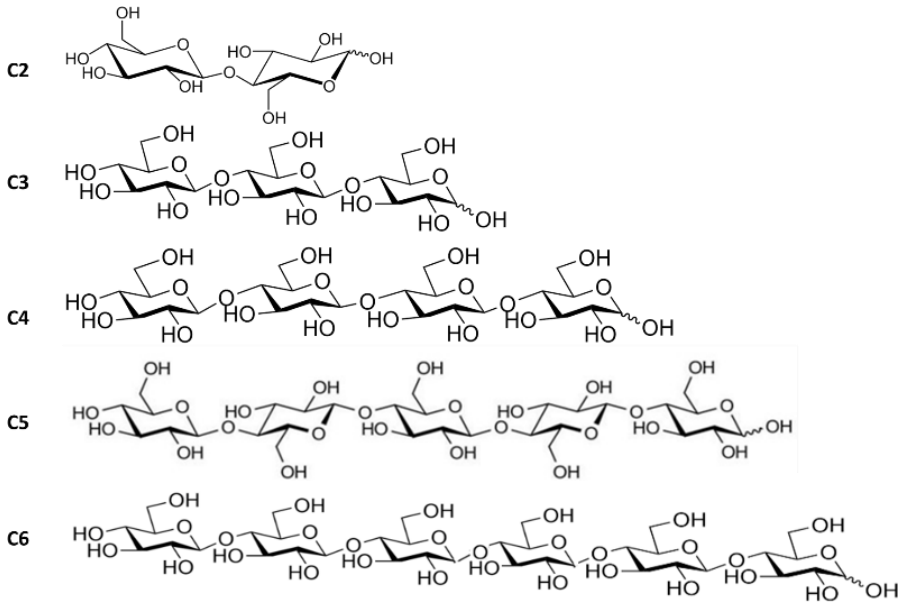
This review shows the importance of COS and XOS in human and animal nutrition, explores their diverse applications, how they are metabolized by the main bacteria present in the intestinal microbiota (*Lactobacillus* and *Bifidobacterias*), what the main metabolites are generated in these fermentation pathways and how they interfere with the host's health. Furthermore, the review discusses the main physical, chemical pretreatments and enzymatic treatments for obtaining COS and XOS, which are products of significant importance that contribute to a million-dollar global market.

2.2 Cellooligosaccharides

COS are oligomers formed by 2 to 9 glucose units, joined by a β-1,4-glycosidic linkage (Figure 2.1) (ÁVILA; DE MÉLO; GOLDBECK, 2023). COS with short chains of up to 6 glucose units are soluble in water, those with 7 to 12 units are partially soluble and those above 12 are

insoluble (standards-based) (ÁVILA et al., 2021). It can be produced by partial cleavage of glycosidic bonds in polysaccharide chains, with cellulolytic enzyme cocktails, chemical reagents, or through the synthesis of monosaccharides or disaccharides by transglycosylation and glycosylation reactions (BARBOSA et al., 2020a; LI et al., 2019).

Figure 2.1 - Chemical structure of cellooligosaccharides. C_2: Cellobiose, C_3: Cellotriose, C_4: Cellotetraose, C_5: Cellopentaose, C_6: Cellohexaose



Source: Author

COS are low-calorie sweeteners with characteristics such as heat and acidic pH resistance, malleability, oil holding capacity, and compression resistance, making them suitable for incorporation into foods, medicines, and food supplements. They also have the potential to be used in the cosmetics industry due to their moisturizing effect and growth inhibition of some pathogenic bacteria, in animal feed, bioenergy production. COS can also be substrates in fermentation for ethanol production, with advantages over glucose, such as shorter fermentation time, lower risk of contamination during the process, and no inhibition by high concentrations of glucose (ÁVILA et al., 2021; BARBOSA et al., 2020b).

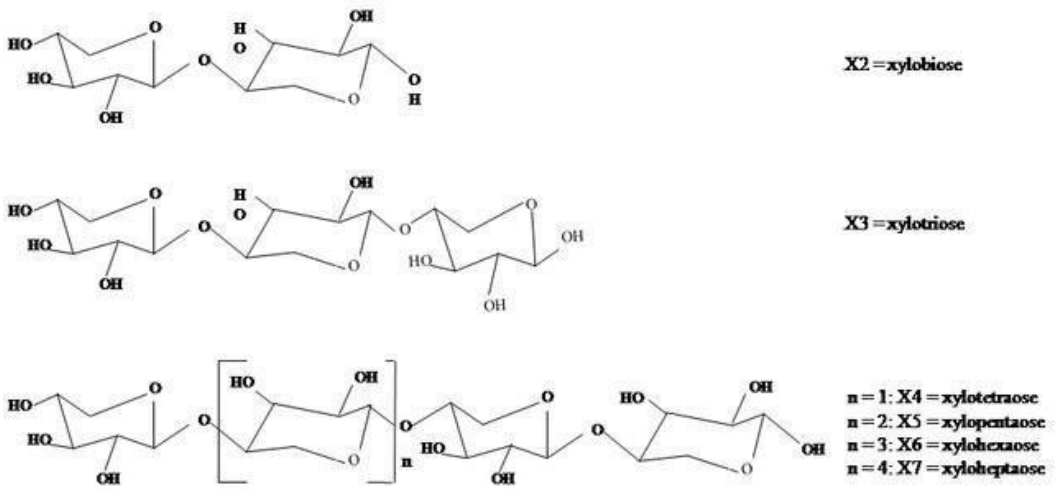
The abundance of cellulose in plants and lignocellulosic by-products is an advantage for COS production. However, one of the most significant challenges concerning other oligosaccharides is the high cellulose recalcitrance, which increases costs and makes it difficult the scale up process (ÁVILA et al., 2021; CHEN; SHROTRI; FUKUOKA, 2021).

Difficulties remain in COS production, underscoring the need for further research to advance the field. Oligosaccharides can contribute to a billion-dollar market, which grows increasingly due to human beings' constant search for improvements in the quality of life. Furthermore, COS are obtained from cellulose, a substance found in nature in large quantities and often discarded, without having its potential adequately explored.

2.3 Xylooligosaccharides

XOS are xylose oligomers, linked through β-(1,4) glycosidic bonds, with a degree of polymerization between 2 and 10 (Figure 2.2), solid and generally colorless, depending on the raw material used in the extraction and the purification process. XOS can have pendant groups such as acetyl, arabinose, uronic acids, which are responsible for the variety of physiological effects of the molecule (MILESSI et al., 2021; MARTINS et al., 2023; PALANIAPPAN; MAPENGO; EMMAMBUX, 2023; MHETRAS; MAPRE; GOKHALE, 2019).

Figure 2.2 - Chemical structure of xylooligosaccharides



Source: Huang et al. (2022).

XOS have physicochemical properties that facilitate their application in foods such as high-water solubility, low viscosity, noncariogenic, pleasant odor, sweet taste and low calories and can be used as sweeteners in various foods and anti-obesity diets. XOS are also resistant to high temperatures and acidic pH, making them suitable for use in juices (MENEZES; DURRANT, 2008; CHEN et al., 2021a; PALANIAPPAN; MAPENGO; EMMAMBUX, 2023). The

consumption of XOS offers a range of health benefits, as described below. Studies suggest that XOS have antioxidant activity, as they increase the amount of antioxidant substances, such as enzyme superoxide dismutase, catalase and glutathione peroxidase. They inactivate the superoxide radical through the breakdown reaction of hydrogen peroxide and hydroperoxide into water and oxygen, which are harmless. In vitro combination of XOS with *Lactobacillus plantarum* can eliminate free radicals (YAN et al., 2022a; IGHODARO; AKINLOYE, 2018).

In a study assessing the antioxidant activity of XOS using the DPPH method, an increase in DPPH radical elimination was observed at XOS concentrations up to 2.5 $mg.mL^{-1}$. Above this concentration, the antioxidant activity remained constant. At a concentration of 2.5 $mg.mL^{-1}$ of XOS with 40% ethanol, the reduction of the DPPH radical was 75% (PASCHOA et al., 2024). In another study, 78% of the DPPH radical was reduced with 2 $mg.mL^{-1}$ of XOS (ÁVILA et al., 2020). The antioxidant activity of these XOS was probably related to compounds in the solution such as phenolic compounds, uronic acids, among others.

When beneficial bacteria ferment XOS, organic acids can be formed, which improve the immune system and regulate antibody production (YAN et al., 2022b). XOS consumption also regulates the expression of pro-inflammatory mediators, suppressing the expression of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), proteins that trigger inflammatory molecules (CHEN et al., 2021b; JANG et al., 2021; BORASCHI, 2022).

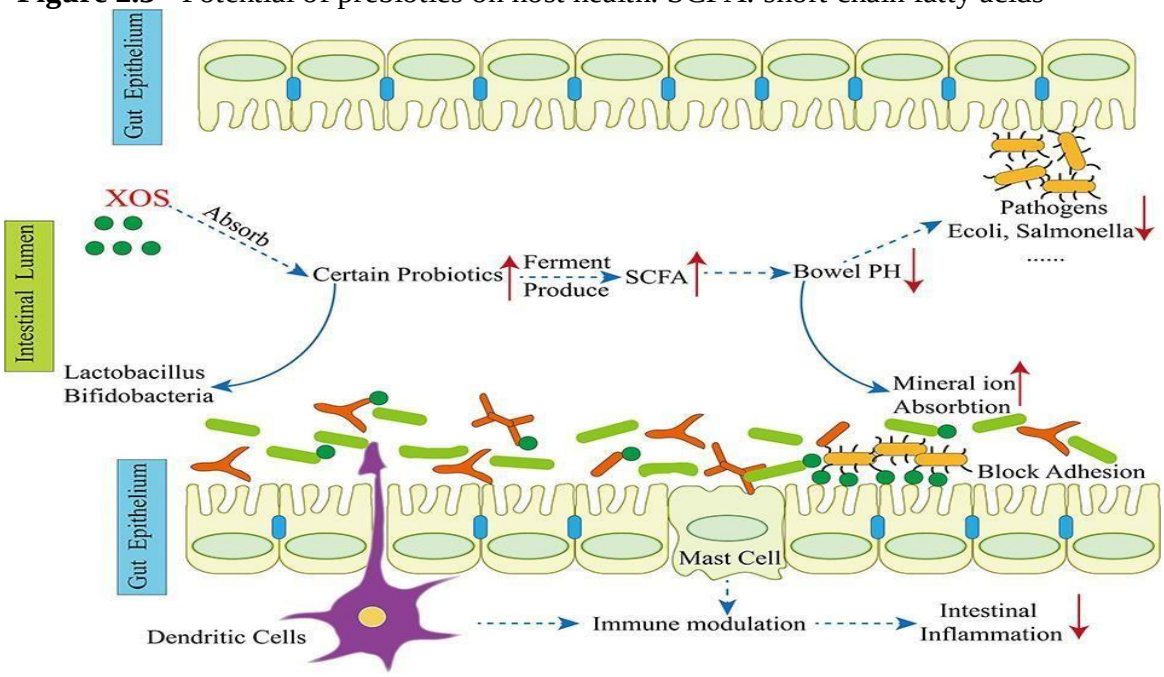
Consumption of XOS can increase the number of *Lactobacilli* in the intestine, which produce lactic acid that can be consumed by butyrate-producing bacteria. Butyrate exerts anti-inflammatory activity by suppressing nuclear factor kappa B (NF-κB), responsible for regulating pro-inflammatory cytokines and increases the expression of peroxisome proliferator-activated receptor (PPAR-γ), which, in addition to inhibiting the expression of inflammatory cytokines, regulate cellular differentiation, lipid and carbohydrate metabolism, attenuate brain degenerative processes and control oxidative stress and neuronal death (DE MAESSCHALCK et al., 2015; VILLAPOL, 2018). XOS can also increase T and B cell activation which are anti-tumor (YAN et al., 2022a).

There are many studies on XOS, which are already produced on a large scale (but only by a few companies). They are widely used in Japan and China as a functional food ingredient. The daily dose required to obtain beneficial health effects is from 1 to 4 g. Doses above 12 g per day can cause undesirable effects such as gastrointestinal discomfort and laxative effect, symptoms common to excessive dietary fiber intake. They are commonly found in fruit juices, dairy products, chewable gums and dietary supplements (JUHÁSZ; PENKSZA; SIPOS, 2020). When

incorporated into animal feed, they have the potential to replace antibiotics, reducing undesirable microorganisms in the intestinal microbiota and reducing gastrointestinal infections (CHEN et al., 2021b).

XOS ingestion decreased the pH of the cecum, possibly due to short-chain fatty acid production, improving the solubility and absorption of minerals. An increase in the height of the duodenal villi may occur, increasing the surface area and contributing to the absorption of minerals such as calcium in the intestine (GAO; ZHOU, 2020). Figure 2.3 summarizes the main effects of XOS consumption on the host's health.

Figure 2.3 - Potential of prebiotics on host health. SCFA: short chain fatty acids



Source: CHEN et al., 2021b

XOS can also be used in cosmetics, due to their moisturizing, emulsifying, stabilizing and antioxidant capacity, Furthermore, prebiotics can improve skin resistance and alleviate irritations, due to the balance of the skin's microbiota (YAN et al., 2022a). The skin's microbiota generally comprise *Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria* and *Firmicutes* and when balanced, they prevent the proliferation of pathogenic bacteria, combating skin diseases such as acne, an inflammation caused by *Propionibacterium acnes*. Bacteriocins produced by resident microbiota can prevent the colonization of invasive species, i.e., *S. lugdunensis* inhibits the pathogens *S. aureus*, *Listeria monocytogenes*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. *S. epidermidis* is capable of destroying *S. aureus* biofilms (SKOWRON et al., 2021; HOU et al., 2022). In a study on premature skin aging, it was observed that consuming XOS in juice for

8 weeks was able to improve the moisture, elasticity and radiance of the participants' skin (HUANG et al., 2022).

XOS have a high market value, estimated between 25 and 50 dollars per kilo, depending on their purity, and as an advantage, they can be produced with agro-industrial by-products (MHETRAS; MAPRE; GOKHALE, 2019).

There are several studies on XOS production, which are commericialized on a large scale and are expected to reach a global market of approximately $144.50 million by 2033, with an annual growth of around 7% (TAMAYO-PEÑA et al., 2024). The global market value reached by oligosaccharides in 2023 was approximately 7.37 billion dollars with a 10.4% annual growth rate (ŠEKULJICA et al., 2023; YAN et al., 2022a).

Methods need to be developed to increase XOS production and reduce costs. Some possible strategies are the following: using accessible and cheap by-products from lignocellulosic materials, such as agricultural and industrial sectors; utilizing more specific and efficient enzymes, which can be produced through genetic engineering; developing techniques for reusing enzymes; having greater control of process conditions, such as temperature, pH and time; and improving purification processes.

2.4 Prebiotic activity of COS and XOS

COS with a chain comprising up to 6 glucose units and XOS are soluble in water and not digested by humans, but when ingested, they have the potential to act as a prebiotic, that is, a digestible compound that is fermentable by some probiotic microorganisms. Probiotics are live microorganisms that provide benefits to human or animal health, such as *Lactobacillus* and *Bifidobacterium* (largest groups) and yeasts of the genus *Saccharomyces.* They inhibit the growth of some pathogenic microorganisms due to acid and bacteriocin production, which are proteins produced by some bacterial strains, with bacteriostatic or bactericidal activity (FORSAN; DE FREITAS; BRIENZO, 2023; DAHIYA; NIGAM, 2022; MILESSI et al., 2021; ÁVILA et al., 2021).

Lactobacillus spp. are facultative anaerobic bacteria, gram-positive, rod-shaped, capable of fermenting carbohydrates and producing lactic, acetic and propionic acid. The main species of Lactobacillus are *L. acidophilus, L. plantarum, L. brevis, L. paracasei, L. casei, L. fermentum, L. gasseri, L. helveticus, L. reuteri* and *L. rhamnosus* (DEMPSEY; CORR, 2022). *Bifidobacterium* spp. is nutritionally demanding, gram-positive, anaerobic bacteria, found in human and warm-

blooded animal intestines. The main species are *B. bifidum, B. longum, B. infantis, B. breve, B. adolescentis, B. angulatum, B. catenulatum*, and *B. dentium*. They can utilize non-digestible oligosaccharides and produce acetic and lactic acid (SHORI, 2022; HOOVER, 2014).

Some bacteria of the genus *Lactobacillus spp*. and *Bifidobacterium spp*. control the pH of the large intestine due to acid production, inhibiting the growth of pathogens and putrefactive bacteria. Furthermore, these bacteria offer several health benefits, such as the suppression of pro-carcinogenic enzymes, degradation of nitrosamines and aflatoxins that cause gastric and colon cancer, improvement in the immune system, production of antibodies, regulation of intestinal homeostasis and vitamin production (SHORI, 2022; WICIŃSKI et al., 2020; LEE et al., 2022; ERDOĞAN; ERTEKIN FILIZ, 2023; XUE et al., 2023; RASTOGI; SINGH, 2022).

Several studies report numerous health benefits associated with increased probiotics through the consumption of prebiotics. These include the restoration of intestinal microbiota following antibiotic use in the digestion of individuals with lactose intolerance (MILESSI et al., 2021; LATIF et al., 2023; GUL; DURANTE-MANGONI, 2024). Probiotics also improve the immune system by distinguishing between beneficial and pathogenic microorganisms, aiding in fiber digestion, and contributing to the production of certain vitamins that humans cannot synthesize. Moreover, they promote the production of short-chain fatty acids (SCFAs), which are metabolites generated through anaerobic fermentation. The main SCFAs are acetate, butyrate and propionate. Acetate is one of the most produced SCFAs in the human intestine and is associated with enteric bacteria such as *Lactobacillus* spp., *Bifidobacterium* spp., *Ruminococcus* spp., *Akkermansia muciniphila* and *Prevotella* spp. It reduces appetite, helping to treat obesity, lowering cholesterol and blood glucose levels (GONÇALVES et al., 2023; DAHIYA; NIGAM, 2022; NOGAL et al., 2021).

Butyrate, a source of energy for intestinal epithelial cells, helps maintain the integrity of the intestinal barrier, reduces high blood pressure and the risk of cardiovascular diseases. It has also been reported to present anti-inflammatory and anti-cancer properties (HOU et al., 2022; ZHONG et al., 2020; SCHWAIGER et al., 2022). The main producing bacteria are: *Faecalibacterium prausnitzii*, species of *Roseburia* and *Clostridium* (MOHAMED ELFADIL et al., 2023).

Propionate has anti-inflammatory activity, is also associated with cholesterol absorption reduction, improves glucose tolerance and insulin sensitivity and inhibits colorectal cancer (XIONG et al., 2022). Propionate can be produced by bacteria *Akkermansia muciniphila* (PATEL; SESHADRI; DALAI, 2022).

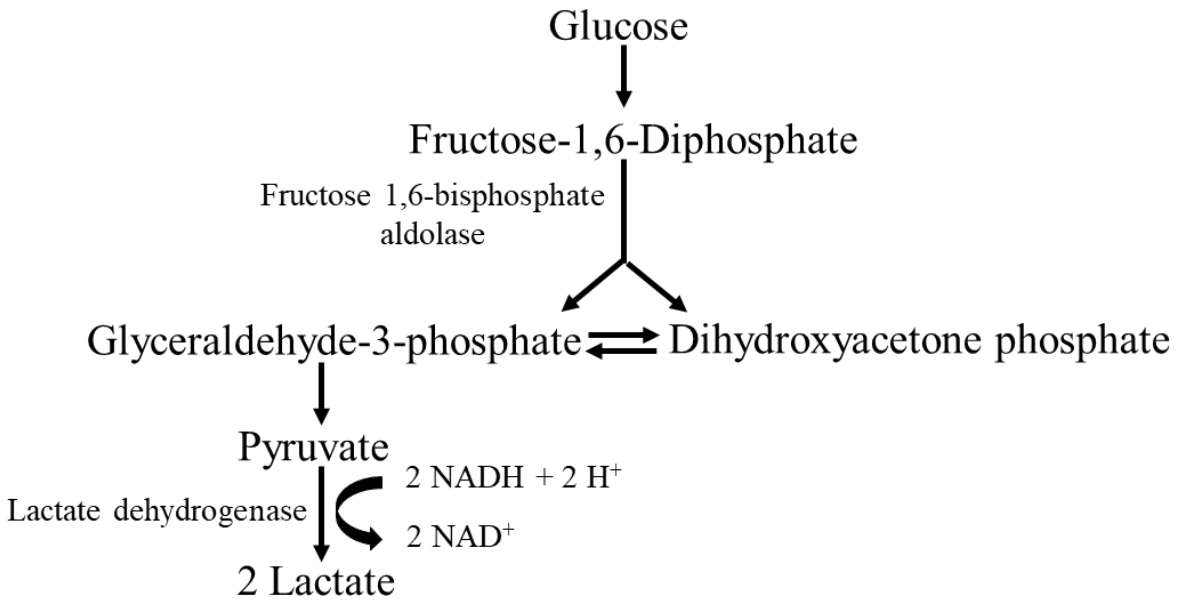
2.5 Glucose metabolism by *Lactobacillus*

Bacteria of the genus *Lactobacillus* are called lactic acid bacteria (LAB), due to their ability to ferment sugars, forming lactic acid as a metabolite (WANG, Y. et al., 2021). *Bifidobacteria* are often included in this group, as they are also capable of producing lactic acid in fermentation (POKUSAEVA et al., 2011).

LAB are facultative anaerobic, gram-positive, non-spore forming, immobile and grow at temperatures between 20 and 45 ºC and pH between 5.5 and 6.5 (PACHLA et al., 2018; SOUZA; DE OLIVEIRA; DE OLIVEIRA, 2023). They are of great importance in the food industry and as probiotics. They can degrade non-digestible polysaccharides and produce short-chain fatty acids, vitamins, bacteriocins and lactic acid, substances that acidify the food, improve their nutritional properties, increase shelf life and improve the flavor of fermented foods. Lactic acid produced by LAB is also used as an emulsifying and moisturizing agent in cosmetics, pharmaceutical processes, the tanning industry, and in biopolymer production, i.e., polylactic acid. It has the potential to replace petroleum-derived polymers, among other functions (WANG, Y. et al., 2021; PAPAGIANNI, 2012).

LAB need complex nutrients to grow, such as vitamins, carbohydrates, mineral salts and amino acids. They are divided into three groups according to the final product formed during fermentation (KWOJI et al., 2022). The first is the homofermentative LAB group, (e.g., *Lactiplantibacillus plantarum, Lacticaseibacillus casei, Lactococcus lactis, Lactobacillus acidophilus, Lacticaseibacillus rhamnosus, Ligilactobacillus salivarius, Lactobacillus helveticus, Lactobacillus Delbrueckii*) (OJO; DE SMIDT, 2023; STEPHEN; SALEH, 2023). They convert hexoses such as glucoses mainly into lactic acid (Figure 2.4) through glycolysis (Embden Meyerhof Parnas pathway). This process can occur under aerobic or anaerobic conditions, conditions and involves the enzyme fructose 1,6-bisphosphate aldolase which converts almost all glucose into energy. Pentoses can also be used by some LAB, through the same process mentioned. During LAB homofermentation, theoretically two molecules of lactic acid are formed per mol of glucose consumed, and 2 mol of ATP. However, the yield varies according to the carbon source consumed (ZAROUR et al., 2017; OJO; DE SMIDT, 2023; STEPHEN; SALEH, 2023; GIACON et al., 2022).

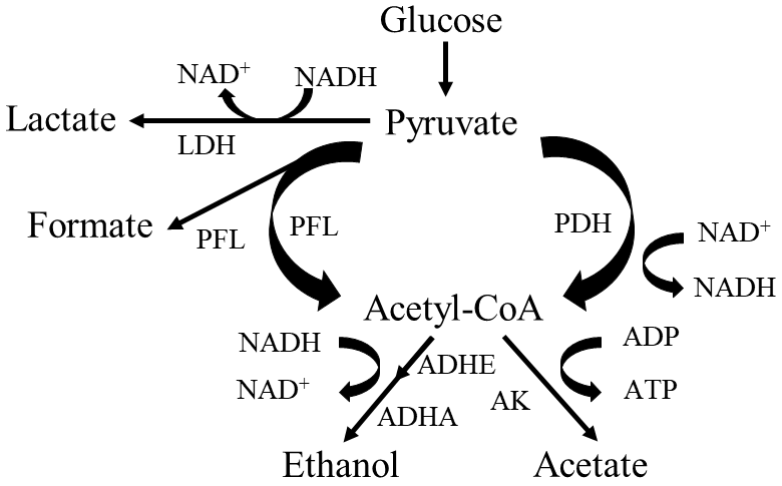
Figure 2.4 - Homolactic fermentation of glucose in lactic acid bacteria (LAB) by glycolysis (Emben–Meyerhof–Parnaspathway)



Source: Author

Under stress conditions, such as glucose limitation, changes in pH and temperature of the medium, or growth on other carbon sources, the homofermentative pathway is used, but pyruvate is metabolized differently through mixed acid fermentation pathways, which can still produce lactic acid, ethanol, acetate, and formate (Figure 2.5). Bacteria with this metabolism are *Lactococcus lactis* and bacteria of the genera *Citrobacter, Yersinia, Salmonella, Escherichia, Proteus, Shigella* and *Vibrio* (CIANI; COMITINI; MANNAZZU, 2008; OJO; DE SMIDT, 2023). In the absence of oxygen, pyruvate is converted to lactic acid and metabolized by pyruvate formate lyase (PFL) to acetyl-CoA and formate. In the presence of oxygen, pyruvate formate lyase (PFL) is inactivated, and pyruvate is metabolized to acetyl-CoA, NADH and carbon dioxide by pyruvate dehydrogenase (PDH) (HOFVENDAHL; HAHN–HÄGERDAL, 2000; JOHANSON et al., 2020). When PFL is activated, acetyl CoA is metabolized to acetate via phosphotransacetylase (PTA) and acetate kinase (AK). In the case of PDH, acetyl CoA is metabolized into ethanol via acetaldehyde dehydrogenase (ADHE) and alcohol dehydrogenase (ADHA). Acetate formation generates additional ATP, while ethanol formation regenerates NAD^+ (JOHANSON et al., 2020; FUKUDA; ASO; NOLASCO-HIPÓLITO, 2023).

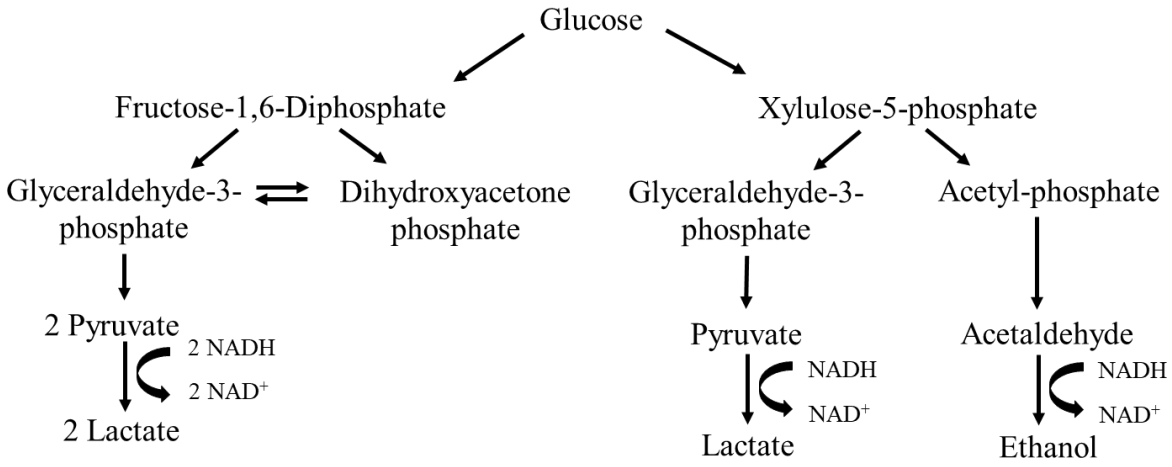
Figure 2.5 - Glucose fermentation route by lactic acid bacteria (LAB): mixed acid fermentation pathway. PDH: pyruvate dehydrogenase, PFL: pyruvate formate lyase, and LDH: lactate dehydrogenase, ADHE: acetaldehyde dehydrogenase, ADHA: alcohol dehydrogenase, AK: acetate kinase



Source: Author.

The third group is the heterofermentative LAB, which in addition to lactic acid produce other products such as carbon dioxide, aroma compounds and acetic acid (ZAROUR et al., 2017). They are divided into two groups, facultative heterofermentative such as *Lactobacillus plantarum, Lactobacillus alimentarius, Lactobacillus casei, Lactobacillus lactis* and *Lactobacillus pentosus* and obligatory heterofermentative, such as *Lactobacillus brevis, Lactobacillus reuteri and Lactobacillus fermentum* (OJO; DE SMIDT, 2023). Facultative heterofermentative LAB have homofermentative metabolism (glycolysis), and ferment hexoses such as glucose, almost entirely into lactic acid. When there is a shortage of glucose, the metabolism is changed to obligatory heterofermentation by the phosphoketolase (PKP) pathway, and hexoses are fermented, generating equimolar amounts of lactate, acetate, ethanol and carbon dioxide and 1 mol of ATP per mol of hexose. Pentoses can be fermented generating lactic and acetic acid (Figure 2.6) (DE ANGELIS; GOBBETTI, 2016; MORA-VILLALOBOS et al., 2020; GIACON et al., 2022).

Figure 2.6 - Heterolactic fermentation of glucose in lactic acid bacteria by the phosphoketolase pathway

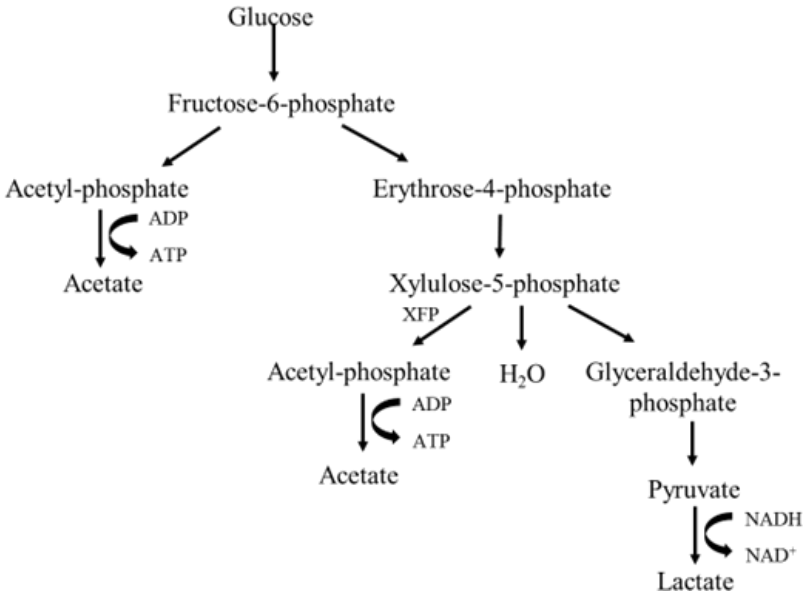


Source: Wee, Kim, Ryu (2006).

2.6 Glucose metabolism by *Bifidobacteria*

Bifidobacteria break down carbohydrates into monosaccharides through enzymes. Monosaccharides (hexoses) are fermented by the pathway called fructose-6-phosphate shunt or 'bifid shunt' (POKUSAEVA; FITZGERALD; VAN SINDEREN, 2011). In this pathway, glucose is isomerized to fructose-6-phosphate, which is cleaved by the enzyme phosphoketolase (XFP) in acetyl phosphate and erythrose-4-phosphate. Acetyl phosphate and ADP are converted into acetate and ATP by the enzyme acetatekinase (ACKA). Erythrose-4-phosphate reacts with another fructose-6-phosphate molecule, forming xylulose-5-phosphate which is cleaved by XFP to acetyl phosphate and glyceraldehyde 3-phosphate. The first is converted into acetate and ATP and the second is converted by the EMP pathway into lactate (Figure 2.7) (WOLFE, 2015).

Figure 2.7 - Glucose metabolism by *Bifidobacteria* through the fructose-6-phosphate pathway or 'bifid' shunt



Source: Author.

Theoretically 2.5 moles of ATP, 1 mol of lactate and 1.5 mol of acetate are produced from every 1 mol of glucose. These amounts may vary according to the species of *Bifidobacteria*, its growth phase and the type of fermented carbohydrate (O'CALLAGHAN; VAN SINDEREN, 2016).

2.7 Xylose metabolism by *Lactobacillus* and *Bifidobacteria*

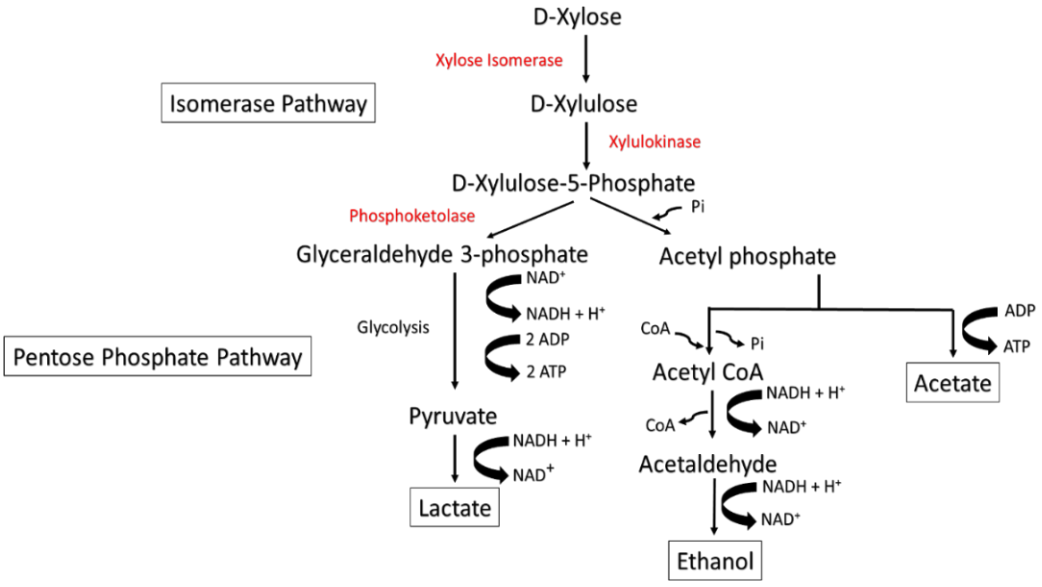
L. plantarum and *B. breve* can ferment hexoses but only a few can use pentoses oligomers, such as XOS. The use of XOS by microorganisms varies according to their degree of polymerization and the strain of bacteria. Microorganisms intracellularly cleave XOS into xylose using various enzymes such as β-xylosidases, exo-xylanases and α-L-arabinofuranosidases, α-glucuronidase, α-L-arabinosidase and acetyl xylan esterase (VIVEK et al., 2019; ILIEV et al., 2020).

A study showed that *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus lactis* are capable of growing in a medium containing XOS and producing mainly lactic, formic and acetic acid and ethanol. These are typical products of mixed acid fermentation, which occur during glucose deprivation in some microorganisms (ILIEV et al., 2020). *B. adolescentis* is able to ferment XOS, producing acetate, lactate and formate (GULLÓN et al., 2008). Strains of *B.*

breve, *B. longum*, *B. animalis*, *B. infantis*, *B. bifidum*, *B. thermophilum*, *L. brevis*, *L. casei*, *L. fermentum* and *L. reuteri* are also able to grow in medium containing XOS (SAVILLE; SAVILLE, 2018).

Bacteria usually ferment xylose through the isomerase pathway. Xylose is converted by xylose isomerase (XI), into D-xylulose, which is phosphorylated into D-xylulose-5-phosphate by xylulose kinase (XK), entering the pentose phosphate pathway, where D-xylulose-5-phosphate is cleaved by phosphoketolase into glyceraldehyde-3-phosphate and acetylphosphate. The first is converted into pyruvic acid and then into lactic acid and the second is converted into acetyl CoA and acetic acid. Moreover, quimolar amounts of lactic and acetic acid are formed (Figure 2.8) (JAGTAP; RAO, 2018; DOMINGUES et al., 2021).

Figure 2.8 - Metabolic pathways of D-xylose metabolism in lactic acid bacteria



Source: Author.

2.8 Process to obtain COS and XOS

Physical-chemical treatments with or without the addition of reagents as a catalyst are effective in hydrolyzing hemicellulose and partially cellulose, producing xylose and glucose oligomers and sugar monomers. To obtain high yields, suitable conditions such as temperature, reaction time, reagent concentration and solids load need to be combined (ÁVILA et al., 2021). The main methods for producing COS are acid hydrolysis and enzymatic hydrolysis (ÁVILA; DE MÉLO; GOLDBECK, 2023). The main methods for COS production are summarized in Table

2.1.

Table 2.1 - Main methods for COS production

Treatment	Advantages	Disadvantages	References
Autohydrolysis	• Menor degradation of cellulose • Lower environmental impact • Lowest cost • The absence of chemical reagents	• Production of monosaccharides • Formation of sugar degradation products using severe conditions (high temperature and reaction time). • Need for special equipment	• Pereira et al., 2022b • Henriques et al., 2021
Acid	• Low cost (some acids) • Solubilization of hemicelluloses, increased accessibility of cellulose to enzymes • Cellulose breakage in COS	• Sugar degradation • Formation of monomers • Equipment corrosion • Environmental Impact • Difficult to recycle the reagent	• Melati et al., 2019 • Oriez et al., 2019 • Candido et al., 2020 • Jin et al., 2022
Alkaline	• Solubilizes hemicellulose • Reduces cellulose crystallinity and degree of polymerization • Facilitates enzymatic hydrolysis for COS production • Milder conditions compared to other types of treatments, with reduced costs • Does not degrade carbohydrates	• Extensive washing and neutralization are required prior to enzymatic hydrolysis • Suitable for biomass with low amounts of lignin.	• Javanmard et al., 2024 • Martins et al., 2021 Sasmal & Mohanty, 2018 • Shimizu et al., 2020
Oxidative	• Effective to remove hemicellulose and lignin from biomass • Milder temperature and concentration conditions • Atmospheric pressure • Use of hydrogen peroxide (low environmental impact) • Less formation of inhibitory compounds • Less degradation of polysaccharides • Solubilizes hemicellulose and lignin from biomass	• Low selectivity, resulting in cellulose degradation • Chemical reactions with carbohydrates and cell wall components - production and accumulation of inhibitory products • Expensive reagents on an industrial scale due to the large volume of gas required	• Zhou et al., 2023 • Ho et al., 2019 • Donkor et al., 2022
Enzymatic	• More ecological method • Without formation of toxic compounds • It is possible to obtain oligosaccharides and little monosaccharide • Products formed are more suitable for application in medicines and foods	• Enzyme purification procedure is expensive and complex • Expensive and time-consuming treatment for large-scale applications	• Schwaiger et al., 2022 • Forsan et al., 2021b • Ghosh et al., 2021 • Henriques et al., 2021

Source: Author

2.8.1 Autohidrolysis or Liquid hot water (LHW)

XOS can be produced by the autohydrolysis method, which consists of using water at high temperatures, releasing acetic acid from the cleavage of the bonds between hemicellulose and acetyl, making the medium acidic, and enhancing the hydrolysis of the glycosidic bonds of the xylan chain (SUREK; BUYUKKILECI, 2017; FORSAN et al., 2022). Using this method, XOS was produced from banana peel and guava bagasse, at 160 ºC for 15 min and converted 32.6% and 26.87% of xylan into XOS, respectively (PEREIRA et al., 2022b). During the autohydrolysis of hazelnut pruning residues at 190 ºC for 5 minutes, the main component extracted from the biomass was xylan, which was hydrolyzed into XOS, reaching a concentration of 7.3 $g.L^{-1}$. The solid residue had a cellulose and lignin concentration of 57.7% and 39.6%, respectively, enabling future enzymatic hydrolysis to produce COS (SUREK; BUYUKKILECI, 2017).

This method minimizes the degradation of cellulose, which is almost completely retained in the solid fraction, as well as lignin and makes it more accessible to enzymes, facilitating the production of COS by enzymatic hydrolysis (GU et al., 2022).

The main advantages are lower environmental impact, reduced process costs, and the absence of catalytic reagents avoiding the need for subsequent recovery and neutralization processes. Under moderate conditions, xylan solubilization and depolymerization occur, resulting in XOS of varying sizes that generally have a more conserved structure compared to xylan (PEREIRA et al., 2022b). As a disadvantage, there is the production of monosaccharides such as xylose, arabinose, mannose and the formation of sugar degradation products using severe condition (high temperature and reaction time). In addition, equipment that can withstand high pressures and temperatures is required (HENRIQUES et al., 2021).

Neto et al. (2020) reported the optimization of XOS production by hydrothermal pretreatment from *Eucalyptus* by-products. Process variables (temperature, time, and mass load) were evaluated using a central composite experimental design comprising a 2^3-factorial design with six axial points that were used to optimize the conditions for maximum XOS production. The optimized condition was as follows: temperature of 161 °C, reaction time of 65 min, and consistency of 10 % (mass/mass) resulting in a xylan conversion to XOS of 42.3% (mass/mass).

Dias et al. (2022) reported the optimization of XOS production by hydrothermal pretreatment of two by-products of sugarcane bagasse and straw. A central composite rotational design in conjunction with response surface methodology was used to optimize the conditions for maximum XOS production. The xylan conversion to XOS was 24.8% (mass/mass) and 45.3%

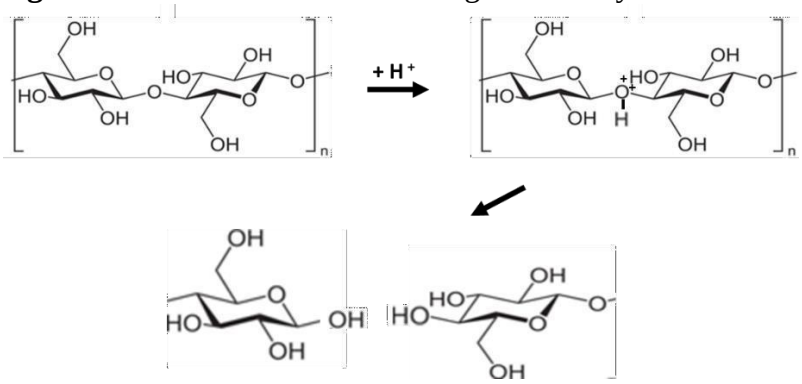
(mass/mass) for sugarcane bagasse and straw, respectively. The developed mathematical models were statistically adequate to predict xylan conversion to XOS, and the by-products were promising to produce XOS.

2.8.2 Acid hydrolysis

Acid hydrolysis treatment generally causes the breakdown of glycosidic bonds, solubilizing hemicelluloses, and increasing cellulose accessibility to enzyme action (BRIENZO et al., 2015; SHIMIZU et al., 2020). The lignin is modified with little or reduced solubilization. Furthermore, most of it remains in the material and is potentially deposited on the material surface (DE AZEVEDO et al., 2024). Due to the cleavage of some ester and ether bonds in the lignin molecule, low molecular weight fragments with a greater quantity of hydroxyl groups are generated. High acid concentrations, high temperatures, or long reaction times can cleave cellulose chains. However, such conditions can also result in sugar degradation (MELATI et al., 2019).

During the acid treatment process, hydronium ions from the acid cleave the β-1,4 glycosidic bonds present in the solubilized xylan (the most abundant hemicellulose), releasing XOS and monosaccharides such as xylose. Glucose (in small amounts) can also be released from the amorphous regions of cellulose, which are disordered and less compact compared to the crystalline regions (Figure 2.9) (ALMASHHADANI et al., 2022; GARGIULO et al., 2022). The acid catalyzes the breakdown of the cellulose chain, through the reaction of the H^+ proton with oxygen between the glucose molecules, breaking the bond between carbon and oxygen and forming a conjugated acid that, when reacting with water, releases sugar and proton. The long cellulose chain can be broken into smaller chains releasing COS, and sugar monomers (SASMAL; MOHANTY, 2018).

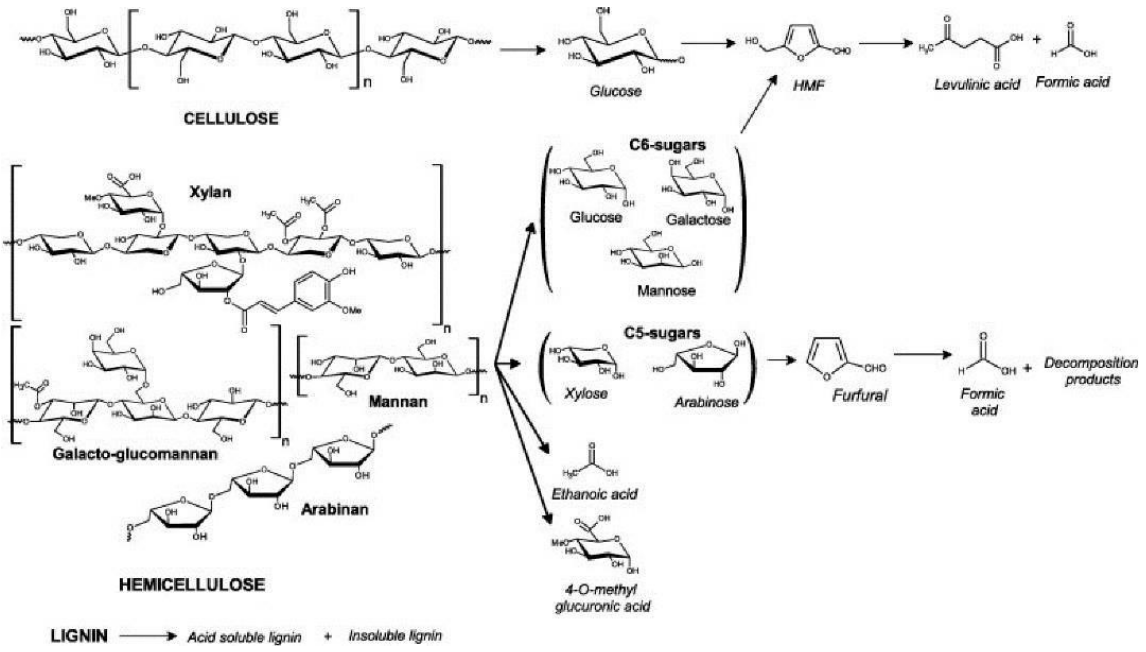
Figure 2.9 - Cellulose chain breaking reaction by acids



Source: Author.

Mineral acids can be used, mainly sulfuric acid due to its efficiency in hydrolysis and low cost. Other acids such as nitric, hydrochloric and phosphoric acid have also been studied but compared to sulfuric acid they are expensive, less efficient. Moreover, in the case of phosphoric acid, there are still few studies for comparison (ORIEZ; PEYDECASTAING; PONTALIER, 2019). Depending on study conditions, cellulose may decompose into monomers of glucose and hemicellulose into monomers of xylose, glucose, mannose, arabinose and galactose. These compounds can be degraded into molecules such as levulinic and formic acid, furfural, 5-hydroxymethyl furfural, among others, which may inhibit fermentation processes and cause loss of sugars (Figure 2.10) (CANDIDO et al., 2020). Furthermore, inorganic acids are corrosive and difficult to recycle. As an alternative, organic acids such as acetic, maleic and oxalic acid have been studied, as they are less corrosive and reduce the formation of sugar degradation products. According to studies, organic acids are effective in breaking down the lignocellulosic structure of materials such as sugarcane, maple, corn waste and hardwoods (JIN et al., 2022; FORSAN et al., 2021a; LIU et al., 2020). By-products, generated in a process, can be removed in processes, such as membrane separation (HENRIQUES et al., 2021), or detoxification with adsorbents compounds (CANDIDO et al., 2020).

Figure 2.10 - Chemical reaction between cellulose and hemicellulose with acids, with formation of degradation products



Source: Oriez; Peydecastaing; Pontalier (2019).

The choice of ideal conditions for acid pretreatment depends on the product of interest. To maximize the production of oligomers and reduce the formation of monomers and degradation products, ideal conditions are required, which depend on parameters such as acid concentration, temperature, reaction time and solid-liquid ratio (FORSAN et al., 2021a). Another factor that can influence hydrolysis is the size of the biomass particles (FERNANDES et al., 2020; ALVES et al., 2021). Hydrolysis with dilute acid (concentration up to 8% w/w) is generally carried out at high temperatures, between 100 and 200 ºC. In the hydrolysis process of banana peel and guava bagasse with 3% sulfuric acid, 37.69% of xylan was converted into XOS for banana peel and 59.60% for guava bagasse, with reaction times of 15 minutes at 160 ºC for banana and 100 ºC for guava (PEREIRA et al., 2023). On the other hand, using concentrated acid, reactions occurred at temperatures below 60 ºC (ORIEZ; PEYDECASTAING; PONTALIER, 2019).

2.8.3 Alkaline pretreatment for solubilizing hemicellulose and reducing cellulose recalcitrance

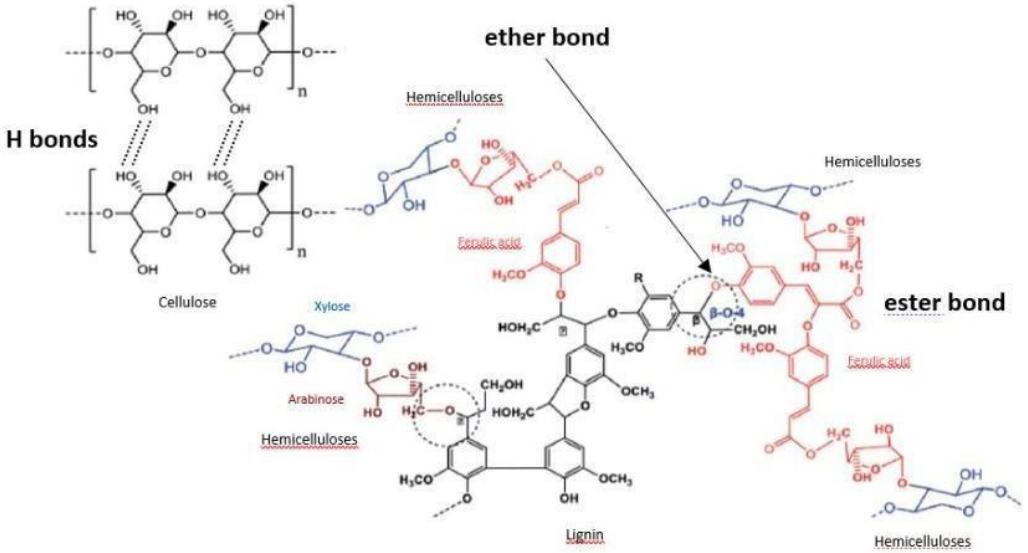
Alkaline pretreatment of the lignocellulosic biomass is chosen as a pretreatment to solubilize hemicellulose, reducing cellulose crystallinity and facilitating subsequent enzymatic hydrolysis for COS production. Solubilized hemicellulose can be hydrolyzed to XOS by other chemical or biological treatments. It is a method that can be carried out under milder conditions compared to other types of pretreatments at a reduced cost. Generally, the reagents used are calcium oxide, sodium, potassium and ammonium hydroxide (MARTINS et al., 2021; SASMAL; MOHANTY, 2018). Hemicellulose solubilization can be improved using hydrogen peroxide in an alkaline medium, resulting in a better quality of hemicellulose in terms of solubility, purity, and enzyme hydrolysis/action (MELATI et al., 2021; BUENO; DE FREITAS; BRIENZO, 2023).

Most bases react directly with lignin, solubilizing it, without affecting carbohydrates in terms of degradation, which is desirable in biofuel and prebiotic production processes (SASMAL; MOHANTY, 2018). Part of the hemicellulose is removed, with low cellulose solubilization under medium conditions, which is more accessible to cellulolytic enzymes (SHIMIZU et al., 2020).

The hydroxyl base weakens the hydrogen bonds between cellulose molecules, and between cellulose and hemicellulose. There is swelling of cellulose, an increase in the contact surface, a decrease in crystallinity and a degree of polymerization (ZHANG, 2021). There is also the cleaving of bonds between lignin and polysaccharides, between hemicellulose and its substituent groups (acetate groups, uronic acids) and breaking of ester bonds between hemicellulose and phenolic monomers (coumaric and ferrulic acids) (Figure 2.11). The undissolved hemicellulose undergoes

structural modification, which enhances its digestibility (ORIEZ; PEYDECASTAING; PONTALIER, 2020).

Figure 2.11 - Effect of alkaline pretreatment on biomass



Source: Oriez; Peydecastaing; Pontalier (2020).

In the study performed by Shimizu et al. (2018), banana pseudostem was pretreated with acid (H_2SO_4), alkali (NaOH) and peroxide (H_2O_2). All the hemicellulose was removed with the acid. Using alkali and peroxide, the amount of hemicellulose was reduced to 4.38% and 8.68%, respectively. Concerning lignin, there was an increase from 17.26% to 40.0% using the acid treatment and a reduction in percentage to 7.65% and 7.17%, with alkali and peroxide, respectively. There was an increase in cellulose percentage from 60.84% to 75.48% in alkaline pretreatment and from 60.84% to 74.37% in peroxide pretreatment. In the case of acid, there was no change in cellulose proportion. Acid and alkali increased surface area and accessibility to cellulose more efficiently than peroxide, improving enzymatic hydrolysis yields for glucose production.

2.8.4 Oxidative pretreatment

Oxidative pretreatment is effective to remove hemicellulose and lignin from biomass, while cellulose remains in the solid fraction. This pretreatment facilitates subsequent enzymatic hydrolysis treatment. It is generally carried out under milder conditions and there is no or less

formation of inhibitory compounds such as furfural and less degradation of polysaccharides (ZHOU et al., 2023).

Various reagents can be used in this pretreatment, such as hydrogen peroxide (H_2O_2), which under alkaline conditions (pH around 11.6), solubilizes hemicellulose and lignin from biomass, due to the formation of superoxide radicals (O_2^-) and hydroxyl (OH^-). It can be carried out at atmospheric pressure and at under milder temperature and concentration conditions. Hydrogen peroxide can be decomposed into water and oxygen, causing low environmental impact (HO; ONG; WU, 2019).

Organic peracids, such as performic acid and peracetic acid, can also be used. Peracetic acid is more commonly used because it is more easily prepared, in a reaction between hydrogen peroxide and acetic acid and a catalyst (sulfuric acid). The effectiveness of the method depends on the reagent concentration, temperature, reaction time and solid-liquid ratio (ZHOU et al., 2023).

Pretreatment with oxidative reagent such as sodium chlorite with acid such as acetic acid can be used for lignin removal and holocellulose recovery. The reaction between these two compounds releases chlorous acid, which can be decomposed, depending on the pH and temperature of the medium, into chlorine dioxide, chlorate and chloride ions. In this pretreatment, some side groups of xylan can also be removed (acetyl, arabinosyl and methyl glucuronic acid) (MAFEI et al., 2019).

Another reagent used is Fenton's reagent, a mixture of hydrogen peroxide and ferrous ions (Fe^{2+}) in an acidic medium, with the formation of hydroxide radicals (OH^-), which can degrade lignin and hemicellulose, improving cellulose accessibility. It can occur at low temperatures, generally below 55 ºC and a shorter reaction time (JEONG et al., 2021; ZHOU et al., 2023).

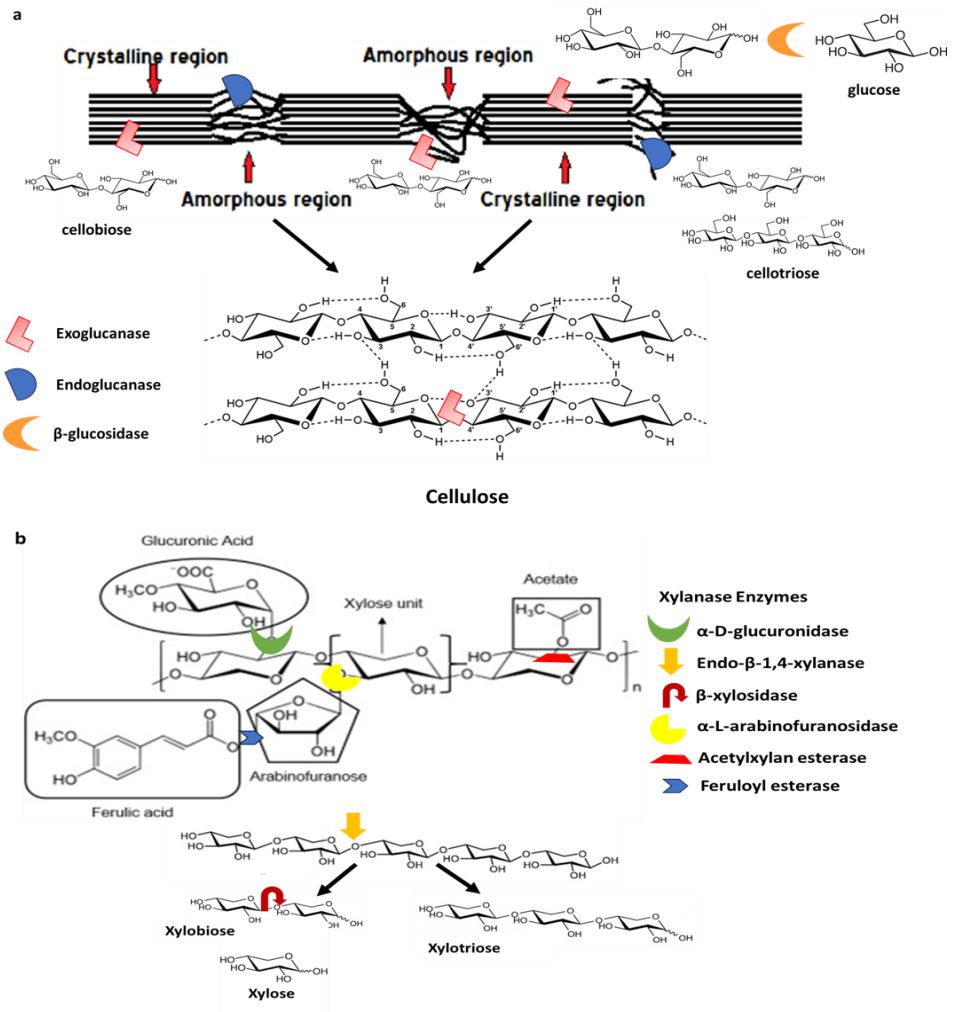
2.8.5 Enzymatic hidrolysis

For the enzymatic depolymerization of cellulose to produce COS, cellulases are used. This enzyme cocktail typically includes the following: endoglucanases, which randomly cleave β-glycosidic bonds in the amorphous regions of cellulose, creating reducing and non-reducing ends and releasing oligomers of varying sizes (primarily cellobiose and cellotriose); exoglucanases (also known as cellobiohydrolases or exo-1,4-β-glucanases), which act at the ends of cellulose chains, releasing primarily cellobiose; β-Glycosidases, which hydrolyze cellobiose into glucose (Figure 2.12a) (BARBOSA et al., 2020a; CHITYALA; NANDANA; JAYACHANDRAN, 2023). Ideally for COS production, an enzymatic cocktail should not contain β-glucosidases. However,

enzyme purification procedure is expensive and complex for large-scale applications (AKHTAR et al., 2016; SCHWAIGER et al., 2022). Optimization of the enzymatic extract can collaborate by decreasing monosaccharide production.

The enzymes endo-β-1,4-xylanases cleave the xylan randomly. Moreover, the β-xylosidases break the chain into xylose monomers. Acetyl xylan esterase hydrolyzes acetylated hemicellulose, releasing acetic acid due to the breakdown of ester bonds. Furthermore, α-D-glucuronidases cleave the bond between xylan and glucuronic acid. α-D-galactosidase hydrolyzes galactose, which is linked to the main xylan chain by an ether bond. In addition, α-L-arabinofuranosidases are responsible for removing arabinose, while feruloyl esterases cleave the ester bond between the xylan chain and ferulic acid (Figure 2.12b) (BHARDWAJ; KUMAR; VERMA, 2019; CHEN et al., 2023; BAJPAI, 2022).

Figure 2.12 – (a) Schematic representation of cellulose chain breakage by exoglucanase, endoglucanase and β-glucosidase enzymes for COS production, (b) Hydrolysis of xylan and its pendant groups by xylanolytic enzymes.



Source: Author.

The advantage of using enzymes to produce COS and XOS over chemical pretreatments is the absence of by-product formation. Enzyme technology produces a hydrolysate rich in oligosaccharides, offering a more eco-friendly method, and the products formed are better suited for application in medical and food products. The disadvantage is that it is expensive and time-consuming (FORSAN et al., 2021b; GHOSH et al., 2021; HENRIQUES et al., 2021).

COS were obtained by enzymatic hydrolysis (endoxylanase 30 $IU.g^{-1}$ and endoglucanase 20 $IU.g^{-1}$ of material) of banana pseudostem and leaves and guava seed cake pretreated with sulfuric acid 20% (w/w). The conversion of cellulose into COS was 53.05%, 79.73% and 59.99%, respectively (FORSAN; DE FREITAS; BRIENZO, 2023). Freeze-dried and ground grape marc was hydrolyzed with endo-1,4-β-d-glucanase from *Trichoderma longibrachiatum* 400 $IU.g^{-1}$ for 24 h. Moreover, 2.5 $g.L^{-1}$ of COS was obtained (LIU; HU; HARITOS, 2024).

De Freitas et al. (2021) produced 11.02 $g.L^{-1}$ of XOS (xylan to XOS conversion was 60.98%) in enzymatic hydrolysis with endoxylanase of *Aspergillus versicolor* 30 $IU.g^{-1}$ of xylan extracted from banana pseudostem with hydrogen peroxide. A similar result was reported by Pereira et al. (2022), equivalent to 11.96 $g.L^{-1}$ of XOS, using xylan extracted from guava seed cake with NaOH, and endoxylanase from *Aspergillus versicolor* 30 $IU.g^{-1}$, 48 h reaction time.

There are several methods for purifying a hydrolysate, generated through the physical-chemical treatments mentioned. The choice of method will depend on the desired purity, cost, yield obtained, and the application of the hydrolysate. Methods such as ultrafiltration, evaporation, adsorption, electrodialysis, liquid-liquid extraction, alkalization, acidification, ion exchange resins, among others, which are capable of removing degradation products, inorganic salts, phenolic compounds, acids, furfural, hydroxymethylfurfural, among others (EGÜÉS et al., 2012; ORIEZ et al., 2019; ORIEZ et al., 2020).

2.9 Emerging technologies in the production of oligosaccharides

Combining physical methods with chemical and biological methods can improve the carbohydrate isolation process or reduce biomass recalcitrance for subsequent production of XOS and COS by hydrolysis. Several technologies have been proposed such as ball milling, genetic engineering, microwave-assisted pretreatment, eutetic solventes and ionic liquids.

2.9.1 Ball milling

Ball milling can reduce the cellulose crystallinity facilitating the enzyme action. A ball mill is comprised of a hollow cylinder filled with steel or rubber balls of various sizes, that rotates around its axis, causing the ball to move, and reducing the particle size of the material through impact. The particle size varies according to the cylinder and ball material, the quantity and size of the balls, the rotation speed and the material to be ground. Mills are generally classified into planetary, horizontal, stirring (SUMANTH KUMAR; JAI KUMAR; MAHESH, 2018; WEI et al., 2022).

Ball milling is an efficient and environmentally friendly form of biomass pretreatment that reduces the size of biomass particles, changes the cellulose from crystalline to amorphous organization, and increases the contact surface area. Ball milling, combined with physical-chemical treatments, facilitates the removal of lignin from biomass, improves biomass digestibility and enzyme accessibility, thereby increasing the efficiency of enzymatic hydrolysis and the yield in oligosaccharide production (QU et al., 2017).

It was possible to convert 79.73% of cellulose into COS using banana leaves pretreated with sodium hydroxide, ground in a ball mill and hydrolyzed with xylanase and endoglucanase (FORSAN et al., 2023).

In COS production using sugarcane bagasse biologically pretreated with *Coniophora puteana*, ground in a ball mill and hydrolyzed with cellulase 50 $IU.g^{-1}$, a conversion of cellulose into COS of 36.65% was obtained. When the material was subjected to the same conditions, but only ground in a knife mill, the conversion was 26.23% (FELIPUCI et al., 2024).

2.9.2 Genetic engineering

The use of enzymes is highly promising for COS and XOS production on an industrial scale, leading to growing interest in genetic engineering and metagenomics research. Through genetic engineering, it is possible to modify genes of microorganisms or polypeptide chains of enzymes to produce enzymes with improved properties, functional stability, better acid and high-temperature tolerance, increased specificity, and catalytic efficiency. Metagenomics aids in the discovery of genes for enzymes with desired properties, such as thermal and pH stability (DONG

et al., 2023; SOUSA et al., 2022).

Through genetic engineering, GH62 arabinofuranosidases from *Aspergillus fumigatus* can be altered, which are enzymes that release α-l-arabinofuranosyl residues, increasing their thermostability, reducing their denaturation and facilitating their application in industrial processes (WILKENS et al., 2017; MARTINS et al., 2024).

2.9.3 Microwave-assisted pretreatment

Using microwaves has been explored in studies as an emerging and environmentally friendly technology to assist in the production of biofuels, biodiesel and convert biomass into valuable products (USMANI et al., 2023).

High temperature and pressure conditions are commonly used in pretreatments to delignify biomass and decompose polysaccharides. As a disadvantage, due to the dehydration of sugars, undesirable by-products such as furfural and HMF are generated, which inhibit fermentative processes. Furthermore, these conditions make the process expensive. As an alternative, the use of microwaves has been suggested, as they offer several advantages such as greater energy efficiency, as the heating is carried out inside the material and not transferred through the surface as in conventional treatments; the entire volume of biomass can be heated and the heating canbe stopped immediately when needed; it is a method that can be faster, with less energy expenditure and greater efficiency; and it can be used with other treatments to improve effectiveness (MIKULSKI; KŁOSOWSKI, 2023; JABLONOWSKI; PAULY; DAMA, 2022).

2.9.4 Deep eutectic solvents (DES)

DES is a mixture of hydrogen bond acceptors and donors, which efficiently convert lignocellulosic biomass. The intramolecular hydrogen bonds of the eutectic mixture can break the hydrogen bonds present in the biomass, solubilizing it. These solvents have desirable characteristics such as low toxicity, thermal resistance, high biodegradability, high stability, reduced cost and can be easily synthesized (AMESHO et al., 2023).

Studies have shown that some DES are capable of dissolving cellulose and can also transform it into nanocrystals that can be used to stabilize water and oil emulsions. In addition, some DES can dissolve lignin, increasing the yield of subsequent hydrolysis with enzyme (CHEN; MU, 2019). They are composed of a cation (generally ammonium, phosphonium or sulphonium)

and a Lewis base, in variable proportions, generating an infinite number of possibilities. DES can be produced for a specific application, with adjustments in density, acidity, viscosity, etc. Some examples of mixtures are: choline chloride and zinc chloride, choline chloride and carboxylic acid, choline chloride and amides (DEL MAR CONTRERAS-GÁMEZ et al., 2023).

2.9.5 Ionic liquids

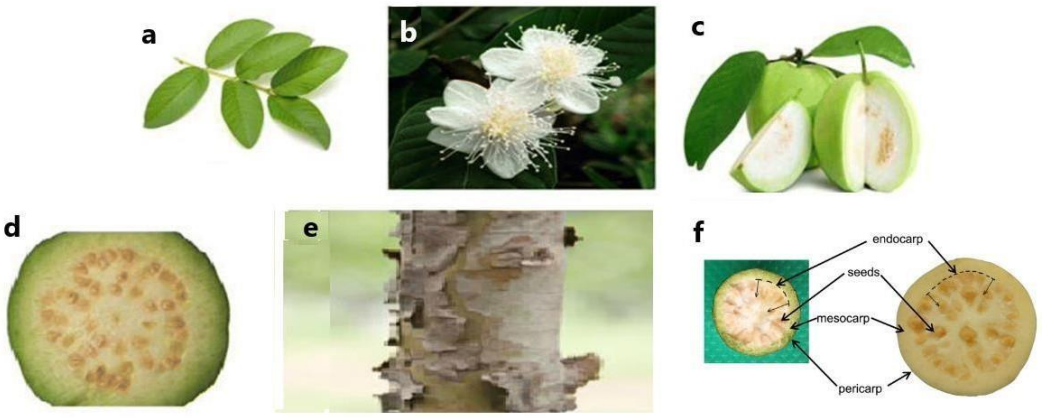
Ionic liquids (ILs) are formed by cations and anions, which can form hydrogen bonds with cellulose at high temperatures. They are promising for dissolving and converting biomass. ILs based on imidazolium are capable of dissolving cellulose. They have advantages such as high stability and dissolution capacity, effectiveness in separating lignin, thermal stability, low vapor pressure (less air pollution), among others. However, they are expensive, not environmentally friendly, they can evaporate, absorb water from the air, are toxic and their synthesis requires a substantial amount of energy (DONG; ZHANG; WANG, 2016; HASANOV; RAUD; KIKAS, 2020; CHEN; MU, 2019; OCRETO et al., 2022). Because they are formed by cations and anions, there are countless possibilities for mixing, so ionic liquids can be designed for a specific application (DONG; ZHANG; WANG, 2016).

2.10 Banana and guava biomasses for COS and XOS production

COS and XOS can be produced with lignocellulosic materials such as guava and banana by-products. The guava (*Psidium guajava* L.) is cultivated in more than 60 countries, with a production of around 55 million tons in 2019. The world's largest producers are India, China, Kenya, Brazil and Mexico. Its cultivation is of great economic importance, due to its low cost, high yield, tolerance to saline soils and to several products that can be obtained and marketed (ANGULO-LÓPEZ et al., 2021; KUMAR et al., 2022; ARÉVALO-MARÍN et al., 2021).

Guava tree is a shrub that can reach a height of approximately 7 meters, with broad leaves, white flowers with 4 to 6 petals, and guavas measuring between 3 and 6 cm, with small, edible seeds that correspond between 6-12% of the total guava weight. In the figure 2.13 it is possible to see the different parts of the guava tree (leaves, flowers, trunk and fruit) and the parts that make up the guava (NASEER et al., 2018; SHARMA; BORAH, 2021).

Figure 2.13 - (a) Leaves (b) Flowers (c) Fruit (d) Seeds in the fruit (e) Bark (f) guava structures



Source: Adapted from Elizalde-González; Segura-Rivera (2018); Naseer et al. (2018).

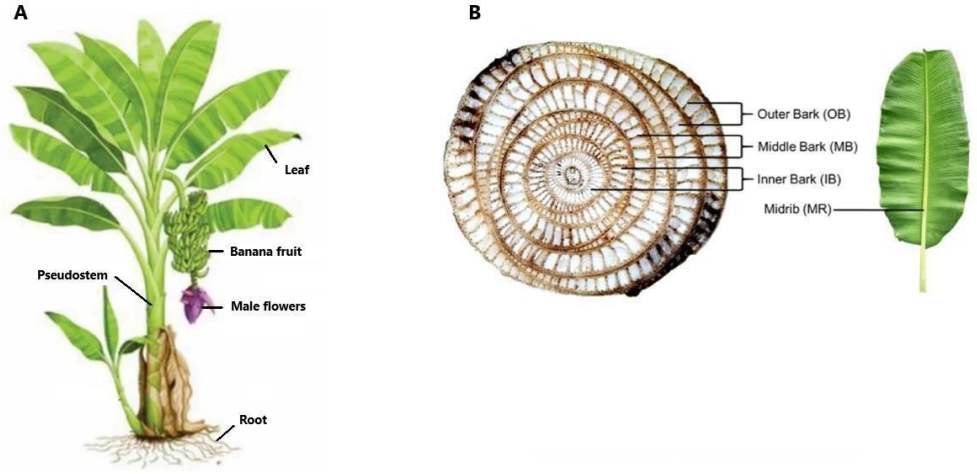
The leaves, bark, seeds and fruits of the guava tree have several medicinal properties, because they are rich in food fibers, pectin, vitamins A and C (more than oranges), minerals like calcium, phosphorus and iron, proteins, lipids, polysaccharides, phenolic compounds, flavonoids, lycopene, carotenoids, essential oils, tannins and triterpenes. Guavas are used in the treatment of gastrointestinal and respiratory disorders, bacterial and fungal infection, to increase platelets in patients with dengue, treatment of diabetes and hypertension and their high concentration of ascorbic acid and citric acid has anti-mutagenic activity. Furthermore, guava has anti-inflammatory, antioxidant, immunomodulatory, and antimicrobial properties (KUMAR et al., 2021; NASEER et al., 2018; KUMAR et al., 2022; JAMIESON et al., 2023).

It is a fruit widely consumed *in natura*, due to its sweet flavor and aroma, and also used in the production of juice, pulp, jelly, nectar, candies and ice cream. Approximately 70 to 75% of the fruit is used in the manufacture of these products and the remaining by-products, approximately 80 kg per metric ton of guava, composed mainly of seeds and peels, can be used for the production of ethanol, lactic acid, antimicrobial compounds (ARÉVALO-MARÍN et al., 2021; KUMAR et al., 2022; KUMARI; PRASAD; AHMAD, 2020).

The banana (*Musa* spp) is the most cultivated fruit in the world after citrus, accounting for around 16% of fruit production. More than 130 countries grow bananas, with the largest producers being India, China, Indonesia and Brazil. They are widely consumed due to their pleasant texture and flavor, as they are very nutritious, have a low amount of fat, high fiber content, vitamins C and B6 and minerals, they also have phenolic compounds such as catechin, tannins, gallic acid, which have antioxidant activity and has the potential to reduce the risk of cancer and cardiovascular disease (ALZATE ACEVEDO et al., 2021).

There are more than 50 species of banana trees, they have underground stems, from which large leaves are arranged in a spiral, forming a false stem (pseudostem) (Figure 2.14) ALZATE ACEVEDO et al., 2021).

Figure 2.14 - A: Parts of the banana tree, B: Left, banana pseudostem (cross section), Right, banana leaf



Source: Adaptado de Motaleb; Mizan; Milašius (2020); Alzate Acevedo et al. (2021).

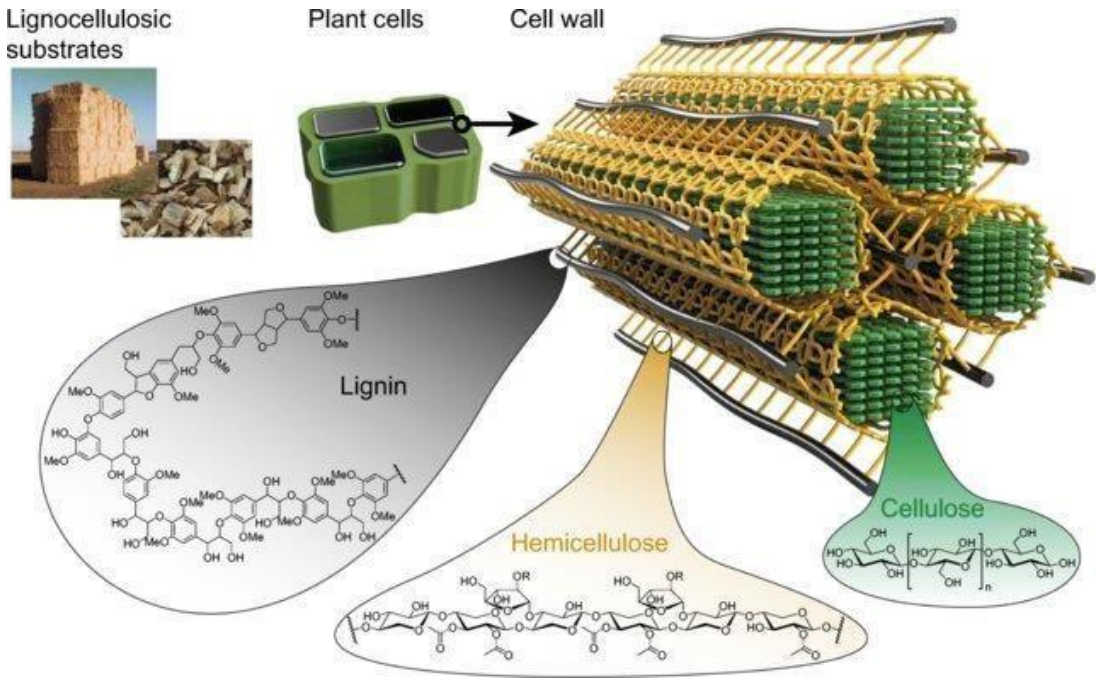
When harvesting one ton of bananas, 100 kg of fruit are rejected and approximately 4 tons of lignocellulosic waste such as peel (440 kg), leaves (480 kg), stems (160 kg) and pseudostem (3 tons) are generated. In general, part of the banana peels is used to feed pigs, the rest of the waste is left to decompose in the fields, but could be used to produce inputs, reducing environmental pollution and adding value to banana farming (FERNANDES et al., 2013).

Lignocellulosic materials have a cell wall that plays an important role in the plant's architecture, providing mechanical support, facilitating the transport of water and nutrients, and defending the plant against biotic stresses (caused by living organisms, such as microorganisms) and abiotic stresses (such as high temperatures and lack of water). It is the most abundant renewable resource on Earth, which has diverse applications, such as animal nutrition, biofuel production, raw material for the paper and textile industry, and heat generation (ZHANG, B. et al., 2021).

The chemical and structural composition of the cell wall of lignocellulosic materials is heterogeneous due to the ability of plants to adapt and evolve in different terrestrial habitats (WANG et al., 2019). The cell wall mainly comprises cellulose (40 to 50%), hemicellulose (15 to 25%), lignin (20 to 25%) and other components such as pectin and glycoproteins (5 to 10%) and

accounts for most of the weight of the dry plant (Figure 2.15) (ZENG; HIMMEL; DING, 2017).

Figure 2.15 - Main components of the plant cell wall

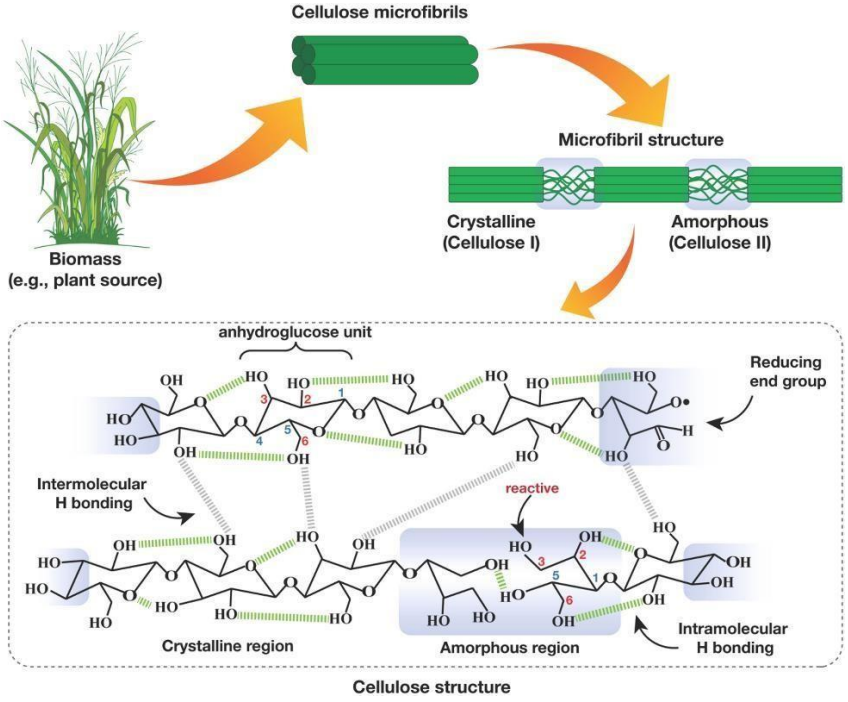


Source: Brethauer; Shahab; Studer (2020).

Cellulose is the main polysaccharide found in plants, as well as in algae, fungi, and certain bacteria, making it the most abundant organic compound on the planet. It is a homopolymer comprising glucose units joined by β-1,4-glycosidic bonds. It has a linear chain, high molecular weight and a degree of polymerization generally between 200 and 1400 glucose units, reaching 10000 glucose units, and formula $(C_6H_{10}O_5)_X$ (IBRAHIM et al., 2019). Cellulose molecules bond through hydrogen bonds between hydroxyl groups (OH) forming rigid microfibrils with crystalline and amorphous regions (Figure 2.16). The crystalline region is highly ordered with strong hydrogen bonds, a compact structure, limiting accessibility to hydroxyl groups that do not react with chemical reagents. The amorphous region comprises less dense cellulose chains compared to the crystalline region, with more accessible hydroxyl groups that can bond with other molecules. These bonds are more easily disrupted by pretreatments (CHAI et al., 2022; RANA; DE LA HOZ SIEGLER, 2023).

Cellulose bonds with hemicellulose through hydrogen bonds and Van der Waals force (MIKI et al., 2020). Between hemicellulose and lignin, there are ether and ester bonds. Furthermore, there is evidence of hydrogen bonds, covalent and ether bonds, between cellulose and lignin (CUI et al., 2022; HUANG et al., 2021; KHODAYARI et al., 2021).

Figure 2.16 - Representation of the cellulose molecule with crystalline regions that are compact and less reactive, amorphous regions that react easily with other molecules, and inter- and intra-chain hydrogen bonds



Souce: Rana; De La Hoz Siegler (2023).

The cellulose is responsible for the mechanical resistance of the plant cell wall due to the ability of the molecules to maintain a semi-crystalline state of aggregation in an aqueous medium (KUMAR GUPTA et al., 2019).

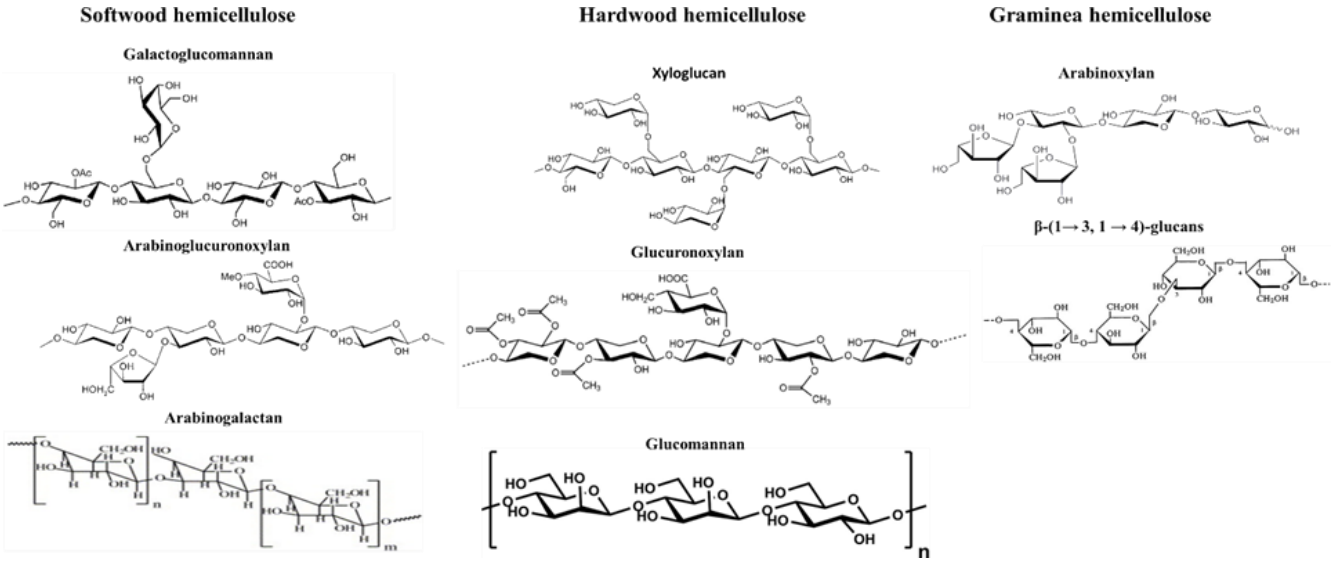
Due to the wide range of applications in chemical industries, cellulose plays an important role. It can be used to produce bioethanol, acetic acid, ether, etc. through hydrolysis with cellulolytic enzymes and fermentation of the glucose released by microorganisms (ZENG; HIMMEL; DING, 2017). It is also used to make paper, cellophane, hydrogels, microcrystalline cellulose, rayon, and cellooligosaccharides. Cellulose is crucial in the diet of animals such as ruminants and termites, which have microorganisms in their intestines and enzymes, such as cellulases that can break down the cellulose molecule. In humans, due to the absence of cellulase, cellulose is not digested, acting as a hydrophilic agent, increasing the volume of feces, and intestinal peristalsis helping to treat constipation (KUMAR GUPTA et al., 2019).

Hemicellulose is the second most abundant polysaccharide in the plant cell wall. Its chain is heterogeneous and comprises 50 to 200 pentose units (monosaccharide consisting of five carbons), such as D-xylose and L-arabinose and hexoses (consisting of 6 carbons), such as D-glucose, D-mannose and D-galactose. These bind to form various polysaccharide structures, such

as xyloglucans (XyG), glucuronoarabinoxylans (GAX), Glucuronoxylans (GX) and galactoglucomannan (GGM) (PAZ-CEDENO et al., 2022).

Due to its amorphous and branched structure with acetyl groups and uronic acids, it has low chemical and thermal stability, which is hydrolyzed more easily compared to cellulose. The removal of cellulose is the first step in facilitating the decomposition of lignocellulosic materials. The composition of hemicellulose and the proportion of constituents depend on the biological source where it is found and the stage of development (MACHMUDAH et al., 2017; WANG et al., 2019; RAO et al., 2023; RAMANATHAN et al., 2022). Hardwoods have hemicelluloses composed mainly of glucuronoxylan and glucomannan and smaller amounts of xyloglucans, which are linked to cellulose by hydrogen bonds. In softwoods, hemicelluloses comprising galactoglucomannan, arabinoglucuronoxylan and arabinogalactan predominate and in grasses, arabinoxylan and β-(1→ 3, 1 → 4)-glucans (Figure 2.17) (RAO et al., 2023).

Figure 2.17 - Main composition of hemicellulose in hardwoods, softwoods and grasses



Source: Author.

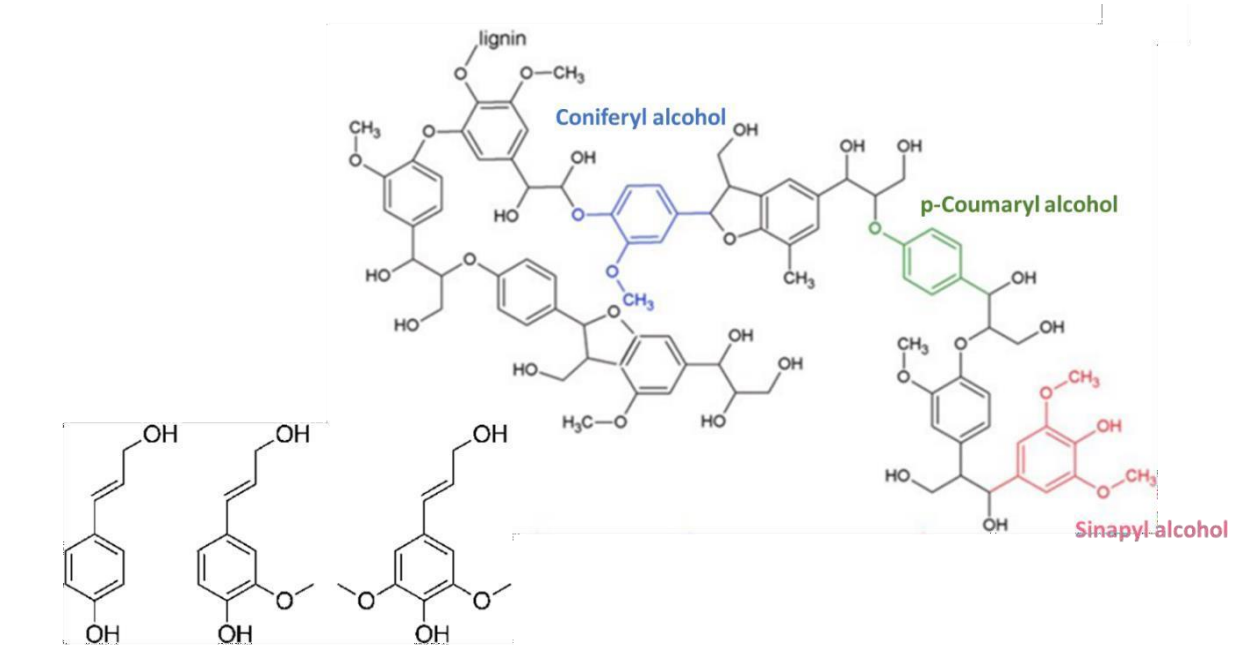
Hemicellulose in the plant cell wall contributes to the flexibility and rigidity of the plant, and due to its hydrophilic nature, contributes to water retention. In addition to the organization of the cell wall, it contributes to the storage of carbohydrates in seeds. Some xyloglucans protect the plant from the invasion of some pathogens. Moreover, mannans can provide strength and hardness (ZHANG, W. et al., 2021). Hemicellulose can be converted into products such as food additives, biofilms, biofuels, alcohol, sorbitol, xylitol, furoic acid, xylose and XOS after chemical or enzymatic treatments (WANG et al., 2019, BRIENZO et al., 2016). Products derived from

hemicellulose can be used in food, cosmetics, pharmaceutical products, toothpaste, and papermaking, among others (HUANG et al., 2021).

Lignin is an aromatic, amorphous, heterogeneous polymer, formed mainly by units of p-coumaryl, coniferyl, and synapyl alcohols, that connect through ether bonds (Figure 2.18). Its structure varies according to the plant species and extraction method and its complexity makes it difficult to isolate so that it can be used as a high-value product (FODIL CHERIF et al., 2020).

In plants, it is responsible for the resistance and rigidity of the root and stem, waterproofing water-conducting tissues, facilitating the transport of water and solutes, and protecting the plant from pathogens. Lignin also protects cellulose fibers from microorganism and enzyme action, making biomass recalcitrant (ZENG; HIMMEL; DING, 2017; BRIENZO et al., 2017).

Figure 2.18 - Representation of the structural formula of lignin with its main constituents: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol



Source: Author.

Lignin acts as a natural barrier, encapsulating carbohydrates and reducing accessibility to enzymes and chemical reagents, hindering carbohydrate conversion into industrially relevant products. Furthermore, it is linked to hemicellulose by numerous types of bonds, hindering its dissolution and extraction. Some studies report that lignin inhibits XOS production by enzymatic hydrolysis due to steric hindrance (LIAO et al., 2023; YING et al., 2022). Therefore, its removal is important for the COS and XOS production process.

Paper and cellulose industries produce around 50 to 70 million tons of lignin per year worldwide. Approximately 98% of this production is used for energy generation or burned for heating. The remaining percentage is used as a dispersant, surfactant and binder in formulations, and to produce polymers, biofuels, chemicals, asphalt and nanomaterials that deliver medicines (YAO et al., 2022).

Table 2.2 shows the chemical composition of some lignocellulosic materials with the potential to be used in COS and XOS production.

Table 2.2 - Chemical composition of lignocellulosic materials

	Cellulose/glucan (%)	Hemicellulose (%)	Lignin (%)	Ashes (%)	Extractives (%)	References
Guava seed cake	37.74	23.0	34.15	1.12	6.22	Forsan; De Freitas; Brienzo (2023)
Guava branch	15.53	11.02	17.77	1.87	-	Camarena-Tello et al. (2015)
Guava leaves	35.36	34.12	35.26	8.2	-	Camarena-Tello et al. (2015)
Guava seed	37.64	18,4	22,10	-	-	Roberto (2012)
Banana pseudostem	48.41	14.67	19.84	8.70	7.27	Forsan; De Freitas; Brienzo (2023)
Banana leaves	24.09	17.29	27.90	10.57	8.33	Forsan; De Freitas; Brienzo (2023)
Banana peel	35.3	19.5	6.7	-	-	Tiwari et al. (2019)

Source: Author
(-): not reported

2.11 Conclusion

Lignocellulosic materials are abundant, renewable and cheap feedstock that can be used to produce various high-value products, such as COS and XOS. These compounds can help prevent and treat numerous diseases and have potential applications in food, pharmaceuticals, chemistry, animal feed production, cosmetics, and other industries, primarily due to their prebiotic and antioxidant properties.

The major challenge in the production of oligosaccharides is to separate the components of the biomass, remove the lignin, and reduce the crystallinity of cellulose so that polysaccharides

become accessible to chemical reagents and enzymes, and are then broken down into oligosaccharides. Several pretreatments are proposed, but they are still complex, expensive and not viable on a large scale. The use of enzymes in this field is very promising, as they typically hydrolyze biomass without generating degradation products and operate under milder temperature and pressure conditions. Additionally, there is no need for complex equipment. The global oligosaccharide market has a multimillion-dollar value, but further research is still needed to refine the techniques used in the production, characterization, and purification of these compounds.

CHAPTER III: CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES PRODUCTION BY A COMBINATION OF MECHANICAL, CHEMICAL, AND ENZYMATIC TREATMENTS OF BANANA PSEUDOSTEM, LEAVES AND GUAVA SEED CAKE

Abstract

This study proposes the use of banana pseudostem and leaves and guava seed cake, waste generated in excess in fruit growing, for the production of cellooligosaccharides (COS), compounds of industrial interest with potential health benefts. The objective was to reduce the recalcitrance of these biomasses through physical and chemical processes. Initially, all biomasses (untreated and chemically pretreated with sulfuric acid, hydrogen peroxide, sodium, and potassium hydroxide) were subjected to the action of endoglucanase enzyme (20, 50, and 100 $IU.g^{-1}$) to determine the optimal condition. Subsequently, the biomasses were hydrolyzed with 30 $IU.g^{-1}$ of endoxylanase for 24 h and 20 $IU.g^{-1}$ of endoglucanase for 6 h. The second stage was carried out under the same conditions mentioned earlier but with the biomasses previously ground in a ball mill. The highest cellulose to COS conversion was 79.73%, achieved from banana leaves, with 20% sodium hydroxide (w/w) at 121 °C/30 min, ball milled, and hydrolyzed with xylanase and endoglucanase. The highest COS yields for banana pseudostem and guava seed cake were 53.05% and 59.99%, respectively, using alkali. The removal of xylan leads to more accessibility of cellulose to endoglucanase action. In addition to obtaining COS, xylooligosaccharides (XOS) were also produced in this process, both with the potential for industrial applications, mainly due to their prebiotic action. In general, the best results were obtained with alkaline pretreatments and always with endoxylanase before endoglucanase. Ball milling was benefcial only for materials pretreated with sulfuric acid and sodium hydroxide.

Keywords: cellulose; endoglucanase, endoxylanase; prebiotic; ball mill; fruit waste.

3.1 Introduction

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) are non-digestible carbohydrates, comprising glucose and xylose units, respectively, linked by β-1,4 glycosidic bonds, with a degree of polymerization between 2 and 6 units, which can be obtained from

lignocellulosic materials. XOS may present side groups, such as acetyl groups, uronic acid, and arabinofuranosyl residues (KENDRICK et al., 2022; PRECUP et al., 2022). COS and XOS are promising substances in the prevention of cardiovascular diseases, colorectal cancer, diabetes, and obesity, increase calcium absorption, act as an anti-inflammatory, antioxidative and are considered prebiotic. They act as a selective substrate for desirable microorganisms such as *Bifidobacterium* and *Lactobacillus* present in the intestinal microbiota, increasing their population and controlling the growth of pathogens (DE FREITAS et al., 2022; DE FIGUEIREDO; DE OLIVA-NETO, 2022). COS demonstrated a prebiotic effect, stimulating the growth of strains of *Clostridium butyricum, Lactococcus lactis*, *Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus plantarum,* and *Lactobacillus gasseri* (JANA; KANGO; PLETSCHKE, 2021; ZHONG et al., 2020). COS has the potential to be applied in the pharmaceutical, food, beverage, cosmetics and animal feed industries. Moreover, it can be applied to biofuel production, as fermentation substrates, and to developing biodegradable materials due to its functional, rheological, and physical-chemical properties. They are resistant to compression and capable of retaining oils and forming powders with high fluidity, characteristics that can improve formulations. As the XOS, they are resistant to heat and acidic pH, resisting hydrochloric acid in the stomach and reaching the intestine intact, where they will serve as a substrate for bacteria (KENDRICK et al., 2022; ÁVILA et al., 2021). XOS have a sweet taste and low viscosity, and can be easily incorporated into foods (FORSAN et al., 2022; YAN et al., 2022a).

COS and XOS can be produced from agro-industrial by-products, such as banana (*Musa spp*) and guava residues (*Psidium guajava* L.), abundant in the harvesting and industrial processing of these fruits (PEREIRA et al., 2022a; PEREIRA et al., 2022c). Brazil is the fourth largest banana producer in the world (SEBRAE, 2023), one of the world's most-consumed fruits. The by-products generated in banana cultivation and harvesting are pseudostems, leaves, peel, and discarded bananas (REDONDO-GÓMEZ et al., 2020). Guava is also a widely consumed fruit in the world and the main residues generated during its processing are seeds, peel, and leaves (KUMAR et al., 2022). Waste discarded in inappropriate places can cause contamination of the soil and water bodies, and spread diseases. Biomass can be reused to manufacture value-added products such as biofuels, nanocellulose fibers, bioplastics, enzymes, food additives and oligosaccharides (REDONDO-GÓMEZ et al., 2020).

COS and XOS can be produced from waste material using pretreatment to remove hemicellulose and lignin and disrupt the crystalline structure of cellulose, exposing it to enzymatic action (MELATI et al., 2019; SHIMIZU et al., 2020; KUNDU et al., 2021). Another strategy to

reduce cellulose crystallinity and degree of polymerization is subjecting it to ball milling, an inexpensive and easily reproducible process, which can significantly enhance enzymatic hydrolysis. The reduction of the particle size and increase of the surface area for enzyme contact show potential for advancing biofuel and bioproduct production (ZHAO et al., 2020; KUMAR et al., 2020). The enzyme used in this study was the commercial cellulase Celluclast 1.5, from Novonesis, widely employed for the hydrolysis of lignocellulosic materials and derived from *Trichoderma reesei*. This enzymatic cocktail is primarily composed of cellobiohydrolases or exoglucanases, which hydrolyze the glycosidic bonds at the end of the cellulose chain, releasing glucose and cellobiose; endo-1,4-beta-glucanases break glycosidic bonds in cellulose randomly, releasing COS of various sizes, and beta-glucosidase breaks cellobiose into glucose. The major advantage of enzymatic hydrolysis is the absence of toxic residues and few or no monomers (ÁVILA et al., 2021; BARBOSA et al., 2020a; SINHA; SHARMA; YAZDANI, 2023; ARSAT; GHAZALI, 2023; EJAZ; SOHAIL; GHANEMI, 2021; ROSGAARD et al., 2007).

Due to the importance of compounds such as COS for human and animal health, there are several studies to produce them. Avila et al. (2021) obtained a yield of 63.56 $mg.g^{-1}$ of COS using sugarcane straw and coffee husks treated with potassium hydroxide 4.5% (w/v), and then applied a combination of enzymatic hydrolysis with cellulase and esterase. Another approach was based on the use of sugarcane straw pretreated with sulfuric acid 0.5% (v/v), and enzymatic hydrolysis with a commercial cocktail of cellulases (Celluclast, Novonesis) (KARNAOURI et al., 2019). The yield obtained was 33.54 mg of COS per gram of pretreated sugarcane straw. Another study obtained 134.48 mg of COS per gram of banana pseudostem hydrothermally pretreated at 150 ºC, applying cellulase from *Trichoderma reesei* ATCC 26,921 (ZHANG, R. et al., 2023). All these yields were lower than that obtained in the present study. Cellulose is abundant in biomass but difficult to hydrolyze from untreated material. The studies reported for COS production resulted in low yield, even using a high enzyme dosage. It is important to develop research to make cellulose exposed to enzymatic action, aiming at high COS yield production.

Cellulose is the most abundant polysaccharide on earth but its natural recalcitrance poses challenges for industrial applications using processes like enzymatic hydrolysis. Decreasing cellulose recalcitrance, whether through physical, chemical, or enzymatic methods, assists biorefineries in extracting the maximum potential from waste biomass. This aids in the production of prebiotics, biofuels, paper, textiles, cosmetics, pharmaceuticals, and more. Prebiotics have numerous health benefits and possibilities for industrial use (PHIROM-ON; APIRAKSAKORN, 2021; DIAZ et al., 2021). The global market for prebiotics is estimated to have been 4.07 billion

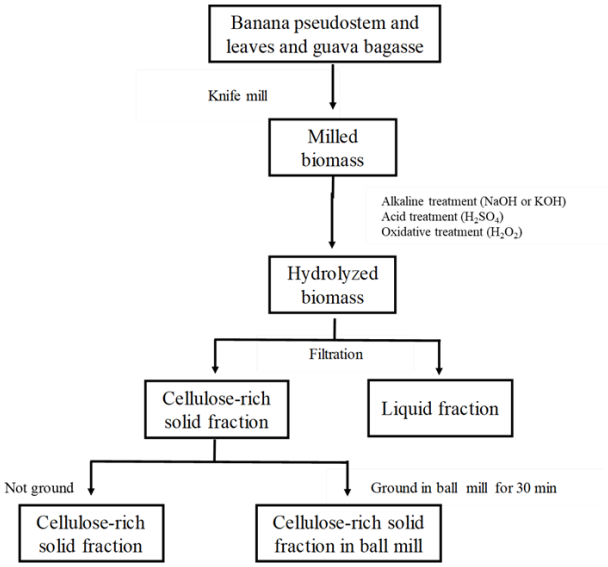
USD in 2017 and reached 7.37 billion USD by 2023 (YAN et al., 2022). Based on this premise, this study aimed to decrease cellulose recalcitrance from banana pseudostem and leaves and guava seed cake, abundant byproducts in Brazil, using chemical treatments. Additionally, the study assessed the impact of ball milling and *Aspergillus versicolor* endoxylanase on reducing cellulose recalcitrance for COS production during enzymatic hydrolysis using Celluclast from Novonesis, a commercial cocktail rich in endoglucanase. The endoxylanase hydrolyzed the xylan still bound to cellulose after chemical treatment, yielding XOS, another compound of industrial interest. The present study showed the advantages of COS production combining ball milling and xylan removal previously to endoglucanase hydrolysis.

3.2 Materials and methods

3.2.1 Sample preparation

The guava seed cake used in this study was supplied by the fruit processing company called Predilecta (Matão, SP). The banana leaf and pseudostem were obtained from a local plantation, Rio Claro, SP, Brazil. The guava seed cake was ground in a knife mill with a 20-mesh sieve (0.8 mm). The banana leaf and pseudostem were exposed to sunlight until excess moisture was lost, cut into small pieces, oven-dried at 60 ºC, until approximately 10% of moisture, and ground in a knife mill with a 20-mesh sieve (0.8 mm) (BARBOSA et al., 2020a). A portion of each sample was further ground in a ball mill for 15 min for later use in the enzymatic hydrolysis (Figure 3.1). The samples were stored in a plastic bag at room temperature.

Figure 3.1 - Flowchart of biomass preparation for enzymatic hydrolysis



Source: Author.

3.2.2 Chemical characterization of untreated and pretreated material

Moisture, ash, and extractive content were determined as reported elsewhere (SINHA; SHARMA; YAZDANI, 2023). About 300 mg of banana pseudostem, banana leaves, and guava seed cake untreated extractive-free and pretreated material were subjected to chemical characterization with 1.5 mL of 72% (w/w) sulfuric acid in a 100 mL Schott Bottle at 45 °C. After 7 min, the reaction was stopped by adding 45 mL of distilled water. The mixture was autoclaved at 121 °C for 30 min (Stermax autoclave), and after cooling it was vacuum filtered through a previously tared sintered glass filter. Tests were performed in triplicate. The solid residue was oven-dried at 105 °C until constant mass for the determination of insoluble lignin. The liquid fraction was used for the determination of acid-soluble lignin content by UV-Vis spectrophotometry at a wavelength of 215 and 280 nm using a blank solution of 4% sulfuric acid. The sum of soluble and insoluble lignin was reported as total lignin. The liquid fraction was also used for the quantification of monosaccharides by high-performance liquid chromatography (HPLC), according to item 3.2.9.

3.2.3 Alkaline pretreatment

About 5 g of the biomass (banana pseudostem and leaf and guava seed cake) milled in a

knife mill with a 20-mesh (0.8 mm) sieve was placed in a Schott flask containing 100 mL of sodium hydroxide or potassium hydroxide 20% (w/w), (mass of alkali per mass of material) (1:20 solid-liquid ratio). The flasks were autoclaved at 121°C for 30 min (Stermax autoclave) (ARSAT; GHAZALI, 2023; EJAZ; SOHAIL; GHANEMI, 2021). The mixture was vacuum-filtered, the liquid fraction was discarded and the solid fraction was washed with water to neutral pH, oven-dried at 45 °C and used for enzymatic hydrolysis and chemical characterization according to item 3.2.9 and. Pretreatments were performed in triplicate.

3.2.4 Acid pretreatment

About 5 g of the biomass (banana pseudostem and leaf and guava seed cake) milled in a knife mill with a 20-mesh (0.8 mm) sieve was placed in a Schott flask containing 100 mL of sulfuric acid 20% (w/w), (mass of alkali per mass of material) (1:20 solid-liquid ratio). The flasks were autoclaved at 121°C for 30 min (Stermax autoclave) (ARSAT; GHAZALI, 2023; EJAZ; SOHAIL; GHANEMI, 2021). The mixture was vacuum-filtered, the liquid fraction was discarded and the solid fraction was washed with water to neutral pH, oven-dried at 45 °C and used for enzymatic hydrolysis and chemical characterization according to item 3.2.9. Moreover, pretreatments were performed in triplicate.

3.2.5 Hydrogen peroxide pretreatment

Banana and guava biomasses were washed with 0.2% (w/v) ethylenediaminetetraacetic acid (EDTA) for 1 h at 90 ºC to remove metal cations. The material was dried at 45 °C for 48 h. About 5 g of material was treated with 100 mL of 6% hydrogen peroxide solution with pH adjusted to 11.6 with 5 mol.L^{-1} sodium hydroxide. The suspension was shaken for 4 h at 120 rpm at 25 ºC on a shaker (Marconi ma 830/a) and vacuum filtered. The liquid fraction was discarded. The solid fraction was washed until neutral pH in the wash water and oven dried at 45 ºC ROSGAARD et al., 2007; PHIROM-ON; APIRAKSAKORN, 2021).

3.2.6 Endo-β-1,4-glucanase activity

A 0.44% carboxymethylcellulose (CMC) solution was prepared in sodium acetate buffer (pH 5.2). The solution was heated to 50 °C and stirred on a magnetic stirrer until dissolution (2 to

3 h). To determine the enzyme activity, 3 test tubes were prepared containing: Blank: 1 mL of distilled water and 1.5 mL of DNS were added to the test tube. The solution was boiled for 5 min. Control: 0.9 ml of substrate (CMC) was added to the test tube. The tube was heated at 50 °C for 5 min and then 1.5 mL of DNS and 0.1 mL of the enzyme was added (Celluclast – Novonesis). The solution was boiled for 5 min. The control flask was made in duplicate. Reagent bottle: 0.9 mL of substrate (CMC) and 0.1 mL of enzyme were added to the third tube and heated at 50 ºC for 5 min. Then, 1.5 mL of DNS was added to stop the reaction and the tube was boiled for 5 min. The reagent bottle was prepared in duplicate. The tubes were read in a spectrophotometer at 540 nm (Bel UV-M51) using the blank to zero the equipment. The enzyme was diluted in sodium acetate buffer pH 5.2 until it was possible to read it in a spectrophotometer. To determine the concentration of reducing sugars, a standard curve was constructed with glucose solutions 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 $mg.mL^{-1}$. The activity of the endoglucanase was 2910 $IU.mL^{-1}$ (International unity per mL) (DÍAZ et al., 2021).

3.2.7 Endo-β-1,4-xylanase activity

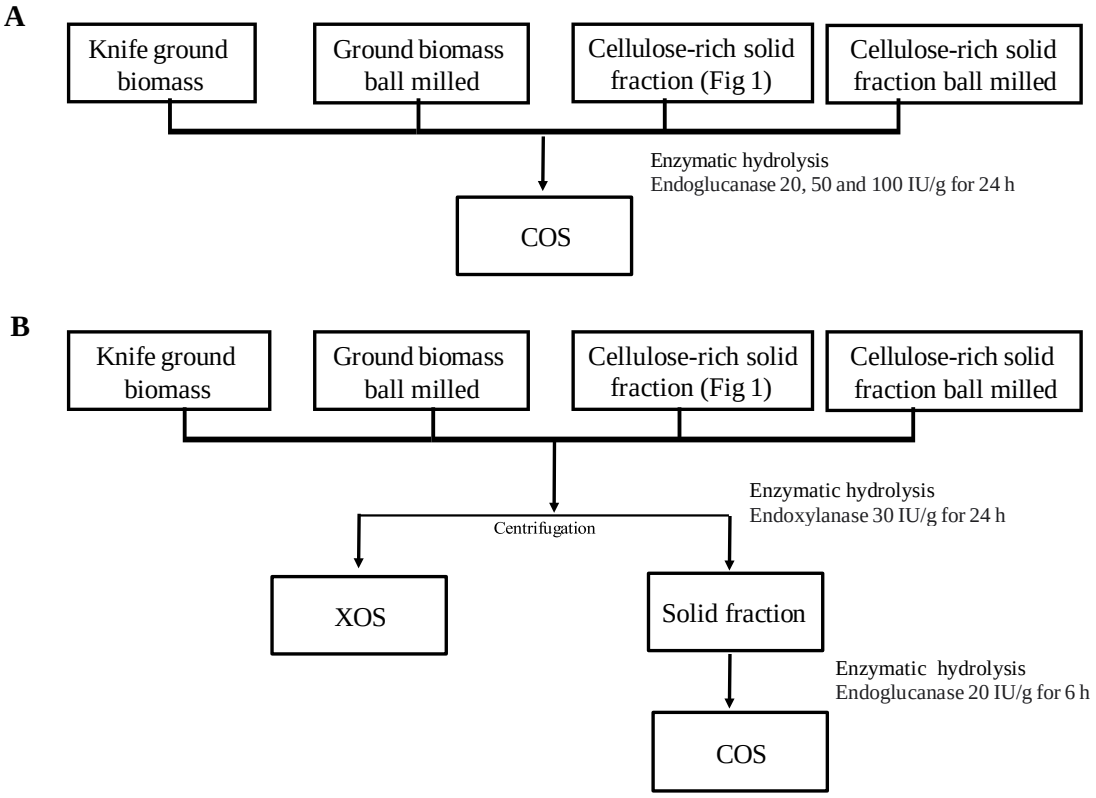
The modified Bailey; Biely; Poutanen (1992) method was used to determine the activity of *Aspergillus versicolor* xylanase produced and purified by De Freitas et al., 2021. A 1% xylan solution (Sigma Aldrich) was prepared with 0.05 $mol.L^{-1}$ sodium phosphate buffer, pH 6. About 750 µL of this solution was added to a test tube with an enzyme volume between 10 and 250 µL (absorbance must not be greater than 1.0) and an aliquot of 250 µL was removed and immediately added to a tube with 250 µL of 3,5-dinitrosalicylic acid (DNS), which was not enough time to generate monomers and oligomers. This solution was boiled and 2.5 mL of distilled water was added. The solution was used as a blank in the spectrophotometer (Bel UV-M51). Furthermore, 250 µL aliquots of the mixture (xylan + enzyme) were removed after 5 min and 10 min in a water bath at 55 ºC and added to tubes containing 250 µL of DNS that were boiled to stop the reaction. Then, 2.5 mL of distilled water was added and the absorbance was read in a spectrophotometer at 540 nm.

To determine the concentration of reducing sugars, a standard curve was constructed with xylose solutions (Merck) 0.2, 0.4, 0.6, 0.8 and 1 $mg.mL^{-1}$. The initial activity of the endoxylanse was 414.4 $IU.mL^{-1}$.

3.2.8 Enzymatic hydrolysis for cellooligosaccharides and xylooligosaccharides production

Untreated and pretreated with sulfuric acid, sodium hydroxide, potassium hydroxide, and hydrogen peroxide biomasses were divided into two groups: unground and ground in a ball mill for 30 min. The enzymatic hydrolysis was performed in two ways (Figure 3.2).

Figure 3.2 - Flowchart of the production process of COS and XOS, using banana and guava biomass, untreated and pretreated with sulfuric acid, hydrogen peroxide and sodium and potassium hydroxide, not ground or ground in a ball mill. A: hydrolysis of samples with endoglucanase. B: Hydrolysis of samples with xylanase and endoglucanase



Source: Author.

First, 0.1 g of each material was hydrolyzed with an endoglucanase load (commercial cocktail Celluclast, Novonesis) of 20, 50 or 100 IU.g^{-1} in 5 mL of 50 mol.L^{-1} sodium acetate buffer, pH 5.2. These enzymatic loads were chosen in accordance with other studies, such as Gomes; Gama; Domingues, 2018 and Felipuci et al., 2024. The mixture was shaken at 170 rpm (Marconi ma 830/A) at 50 ºC for 24 h, and then, the tubes were boiled for 5 min to stop enzymatic action.

Second, about 0.1 g of each material was hydrolyzed with 30 IU.g^{-1} of endo-β-1.4-xylanase purified from *Aspergillus versicolor* in 5 mL of 50 mol.L^{-1} sodium phosphate buffer, pH 6.0, with the agitation of 170 rpm on a shaker (Marconi ma 830/A), at 55 ºC for 24 h and the tubes were

boiled for 5 min to stop the enzymatic action (FERNANDES et al., 2020). The hydrolysate was centrifuged for 15 min at 5000 rpm (Novatecnica, NT815), and the liquid fraction was removed. Then, 5 mL of 50 mol.L^{-1} sodium acetate buffer pH 5.2 was added to the solid fraction, adding 20 IU.g^{-1} of endoglucanase (enzymatic load that generated greater conversion of cellulose into COS, in the previous experiment) and the reaction was performed under agitation of 170 rpm at 50 °C for 6 h. After the reaction, the flask was boiled in water for 5 min to stop the enzyme action (Figure 3.2) (SLUITER et al., 2010).

The liquid fractions were filtered through a syringe filter of 0.22 µm and the colored samples were filtered with a Sep-Pak (Waters) cartridges to remove color. The filtered samples were characterized for glucose, COS, xylose and XOS concentrations by HPLC according to item 3.2.9.

Conversion to COS and XOS was calculated in relation to the cellulose and xylan content in the materials.

3.2.9 Determination of monosaccharides, COS and XOS

The contents of glucose, xylose, arabinose, and acetic acid from the chemical composition characterization were determined by HPLC using a Bio-Rad Aminex HPX87H (300×7.8 mm) column maintained at 50 °C, Waters 2414 Refractive Index Detector at 40 °C, a mobile phase of sulfuric acid 0.005 mol.L^{-1}, a fow rate of 0.6 mL.min^{-1}, and sample injection volumes of 20 µL. The samples were fltered using a syringe flter with 0.22-µm pore size. The glucan, xylan, arabinosyl, and acetyl side-group contents were calculated by multiplying the percentage of glucose, xylose, arabinose, and acetic acid by their hydrolysis factors (0.9, 0.88, 0.88, and 0.72, respectively). COS and XOS concentrations were determined by HPLC using Waters equipment, under the following conditions: Aminex HPX-87C BIO-RAD column (300×7.8 mm); temperature: 80 °C; eluent: ultrapure water with 0.6 mL.min^{-1} fow; sample volume: 20 µL; detector: refractive index at 40 °C; and analysis time: 15 min. Glucose (C1) (Sigma), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5), and cellohexaose (C6) (Megazyme) solutions were used as standards. Samples were fltered using a syringe flter with 0.22-µm pore size. Xylose (X1) (Sigma), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) (Megazyme) solutions were used as standards (FORSAN et al., 2021b; DE FREITAS; BRIENZO, 2022).

3.2.10 Statistical Analysis

The design expert 6.0 was used for the statistical study. Statistical analyzes were performed using the Tukey test to compare means obtained in the chemical characterization of untreated and pretreated material and the means for the conversion of cellulose into COS and xylan into XOS. Statistical analysis considered the confidence level of 95%.

3.3 Results and discussion

3.3.1 Chemical characterization of biomass

The chemical composition of the biomasses used in this study found in the literature was displayed in Table 3.1. It is important to note that depending on the species and the type of process to which these biomasses were submitted, the chemical composition differed. Banana pseudostem presented 48.4% cellulose and 14.6% hemicellulose, which were results similar to those obtained by (KUMAR et al., 2020). However, the amount of cellulose and hemicellulose present in banana pseudostem can be up to 60.8% and 33.5%, respectively (MELATI et al., 2023; ARSAT; GHAZALI, 2023). Banana leaves presented 24.09% cellulose, 17.29% hemicellulose and 27.90% lignin, which were results similar to those obtained by Brienzo; Siqueira; Milagres (2009) for cellulose, similar to those reported by Alves et al. (2021), for hemicellulose and lignin. Cellulose, hemicellulose and lignin were found in the literature with the following values respectively: 20.4%-35.2% (ALVES et al., 2021; TAREEN; PUNSUVON; PARAKULSUKSATID, 2020), 8.6%-25.8% (TAREEN; PUNSUVON; PARAKULSUKSATID, 2020; BRIENZO; SIQUEIRA; MILAGRES, 2009) and 17%-27.9% (BRIENZO; SIQUEIRA; MILAGRES, 2009).

Table 3.1- Chemical composition of banana pseudostem and leaves and guava seed cake from different authors

	Cellulose/glucan (%)	**Hemicellulose[a] (%)**	**Lignin[b] (%)**	**Ashes (%)**	**Extractives (%)**	**References**
Guava seed cake	37.74 ± 0.06	23.0 ± 0.93	34.15 ± 1.81	1.12 ± 0.07	6.22 ± 0.68	Present study[cd]
	34.3	16.12	6.6	-	-	Brienzo et al. (2017)
	37.64	18.4	22.10	-	-	Oriez; Peydecastaing; Pontalier (2019)
Banana Pseudostem	48.41 ± 0.58	14.67 ± 0.16	19.84 ± 1.74	8.70 ± 0.38	7.27 ± 1.17	Present study[d]

Continuation of the table 3.1

	47.39	14.98	7.92	15.44	-	Kumar et al. (2020)
	29	23	19	-	-	De Freitas; Brienzo. (2022)
	23.82	25.69	8.56	7.06	4.25	Kusmiyati; Sukmaningtyas (2018)
	39.12	33.59	10.78	8.2	3.05	Melati et al. (2023)
	60.84	19.62	17.26	-	10.5	Arsat; Ghazali (2023)
Banana Leaves	24.09 ± 1.44	17.29 ± 0.73	27.90 ± 2.43	10.57 ±0.95	8.33 ± 0.44	Present study[d]
	20.4	8.6	24.3	19.4	16.1	Tareen; Punsuvon; Parakulsuksati (2020)
	32.6	12.0	21.8	19.4	-	Gabhane et al. (2014)
	26.7	25.8	17.0	8.7	-	Brienzo; Siqueira; Milagres (2009)
	35.2	20.28	25.25	15.35	7.59	Alves et al. (2021)

Source: Author
(-): not determined
[a]Hemicellulose: sum of xylose, arabinose, and acetyl group
[b]Lignin: sum of soluble and insoluble lignin.
[c]Guava seed cake (oil-extracted)
[d]Extractive free biomass

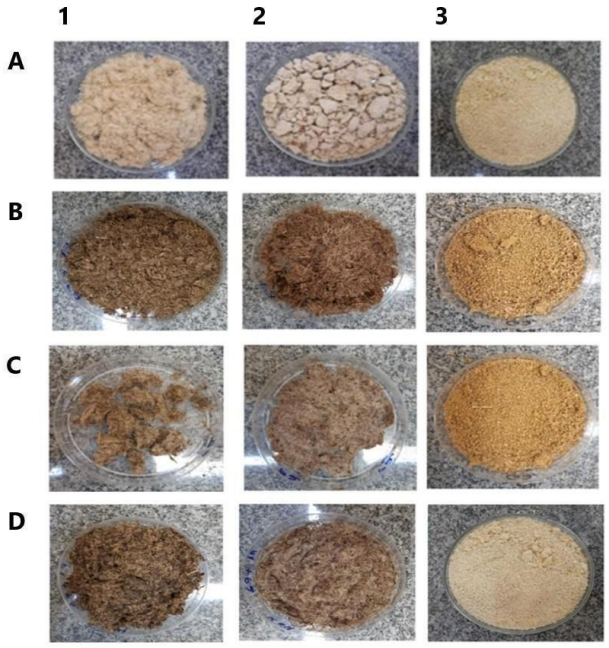
Some studies reported the amount of holocellulose, for example, Bailey; Biely; Poutanen, (1992) reported the composition of guava branches and leaves as 26.56-69.49% for holocellulose, 11.02-34.12% for hemicellulose, 17.77-35.26% for lignin, 1.87-8.20% for ashes. In the present study, the values found for guava seed cake were close to the cited study: 17.17%; 19.96%; 36.33%; 1.12% and 6.22% for cellulose, hemicellulose, lignin, ashes, and extractives (Table 3.1). The variation in the composition of materials is influenced by factors such as plant growth location, soil nutrients, harvest, and climate among others (SHIMIZU et al., 2018).

3.3.2 Chemical characterization of pretreated material

Banana leaf and pseudostem and guava seed cake were pretreated with 6% hydrogen peroxide in an alkaline medium, containing sodium hydroxide, 20% (w/w) sulfuric acid, 20%

(w/w) sodium hydroxide, and 20% (w/w) potassium hydroxide (Figure 3.3). In the presence of hydrogen peroxide, the material acquires a lighter color compared to other pretreatments due to the bleaching reaction.

Figure 3.3 - Pretreated materials. Columns 1, 2 and 3: banana leaves, banana pseudostem, and guava seed cake, respectively. Line A, B, C and D: Hydrogen peroxide, Sulfuric acid, Sodium Hydroxide, and Potassium Hydroxide, respectively



Source: Author.

The highest percentage of mass recovered was obtained in pretreatment with hydrogen peroxide 6% (w/v) in an alkaline medium, except in the case of banana leaves, in which the highest mass recovery obtained was with potassium hydroxide 20% (w/w) (Table 3.2).

Pretreatments cause mass loss due to the solubilization of components present in the material. In this process the breaking of glycosidic bonds occurs, solubilization and removal of hemicellulose, precipitation of lignin due to the breaking of its molecular bonds and condensation, and removal of acetyl groups present in hemicellulose facilitating the activity of hemicellulases. All these changes in the biomass cause an increase in the cellulose percentage, making the material porous, and the cellulose more accessible to the action of enzymes such as cellulases (ARSAT; GHAZALI, 2023; DE FREITAS et al., 2021). The acid pretreatment is more suitable for biomass with lower lignin content, since during the process, most of it remains in the solid fraction and approximately 5 to 10% is solubilized (SCHMATZ; MASARIN; BRIENZO, 2022).

The material with the highest percentage of cellulose (62.85%) was obtained using banana pseudostem treated with 20% (w/w) sulfuric acid. In the materials pretreated with acid, there was an increase in the concentration of cellulose and lignin, and a decrease in hemicellulose, which was solubilized and removed from the biomass (Table 3.2). Arsat; Ghazali (2023) carried out the chemical characterization of banana pseudostem pretreated with sulfuric acid 20% (w/w), at 121 °C for 30 min, and the values found for cellulose, hemicellulose, and lignin were respectively: 63.37%, 4.13%, and 30.08%.

In pretreatment with sodium hydroxide 20% (w/w), the highest concentration of cellulose was obtained using banana pseudostem. The concentration of cellulose, hemicellulose and lignin were 53.75%, 14.21% and 17.6%, respectively. In the pretreatments with sodium hydroxide, there was an increase in the cellulose content in the banana pseudostem and leaf and a decrease in the guava seed cake. The hemicellulose decreased in all cases and the lignin decreased only in the pseudostem. In the alkaline pretreatment with potassium hydroxide, there was an increase in cellulose only for the pretreated leaf, and an increase in the hemicellulose and lignin content in all cases (Table 3.2). The chemical characterization of banana pseudostem pretreated with sodium hydroxide 20% (w/w) at 121 °C for 30 min was previously reported. The values found for cellulose, hemicellulose and lignin were respectively: 70.01%, 9.68% and 10.78% (ARSAT; GHAZALI, 2023). De Freitas and Brienzo (2022) carried out the pretreatment of pseudostem banana using 2 $mol.L^{-1}$ sodium hydroxide, autoclaved for 90 min, mass-to-liquid ratio of 1:4. A material with 51.66% cellulose, 23.29% hemicellulose and 11.44% lignin was obtained. Cellulose concentration can increase by up to 10% in NaOH treatment. In the present study, the cellulose percentage was similar to the cited study. However, there was a greater removal of hemicellulose and a lower removal of lignin, with a concentration of NaOH equivalent to 0.25 $mol.L^{-1}$.

In the study, banana pseudostem was pretreated in several steps using 5% sodium hydroxide, followed by 16% (v/v) hydrogen peroxide in an alkaline medium and 60% (w/w) sulfuric acid (NASCIMENTO et al., 2021). The yield obtained was 0.073 g of cellulose per gram of pseudostem (7.3% extraction yield). The result found was lower than that obtained in the present study considering all biomasses and pretreatments carried out. A study reported hydrolysis of banana pseudostem with sulfuric acid and sodium hydroxide 2% (w/v), at 121°C for 30 min and obtained a material with 52.11% and 50.6%, cellulose, respectively, 28.45% and 28.83%, hemicellulose, 10% and 12% lignin, respectively (LI et al., 2010). Compared to the present study, the percentages obtained for cellulose and lignin were lower, and for hemicellulose higher.

Alkaline pretreatments solubilize and partially remove the hemicellulose and lignin and

swell the cellulose. Sodium hydroxide is widely used in alkaline pretreatments because it is a strong basic catalyst, capable of significantly solubilizing hemicellulose and lignin under optimized conditions (SHIMIZU et al., 2020). In relation to other alkaline pretreatments, it can be observed that sodium hydroxide increases the efficiency of enzymatic hydrolysis, reducing the recalcitrance of the material. It works by breaking ether and ester bonds between lignin and hemicellulose and ester and carbon-carbon bonds in the lignin molecule (FERNANDES et al., 2013).

In the pretreatment with hydrogen peroxide 6% (w/w), the highest percentage of cellulose was obtained with banana pseudostem. The percentage of cellulose, hemicellulose and lignin were 53.57%, 16.46% and 19.21%, respectively (Table 3.2). In this pretreatment, the cellulose amount increased in the pseudostem and the banana leaf and decreased in the guava seed cake, the hemicellulose decreased in all samples and the lignin decreased only in the banana pseudostem (Table 3.2). Arsat; Ghazali (2023) carried out the chemical characterization of banana pseudostem pretreated with hydrogen peroxide 6% (w/w) at 25 °C for 4 h. The values found for cellulose, hemicellulose and lignin were, respectively: 70.99%, 11.31%, and 9.29%. Oxidant reagents combined with an alkaline medium are efficient for the breakdown of lignin and hemicellulose molecules and cause their solubilization, increasing the amount of sugars released after enzymatic hydrolysis. Several studies have been successful with the use of hydrogen peroxide (TARRÉS et al., 2017; ARSAT; GHAZALI, 2023).

Table 3.2 – Chemical composition of untreated and pretreated material with hydrogen peroxide (H_2O_2), sulfuric acid (H_2SO_4), sodium and potassium hydroxide (NaOH and KOH)

Chemical Composition of the untreated and pretreated material (%, dry base)

Material	Pretreatment	Cellulose/ glucan	Xylan	Arabinosyl	Acetyl	Total Hemicellulose	Lignin	Recovered mass (%)
Banana pseudostem	Untreated	48.41^{a} + 0.58	10.48 ± 0.16	3.80 ± 0.11	0.38 ± 0.42	18.5^{a} ± 0.69	19.84^{a} ± 1.74	-
	H_2O_2 6% (w/v)	53.57^{b} ± 0.46	11.97 ± 0.32	4.49 ± 0.12	0	16.46^{b} ± 0.44	19.21^{a} ± 3.25	54.32
	H_2SO_4 20% (w/w)	62.85^{c} ± 0.44	5,17 ± 0.47	0.56 ± 0.09	0.21 ± 0.19	5.94^{c} ± 0.56	34.21^{b} ± 1.27	34.66
	NaOH 20% (w/w)	53.75^{d} ± 1.18	10.26 ± 0.31	3.95 ± 0.40	0	14.21^{d} ± 0.71	17.6^{c} ± 1.99	32.88
	KOH 20% (w/w)	42.35^{e} ± 2.17	21.46 ± 1.11	5.71 ± 0.92	0	27.17^{e} ± 2.03	21.72^{d} ± 0.73	38.16
Banana leaves	Untreated	24.09^{a} ± 1.44	10.62 ± 0.59	6.32 ± 0.62	0.37 ± 0.27	17.31^{a} ± 1.48	27.90^{a} ± 2.43	-
	H_2O_2 6% (w/v)	41.42^{b} ± 2.67	11.19 ± 1.02	5.25 ± 0.32	0	16.45^{b} ± 1.34	44.42^{b} ± 1.38	32.21
	H_2SO_4 20% (w/w)	36.05^{c} ± 1.67	4.31 ± 0.27	0.54 ± 0.04	0.19 ± 0.02	5.04^{c} ± 0.33	41.86^{c} ± 0.39	48.62
	NaOH 20% (w/w)	35.94^{d} ± 2.77	11.70 ± 1.22	2.09 ± 1.12	0	13.79^{d} ± 2.34	30.07^{d} ± 0.47	39.89
	KOH 20% (w/w)	36.39^{ec} ± 0.62	13.94 ± 0.28	7.29 ± 0.58	0	21.23^{e} ± 0.86	30.42^{d} ± 1.53	52.23
Guava seed cake	Untreated	37.74^{a} ± 0.06	19.09 ± 0.93	1.89 ± 0.72	2.02 ± 1.53	23^{a} ± 3.18	34.15^{a} ± 1.81	-
	H_2O_2 6% (w/v)	28.71^{b} ± 4.29	17.91 ± 0.65	1.18 ± 1.89	0	19.09^{b} ± 2.54	38.77^{b} ± 2.41	50.98
	H_2SO_4 20% (w/w)	39.22^{c} ± 3.07	11.16 ± 3.58	0.17 ± 0.26	0 ± 0.08	11.33^{c} ± 3.92	49.66^{c} ± 0.28	43.91
	NaOH 20% (w/w)	34.66^{d} ± 2.51	11.02 ± 1.02	0.92 ± 1.17	0	11.93^{dc} ± 2.19	34.78^{a} ± 0.97	44.85
	KOH 20% (w/w)	31.04^{e} ± 1.97	23.90 ± 1.36	4.43 ± 0.59	0.23 ± 0.03	28.56^{e} ± 1.98	42.96^{d} ± 0.26	45.10

Source: Author

* tests performed in triplicate; (-): not reported; Lignin: sum of soluble and insoluble lignin.

Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 (p<0.05)

The high degree of polymerization and crystallinity of cellulose, and its association with lignin and hemicellulose makes the biomass recalcitrant. Pretreatments (grinding, chemical, and physicochemical) are essential for the efficient posterior enzymatic hydrolysis of cellulose, consequently increasing its reactivity (SHIMIZU et al., 2020).

3.3.3 Enzymatic hydrolysis for COS production

The present study aimed firstly at producing COS from fruit waste by applying sulfuric acid, sodium hydroxide, potassium hydroxide, and hydrogen peroxide as pretreatment previously to enzymatic hydrolysis. The materials were knife milled (20 mesh, 0.8 mm) before pretreatments, and a part was ball milled after the pretreatments. Another strategy was to apply xylanase hydrolysis followed by endoglucanase. This strategy produced XOS, removing hemicellulose from the material, exposing cellulose to the endoglucanase hydrolysis, releasing COS.

The untreated material, knife milled and ball-milled was subjected to enzymatic hydrolysis by both endoxylanase and endoglucanase, (Table 3.3, Figure 3.4). For untreated material (only knife milled), the highest COS yields were 10.10%, 21.72%, and 32.06% for pseudostem (20 $IU.g^{-1}$), leaf (20 $IU.g^{-1}$), and guava seed cake (100 $IU.g^{-1}$), respectively. The enzymatic hydrolysis charge evaluated (20, 50 and 100 $IU.g^{-1}$) showed a small range variation in the COS yield. The highest COS yield of 32.06% for guava seed cake presented a concentration of 1.0 $g.L^{-1}$ in the experimental conditions applied.

The application of endoxylanase resulted in XOS yield of 56.76%, 54.35%, and 52.48%, respectively for BP, BL, and GB. These yields of XOS could be considered high compared to processes that solubilized xylan previously to the enzymatic hydrolysis (OLIVEIRA et al., 2007). The solid residue from the endoxylanase hydrolysis was submitted to a step of hydrolysis with endoglucanase. The COS yield increased 5.7 times for BP and 3.4, and 1.5 times for BL and BG, respectively (Table 3.3). The removal of hemicellulose from the untreated material proved to be a good strategy for subsequent hydrolysis with endoglucanase to produce COS.

The untreated material was ball milled previously to the endoglucanase hydrolysis, increasing the COS yield 1.6 times for BP, and 1.3 times for BL, with no increase for GB. The GB was more resistant to the ball mill, perhaps influenced by the seed structure and lignin content (Table 3.3). For XOS production, the yield of XOS was slight compared to the unmilled (Figure 3.4). COS yield was slightly lower applying the ball mill previously to endoxylanase and andoglucanase. For both XOS and COS production, the ball mill was not efficient in increasing the production yield.

Table 3.3.- Yield of conversion of cellulose into COS and conversion of xylan into XOS after enzymatic hydrolysis of the banana pseudostem (BP) and leaf (BL) and guava seed cake (GSC) untreated

			treated material hydrolyzed with endoglucanase							
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	24	44.90	4.49	0	0	0	3.99	8.48[a]	0.85
	50		47.40	0	0	0	0	3.85	3.85[b]	0.43
	100		50.90	0	0	0	4.69	5.40	10.10[c]	0.98
BL	20		67.20	12.60	0	0	0	9.15	21.74[a]	1.01
	50		60.50	0	0	0	0	2.57	2.57[b]	0.12
	100		68.70	8.17	1.11	0	0	4.29	13.56[c]	0.65
GSC	20		16.00	20.01	0	0	0	8.33	28.34[a]	0.97
	50		13.00	14.64	0	0	0	7.93	22.57[b]	0.83
	100		12.00	0	1.28	0	0	30.78	32.06[c]	1.09
			Untreated material hydrolyzed with xylanase, followed by endoglucanase							
Material	Xylanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5/X_6 (%)		Total XOS (%)	Total XOS ($g.L^{-1}$)
BP	30	24	0	55.15	0	0	1.60		56.76[a]	11.24
BL	30		0.39	0	0	0	0		54.35[b]	11.63
GSC	30		0.04	52.48	0	0	0		52.48[c]	11.55
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	6	6.26	47.61	0	0	0	1.28	48.88[a]	4.65
BL	20		2.63	72.32	0	0	0	0	72.32[b]	3.71
GSC	20		7.46	16.88	31.52	0	0	0	48.40[c]	1.70

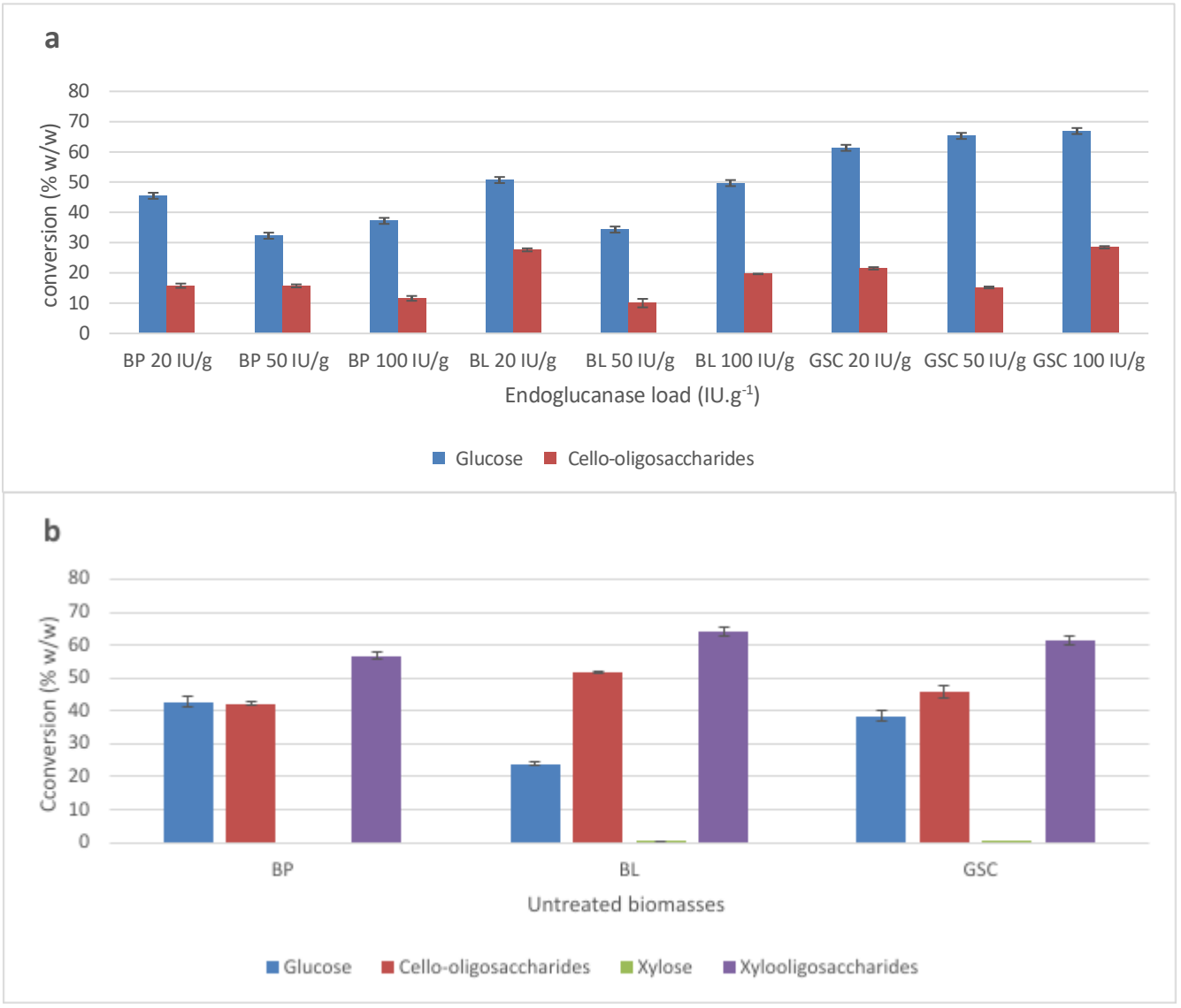
Source: Author

Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 ($p<0.05$)

C_1: glucose; C_2: celobiose; C_3: celotriose; C_4: celotetraose; C_5: celopentaose; C_6: celohexaose; COS: cellooligosaccharides=$C_2+C_3+C_4+C_5+C_6$.

X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=X2+X3+X4+X5/X6.

Figure 3.4 - Yield of COS and XOS after enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake untreated and ground in ball mill. A: Material hydrolyzed with endoglucanase, enzymatic load of 20, 50 and 100 IU.g^{-1} for 24 h. B: Material hydrolyzed with xylanase, enzyme load 30 IU.g^{-1} for 24 h, followed by endoglucanase, 20 IU.g^{-1} for 6 h. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

3.3.1 Alkali pretreatment (sodium and potassium hydroxide)

Similar strategies were applied to the biomass but previously pretreated with sodium and potassium hydroxide. The sodium hydroxide pretreatment previously to the endoglucanase hydrolysis resulted in lower COS yield compared to the enzymatic hydrolysis of the untreated material (Table 3.4). The profile of COS and XOS production with the endoxylanase, endoglucanase, and ball mill before enzymatic hydrolysis was similar for the untreated material.

This profile of XOS and COS production means the trend effect of each process step. In a general comparison, the pretreatment showed benefits for some specific conditions.

The highest COS yields were obtained using biomass pretreated with alkali. The values were 53.05% for banana pseudostem pretreated with potassium hydroxide, 79.73% for banana leaves pretreated with sodium hydroxide, and 59.99% for guava seed cake pretreated with sodium hydroxide (Figure 3.5 and 3.6).

Table 3.4 – Yield of conversion of cellulose into COS and conversion of xylan into XOS after enzymatic hydrolysis of the banana pseudostem (BP) and leaf (BL) and guava seed cake (GSC) pretreated with sodium hydroxide

Materi l hydroly zed with e doglucan se										
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	24	3.99	3.82	0	0	0	1.68	5.50[a]	0.59
	50		6.21	3.81	0	0	0	1.88	5.69[b]	0.63
	100		14.80	4.75	0.24	0	0	3.20	8.20[c]	0.85
BL	20		3.93	3.42	0	0	0	4.03	7.44[a]	0.51
	50		10.30	4.22	0	0	0	4.06	8.27[b]	0.57
	100		20.20	3.71	0.10	0	0	5.35	9.16[c]	0.67
GSC	20		0	0.17	0	0	0	1.22	1.39[a]	0.08
	50		0	0.87	0	0	0	3.86	4.73[b]	0.27
	100		10.60	1.45	0	0	0	3.63	5.08[c]	0.29
Hydrolysis with xylanase, followed by endoglucanase										
Material	Xylanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5/X_6 (%)		Total XOS (%)	Total XOS ($g.L^{-1}$)
BP	30	24	0	57.99	0	0	0		57.99[a]	11.83
BL	30		0	52.70	0	0	0		52.70[b]	11.49
GSC	30		0	55.14	0	0	0		55.14[c]	11.69
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	6	3.26	42.82	0	0	0	0	42.82[a]	4.54
BL	20		0.60	45.67	0	0	0	0	45.67[b]	3.17
GSC	20		0	15.72	29.36	0	0	0	45.09[c]	2.55

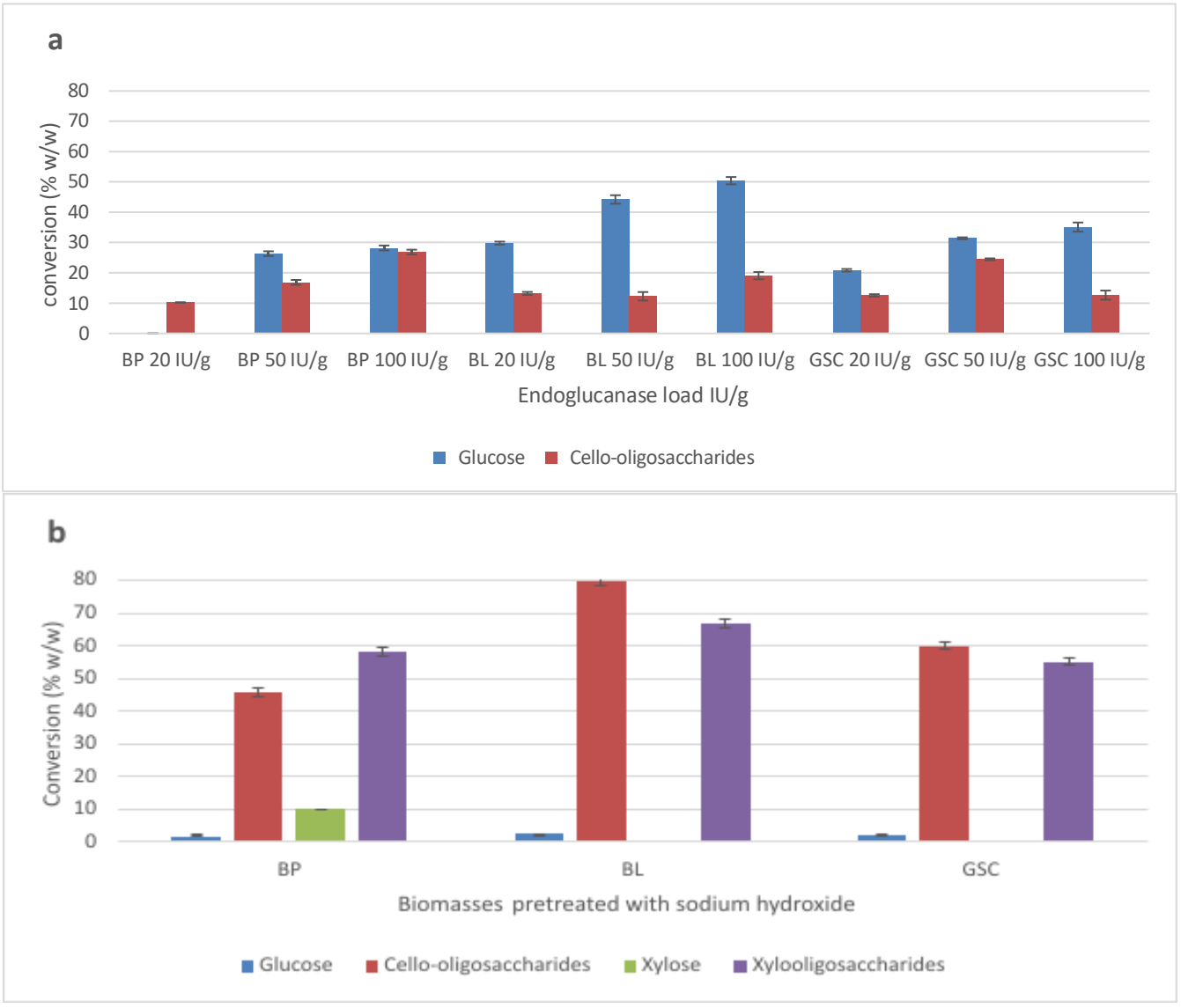
Source: Author

Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 ($p<0.05$)

C_1: glucose; C_2: celobiose; C_3: celotriose; C_4: celotetraose; C_5: celopentaose; C_6: celohexaose; COS: gluco-oligosaccharides=$C_2+C_3+C_4+C_5+C_6$.

X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=X2+X3+X4+X5/X6.

Figure 3.5 - Yield of COS and XOS after enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake pretreated with sodium hydroxide and ground in ball mill. A: Material hydrolyzed with endoglucanase, enzymatic load of 20, 50 and 100 IU.g^{-1} for 24 h. B: Material hydrolyzed with xylanase, enzyme load 30 IU.g^{-1} for 24 h, followed by endoglucanase, 20 IU.g^{-1} for 6 h. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

Sodium potassium as a pretreatment for further endoglucanase hydrolysis resulted in lower COS yield compared to untreated material hydrolysis (Table 3.3 and 3.5). Fruit waste showed a chemical composition characteristic of a lignocellulosic material (Table 3.1). However, the effect of pretreatments with sodium and potassium hydroxide was not efficient in the modification of structure for further enzymatic hydrolysis. For a typical lignocellulosic material, it is expected that the alkaline pretreatment would remove part of the lignin and hemicellulose (SHUIMIZU et al., 2020), increasing accessibility to the cellulose (KUNDU et al., 2021). However, for the fruit waste

(banana and guava seed cake) the effect of the pretreatment was not in the same tendency observed for hemicellulose and lignin (Table 3.1). The pretreatment may have affected the cellulose/glucan content with no strong benefit for further endoglucanase hydrolysis.

Table 3.5 – Yield of conversion of cellulose into COS and conversion of xylan into XOS after enzymatic hydrolysis of the banana pseudostem (BP) and leaf (BL) and guava seed cake (GSC) pretreated with potassium hydroxide

Hydrolysis with endoglucanase										
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	24	3.08	2.38	0	0	0	6.41	8.78[a]	0.77
	50		7.53	4.20	0	0	0	3.15	7.35[b]	0.62
	100		25.00	7.26	0.41	0	0	4.65	12.32[c]	1.05
BL	20		4.25	2.55	0	0	0	2.62	5.17[a]	0.39
	50		8.82	4.37	0.18	0	0	2.95	7.50[b]	0.56
	100		14.00	1.97	0.10	0	0	3.76	5.84[c]	0.44
GSC	20		5.83	1.98	0.00	0	0	1.70	3.67[a]	0.24
	50		17.20	3.60	0.00	0	0	1.34	4.94[b]	0.31
	100		7.24	1.70	0.00	0	0	2.55	4.25[c]	0.28
Hydrolysis with xylanase, followed by endoglucanase										
Material	Xylanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5/X_6 (%)		Total XOS (%)	Total XOS ($g.L^{-1}$)
BP	30	24	0	59.10	0	0	0		59.10[a]	12.06
BL	30		0	57.45	0	0	0		57.45[b]	11.49
GSC	30		0.04	56.49	0	0	0		56.49[c]	11.64
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	6	0.10	53.05	0	0	0	0	53.05[a]	4.50
BL	20		3.36	58.07	0	0	0	0	58.07[b]	4.18
GSC	20		0	16.42	34.39	0	0	0	50.81[c]	3.24

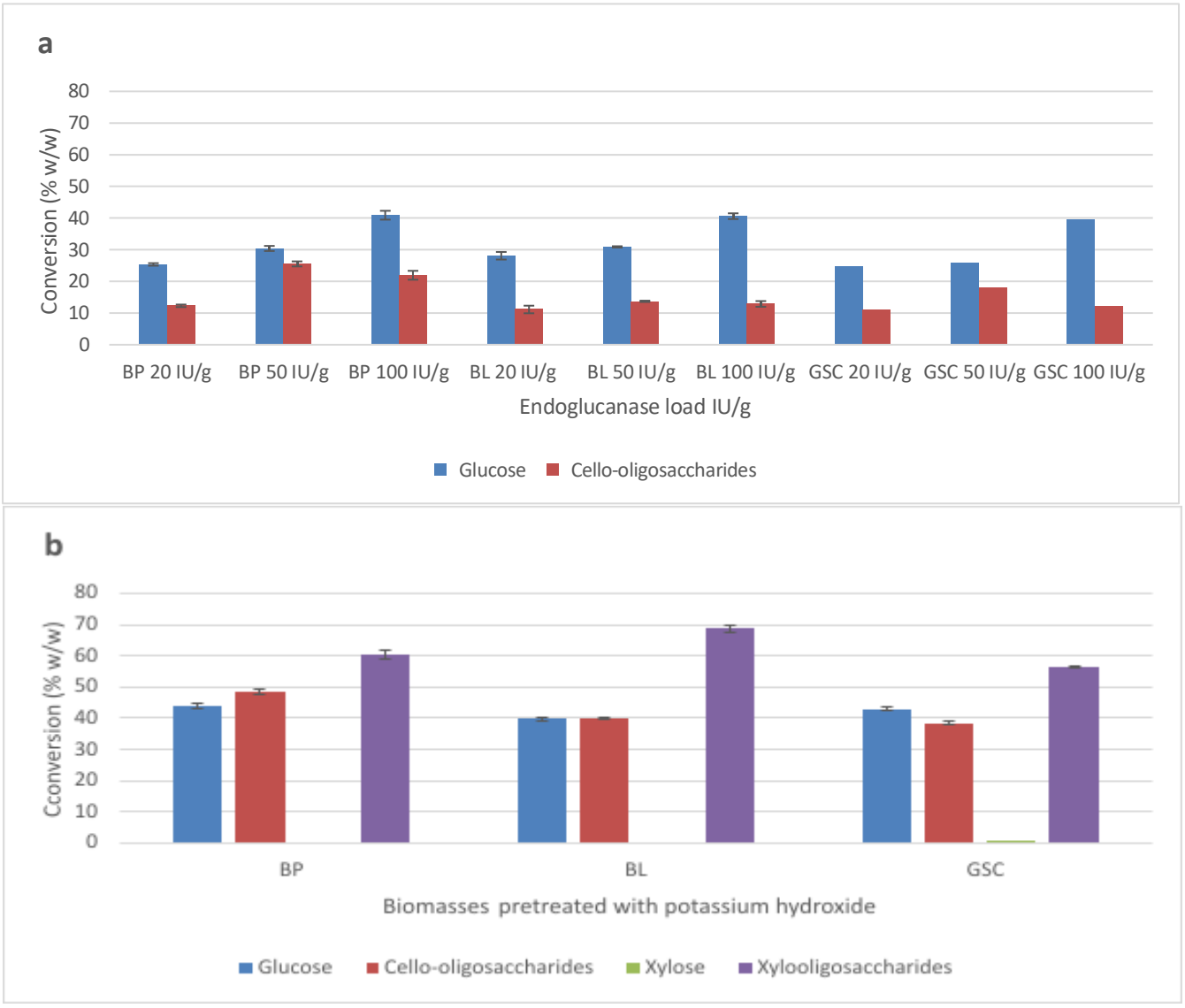
Source: Author

Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 ($p<0.05$)

C_1: glucose; C_2: celobiose; C_3: celotriose; C_4: celotetraose; C_5: celopentaose; C_6: celohexaose; COS: gluco-uoligosaccharides=$C_2+C_3+C_4+C_5+C_6$.

X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=X2+X3+X4+X5/X6.

Figure 3.6 - Yield of COS and XOS after enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake pretreated with potassium hydroxide and ground in ball mill. A: Material hydrolyzed with endoglucanase, enzymatic load of 20, 50 and 100 IU.g^{-1} for 24 h. B: Material hydrolyzed with xylanase, enzyme load 30 IU.g^{-1} for 24 h, followed by endoglucanase, 20 IU.g^{-1} for 6 h. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

3.3.2 Sulfuric acid pretreatment

Sulfuric acid pretreatment resulted in lower COS yield with the endoglucanase hydrolysis (Table 3.6), compared to hydrolysis of untreated biomass. The XOS yield was in the same range, and there was a lower COS yield when endoglucanase was applied after the endoxylanase compared to untreated material.

Table 3.6 – Yield of conversion of cellulose into COS and conversion of xylan into XOS after enzymatic hydrolysis of the banana pseudostem (BP) and leaf (BL) and guava seed cake (GSC) pretreated with sulfuric acid

Hydrolyzed with endoglucanase										
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	24	0.62	1.08	6.69	0	0	0	7.77[a]	1.03
	50		3.46	1.57	0	0	0	1.77	3.34[b]	0.42
	100		6.93	1.81	0	0	0	2.26	4.07[c]	0.53
BL	20		0.91	2.26	0	0	0	5.04	7.30[a]	0.53
	50		5.41	1.80	0	0	0	6.08	7.88[a]	0.57
	100		13.60	3.44	0	0	7.94	0	11.38[b]	0.84
GSC	20		6.12	2.92	0	0	0	2.59	5.51[a]	0.43
	50		3.83	1.18	0	0	0	4.46	5.64[a]	0.42
	100		14.30	2.54	0.13	0	0	4.46	7.13[b]	0.53
Hydrolyzed with xylanase, followed by endoglucanase										
Material	Xylanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5/X_6 (%)		Total XOS (%)	Total XOS ($g.L^{-1}$)
BP	30	24	0.05	60.13	0	0	0		60.13[a]	12.15
BL	30		0	52.58	0	0	0		52.58[b]	11.88
GSC	30		0	57.25	0	0	0		57.25[c]	12.14
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	6	1.40	30.51	0	0	0	0	30.51[a]	3.88
BL	20		3.58	53.01	0	0	0	0	53.01[b]	4.31
GSC	20		0	11.71	19.95	0	0	0	31.66[c]	2.48

Source: Author

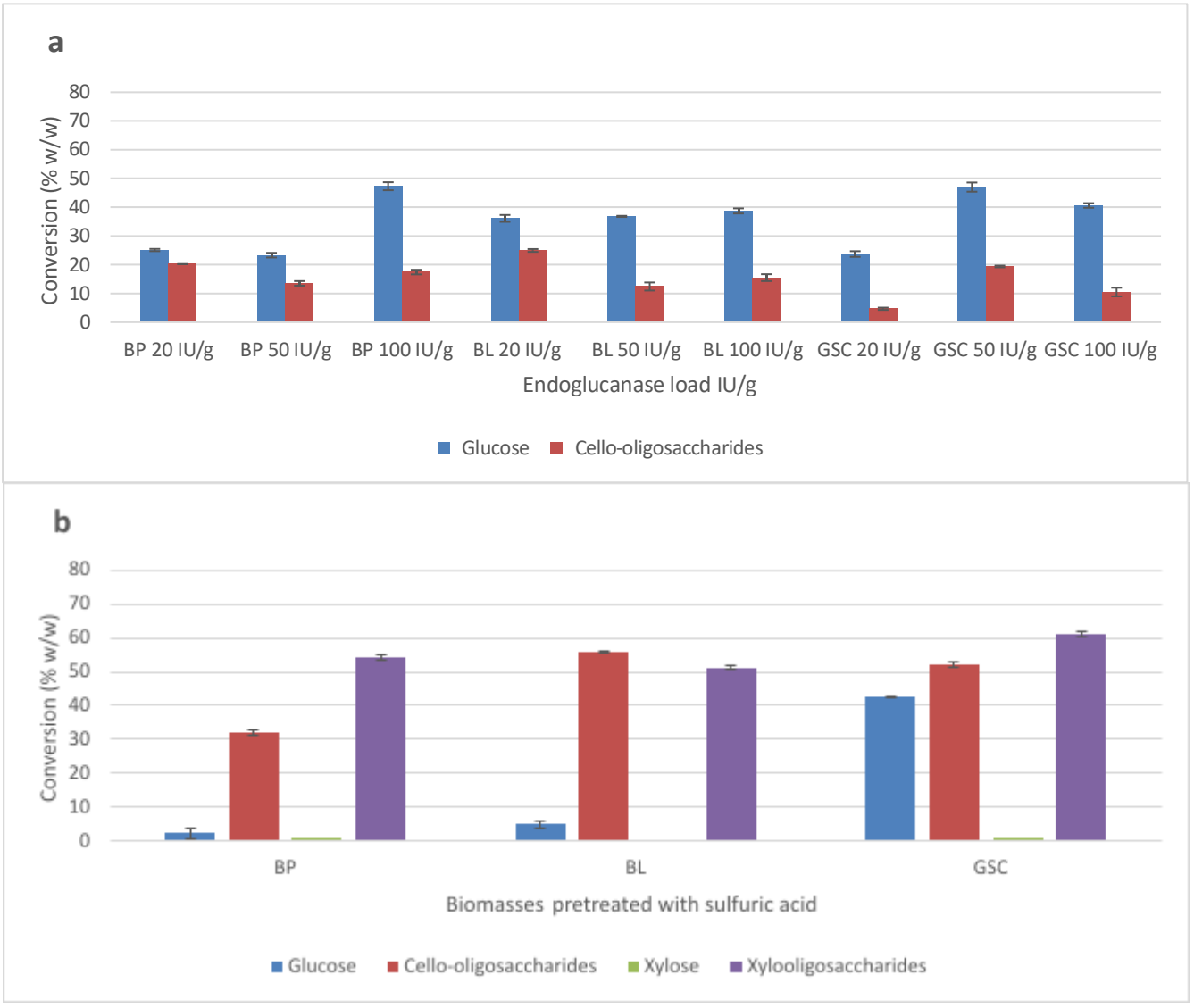
Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 ($p<0.05$)

C_1: glucose; C_2: celobiose; C_3: celotriose; C_4: celotetraose; C_5: celopentaose; C_6: celohexaose; COS: gluco-oligosaccharides=$C_2+C_3+C_4+C_5+C_6$.

X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=X2+X3+X4+X5/X6.

The highest yield was obtained with the material ground in a ball mill, and subjected to xylanase before endoglucanase, however for the pseudostem and banana leaf the yield was similar to the material subjected to the same conditions but not ground in a ball mil (Figure 3.7).

Figure 3.7 - Yield of COS and XOS after enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake pretreated with sulfuric acid and ground in ball mill. A: Material hydrolyzed with endoglucanase, enzymatic load of 20, 50 and 100 IU.g^{-1} for 24 h. B: Material hydrolyzed with xylanase, enzyme load 30 IU.g^{-1} for 24 h, followed by endoglucanase, 20 IU.g^{-1} for 6 h. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

3.3.3 Hydrogen peroxide pretreatment

Hydrogen peroxide pretreatment resulted in low COS yield for all unmilled biomasses (Table 3.7). When the biomasses were ground in a ball mill, there was an increase in COS yield, however the greatest result was obtained with biomasses not ground in a ball mill and subjected to the action of endoxylanase before endoglucanase (Figure 3.8). The higher yield of COS was observed with the lowest endoglucanase charge (20 IU.g^{-1}), while the increase in the enzyme

charge did not collaborate for a higher yield.

Table 3.7 – Yield of conversion of cellulose into COS and conversion of xylan into XOS after enzymatic hydrolysis of the banana pseudostem (BP) and leaf (BL) and guava seed cake (GSC) pretreated with hydrogen peroxide

H drolyzed with endoglucanase										
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	24	41.30	5.98	1.00	0	0	3.41	10.39[a]	1.18
	50		49.70	7.72	0	0	0	2.89	10.61[a]	1.17
	100		50.10	8.29	0	0	4.86	0	13.15[b]	1.43
BL	20		57.70	7.72	0	0	0	3.96	11.67[a]	1.01
	50		62.40	9.49	1.01	0	0	4.54	15.04[b]	1.44
	100		64.20	8.14	0	0	14.28	0.00	22.41[c]	1.87
GSC	20		66.00	0	0	0	0	2.04	2.04[a]	0.12
	50		75.10	10.90	0	7.09	0	3.72	21.71[b]	1.26
	100		69.30	7.84	0	0	0	3.75	11.59[c]	0.79
Hydrolyzed with xylanase, followed by endoglucanase										
Material	Xylanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5/X_6 (%)		Total XOS (%)	Total XOS ($g.L^{-1}$)
BP	30	24	0	58.10	0	0	0		58.10[a]	11.97
BL	30		0	12.69	30.58	0	0		43.28[b]	9.0
GSC	30		6.78	13.91	27.58	0	0		41.49[c]	8.30
Material	Endoglucanase enzymatic load ($IU.g^{-1}$)	Reaction time (h)	C_1 (%)	C_2 (%)	C_3 (%)	C_4 (%)	C_5 (%)	C_6 (%)	Total COS (%)	Total COS ($g.L^{-1}$)
BP	20	6	4.60	31.81	0	0	0	0	31.81[a]	3.47
BL	20		4.32	75.95	0	0	0	0	75.95[b]	6.35
GSC	20		0	8.67	28.24	0	0	0	36.91[c]	2.20

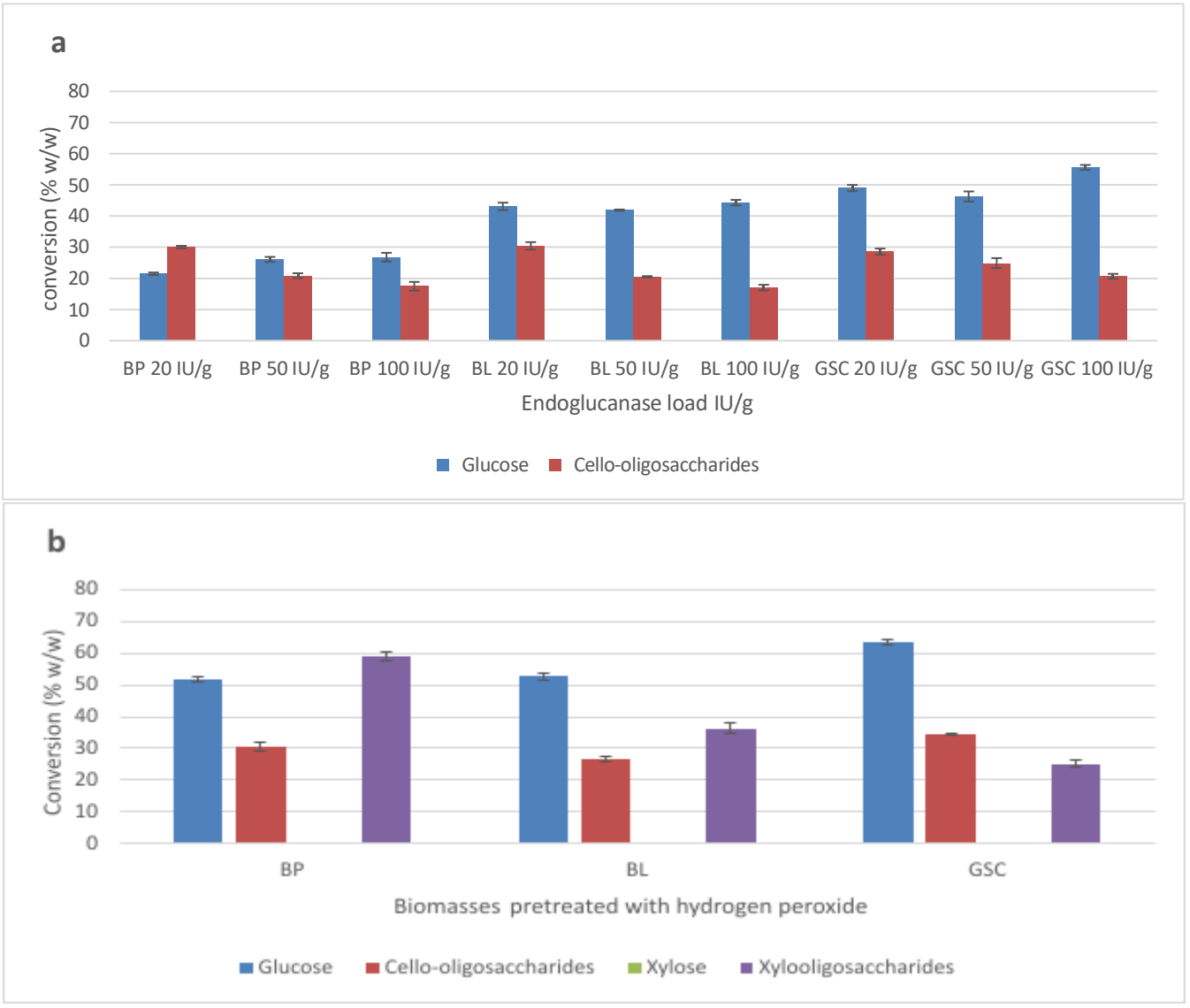
Source: Author

Equal letters, in the same column for each biomass, represent the same statistical similarity, less than 0.05 ($p<0.05$)

C_1: glucose; C_2: celobiose; C_3: celotriose; C_4: celotetraose; C_5: celopentaose; C_6: celohexaose; COS: gluco-oligosaccharides=$C_2+C_3+C_4+C_5+C_6$.

X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5/X6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=X2+X3+X4+X5/X6.

Figure 3.8 - Yield of COS and XOS after enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake pretreated with hydrogen peroxide and ground in ball mill. A: Material hydrolyzed with endoglucanase, enzymatic load of 20, 50 and 100 IU.g^{-1} for 24 h. B: Material hydrolyzed with xylanase, enzyme load 30 IU.g^{-1} for 24 h, followed by endoglucanase, 20 IU.g^{-1} for 6 h. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

It was observed that the smaller particle size obtained after grinding in a ball mill and the use of the enzyme xylanase were responsible for the increase in the COS production. In some cases, glucose also increased, probably due to the increase in the contact surface and greater exposure of cellulose to the endoglucanase caused by the removal of xylan.

A COS production was reported using sugarcane straw pretreated with sulfuric acid and hydrolyzed using the commercial cocktail Celluclast (ZHAO et al., 2020). The material was pretreated with sulfuric acid 0.5% (v/v), solids concentration of 9% (w/w) at 140 ºC for 15 min

and then it was submitted to enzymatic hydrolysis with Cellulast 10 $IU.g^{-1}$, in 0.1 $mol.L^{-1}$ sodium phosphate buffer (pH 5), temperature of 50 ºC, substrate concentration of 1% (w/v) for 48 h. The amount of COS obtained was approximately 33.54 mg of COS per gram of pretreated sugarcane straw. In the present study, the highest yield of COS obtained (79.73% for banana leaves pretreated with sodium hydroxide) was equal to180.5 mg of COS per gram of material, that is, it was 5 times greater than the study reported. Probably, the lower COS concentration obtained in the study performed by Barbosa et al. (2020a) was due to the low acid concentration, which was insufficient to make cellulose more accessible to the enzyme (Cellulast cocktail) and the enzyme concentration was not able to hydrolyze cellulose efficiently. The study was also conducted with ionic liquid and the commercial cocktail Celluclast 10 $IU.g^{-1}$. The biomass was pretreated with 2-hydroxyethylammonium acetate and 2-hydroxyethylammonium hexanoate at 130 °C for 4 h. There was a production of approximately 90 $mg.g^{-1}$ of COS, an amount about 2.7 times higher than the value reported with the use of sulfuric acid and 2 times lower than the present study.

Enzymatic hydrolysis was applied after hydrothermal pretreatment to obtain COS of banana pseudostem (KUMAR et al., 2020). The yield of oligosaccharides obtained from the material without pretreatment did not exceed 3.2%. When the material was hydrothermally pretreated at 150 ºC, the yield increased to 28% (of which 72% was in the form of COS, that is 134.48 mg of COS per g of pseudostem), with 1 $FPU.g^{-1}$ of cellulase for 12 h. The yield was lower than that obtained in the present study, as less than 28% of cellulose was converted into COS. Hydrothermal pretreatment was less efficient than chemical pretreatments according to the conditions applied.

COS were produced using sugarcane straw and coffee husks delignified with sodium chlorite, treated with potassium hydroxide 4.5% w/v at 121 ºC for 30 min and hydrolyzed with a mixture of commercial enzymes containing cellulase and esterase (ÁVILA et al., 2021). The maximum yield obtained was 63.56 $mg.g^{-1}$ of substrate and low glucose concentration. In the present study, when potassium hydroxide was used in the pretreatment, the maximum yield was 53.05% for banana pseudostem (Table 3.5), that is, 230 $mg.g^{-1}$, a value 3.6 times greater than that of the cited study. This was probably due to the potassium hydroxide effect in the pretreatment, and the use of xylanase previously to the endoglucanase.

Xylanase was applied in the present study aiming at xylan removal, and consequently, exposing cellulose to further action of endoglucanase. The removal of components such as xylan and lignin can increase the cellulose surface area exposed, and consequently increasing the enzymatic action (SHIMIZU et al., 2020; KUNDU et al., 2021). Acid pretreatment can cause lignin reallocation/precipitation under the fiber, also contributing to cellulose exposure (CAMARENA-

TELLO et al., 2015).

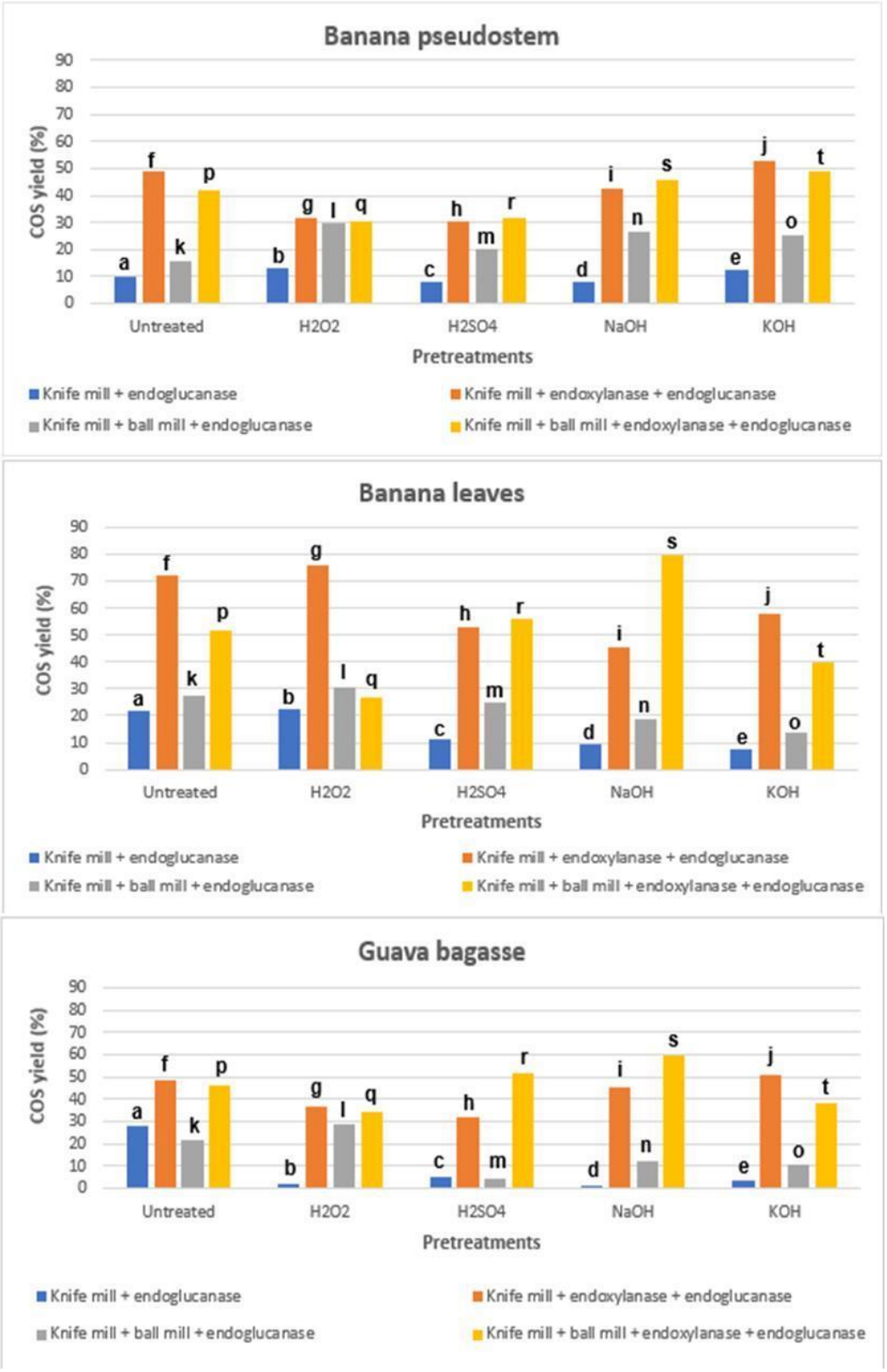
In general, the conversion yield of xylan into XOS in the present study was above 50%, reaching a maximum percentage of 68.76% in the hydrolysis of banana leaf ground in a ball mill and pretreated with potassium hydroxide. After the materials were ground in a ball mill, there was a tendency to increase the yield of XOS probably by increasing the contact surface, allowing for a better interaction with the endoxylanase.

It was possible to convert 67.43%, 69.71% and 61% of xylan into XOS in the hydrolysis of sugarcane bagasse and leaf and banana pseudostem, respectively, using purified endoxylanase from *Aspergillus versicolor* (the same enzyme as the present study), using an enzymatic load of 30 UI.g^{-1} for 24 h (OLIVEIRA et al., 2007; FERNANDES et al., 2020). The use of endoxylanase has decreased the recalcitrance of the material with xylan solubilization, leaving the cellulose more exposed and accessible to endoglucanase, and enabling the production of XOS and COS in the same reaction.

COS were produced with a predominance of cellobiose and cellohexaose. Endoglucanases randomly break β-glycosidic bonds mainly from the amorphous region of cellulose, releasing oligomers of varying sizes, especially cellobiose and cellotriose. Using the material without pretreatment, there was the formation of cellobiose and cellohexaose in all conditions. However, high glucose formation was observed, except in the non-milled and submitted to xylanase previously to the endoglucanase material. After the hydrolysis of the material pretreated with hydrogen peroxide, there was a formation of cellobiose and glucose in all assays. When the non-milled material was subjected to the action of xylanase and endoglucanase, there was lower glucose formation. Using the material pretreated with sulfuric acid and sodium hydroxide, there was a formation of cellobiose in all the assays and low glucose formation, except in the material ground and hydrolyzed with endoglucanase only.

To facilitate the understanding of the results, graphs were created with the best yields for each material submitted to the different pretreatments (Figure 3.9). It can be observed that the untreated material submitted to enzymatic hydrolysis resulted in a high amount of COS. The combination of endoxylanase and endoglucanase resulted in a higher yield of COS. The ball mill had a positive effect for COS yield for material pretreated with sulfuric acid and sodium hydroxyde. The conversion yield with banana pseudostem and leaf and guava seed cake untreated was lower, 4.2, 7.4 and 11.6%, respectively compared to the maximum yields obtained with pretreated materials. Economic feasibility studies would be necessary to confirm whether chemical pretreatment is viable in these cases (Figure 3.9).

Figure 3.9 – Higher conversion yields of cellulose into COS (%, m/m), with biomass submitted to several pretreatments



Source: Author

Equal letters, in the same pretreatment indicated by colour, represent the statistical similarity, less than 0.05 (p<0.05)

3.4 Conclusion

The objective of the present study was to produce COS through enzymatic hydrolysis using endoglucanase. The study assessed the influence of chemical pretreatments, ball milling, and the use of endoxylanase to reduce cellulose recalcitrance and remove bound xylan. The highest yields for banana pseudostem, banana leaves, and guava seed cake were achieved with alkali pretreatment and the application of endoxylanase before endoglucanase. However, the ball mill was only effective for materials pretreated with sulfuric acid and sodium hydroxide. In the future, these methods could be improved for large-scale COS and XOS production in the fruit beverage and food industries, imparting nutritional advantages to food products.

CHAPTER IV: ANTIOXIDANT ACTIVITY OF CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES FROM BANANA LEAVES AND PSEUDOSTEM AND GUAVA SEED CAKE BY DPPH METHOD

Abstract

Free radicals are molecules generated in our body during some biochemical processes and in excess they can cause various diseases, therefore their generation needs to be controlled. One of the ways is through the consumption of substances with antioxidant capacity, that is, capable of containing free radicals. This study tested the antioxidant activity of cellooligosaccharides and xylooligosaccharides solutions, extracted from banana leaf and pseudostem and guava seed cake, unfiltered and filtered in a Sep-Pak cartridges, from its monomers, glucose and xylose (commercial), and cellobiose (commercial) using the radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH). It was possible to observe antioxidant activity with unfiltered COS and XOS and absence of activity with filtered solutions. It is possible to conclude that the antioxidant activity is due to the presence of compounds dissolved in the oligosaccharide solutions, such as lignin, extractives and carboxylic acids, which were qualified by the Folin-Ciocalteu method and scanning spectrophotometry.

Keywords: Enzymatic hydrolysis; Folin-Ciocalteu; Free radicals; Scanning spectrophotometry.

4.1 Introduction

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) are oligomers of glucose and xylan that have the potential to be used as immunostimulant, anticancer, prebiotic, in reducing cholesterol and blood glucose levels and as an antioxidant (FUSO et al., 2023). Antioxidants can be produced by our body, such as the enzymes coenzyme Q10, glutathione peroxidase, catalase, glutathione S transferase and superoxide dismutase. Furthermore, they can also be obtained through the diet, with the consumption of foods such as fruits, vegetables, legumes, which contain substances such as ascorbic acid (vitamin C), vitamin A, tocopherol (vitamin E), polyphenols, selenium, flavonoids, isoprenoids, lycopene, ß-carotene, among others, or industrially produced and added to foods and products such as fuels and cosmetics, preventing oxidation reactions and helping to preserve (KIRAN; OTLU; KARABULUT, 2023; KHAN et al., 2018; MALCANGI et

al., 2023). Antioxidants are capable of inhibiting the oxidation of molecules and reduce damage caused by free radicals (CHAUDHARY et al., 2023; XU et al., 2022; RAHAMAN et al., 2023).

Free radicals are unstable atoms or molecules that are highly reactive and short-lived due to unpaired electrons in the valence shell and are capable of existing independently. Due to their instability, they react with other compounds by capturing their electrons and transforming them into free radicals, generating a chain reaction that can cause damage and cell death (KHAN et al., 2018; PHANIENDRA; JESTADI; PERIYASAMY, 2015). They are produced by our cells during natural biological processes such as breathing, converting fats into energy, food digestion, and during the metabolism of drugs and alcohol (SHARIFI-RAD et al., 2020). Free radicals in moderate amounts can be beneficial, as they are part of several biological processes, such as the destruction of bacteria by phagocytes and macrophages, cell signaling, in the maintenance of the body's homeostasis and in various cellular functions. (WANG et al., 2021; SHARIFI-RAD et al., 2020).

When our body cannot destroy them by the natural antioxidant system or when they are produced in excess, as in the case of excessive exposure to solar radiation, pollution, consumption of some foods, alcohol or medicines, cigarette smoke, among others, a process called oxidative stress occurs, causing cellular and molecular damage, alteration of deoxyribonucleic acid (DNA), in normal cell division, in energy generation, and in lipids, proteins, cell membranes, and consequently, diseases such as cancer, degeneration of the nervous system, diabetes mellitus, rheumatoid arthritis, cardiovascular and neurodegenerative diseases, cataracts and premature aging (PHAM-HUY; HE; PHAM-HUY, 2008; SHARIFI-RAD et al., 2020; HAJAM et al., 2022; BABILI, et al., 2022; PHANIENDRA; JESTADI; PERIYASAMY, 2015).

During aging, there is a decrease in the activity of enzymes that fight free radicals, therefore, it is important to consume foods and substances that have antioxidant properties. In this context the COS, XOS, and oligosaccharides in general has the potential to act as antioxidants, but the mechanism is still unknown. According to some studies on XOS, it was observed that the properties that interfere with the antioxidant activity are: higher degree of polymerization and degree of substitution (type and amount of substituent groups in the chain). XOS with uronic acids such as galacturonic or glucuronic acid scavenge free radicals more effectively. Regarding oligosaccharides in general, the acetyl group can increase or decrease the antioxidant activity. Some studies associate this activity with the presence of compounds such as phenols, which are also extracted in the process (HUANG et al., 2019; FUSO et al., 2023).

To test the antioxidant activity, the assay with 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is one of the most used methods because it is accurate, easy, fast, reliable, economical, and with

reproducible results (BABILI, et al., 2022). The DPPH method evaluates the necessary concentration of substances with the antioxidant potential to reduce the initial concentration of DPPH radicals by 50% (IC_{50}). Radical reduction is measured by spectrophotometry before and after the addition of the studied substances (TAKATSUKA et al., 2022; GULCIN; ALWASEL, 2023).

Regarding COS, studies are of scarce, therefore, the present study aimed to analyze its possible antioxidant activity, using the DPPH method. XOS activity was also measured and compared with literature data. XOS and COS were produced using guava seed cake and banana pseudostem and leaves, very abundant biomasses in Brazil, pretreated with alkali and enzymatic hydrolysed (endoxylanase and endoglucanase). The antioxidant activity was measured in raw hydrolysed and purified hydrolysed with a Sep-Pak cartridges (removal of color compounds, phenols, and aromatic).

4.2 Materials and methods

4.2.1 Sample preparation

The guava seed cake was donated by the company Agttec (Dois Corregos-SP), after oil extraction. The banana pseudostem and leaves were collected in local plantation at Rio Claro (22°23'59"S 47°34'18"W). All biomasses were dried at 60 ºC and ground in a knife mill to 20 mesh (FERNANDES et al., 2020). This material was used pretreatment and for enzymatic hydrolysis for COS and XOS production.

4.2.2 Chemical characterization of biomass and pretreated material

Biomass extractives were removed by Soxhlet, using water and ethanol for 8 h. The material was dried at 60 ºC, and about 300 mg were chemically characterized with 1.5 mL of 72% (w/w) sulfuric acid for 7 min at 45 ºC. After the reaction period, 45 mL of distilled water was added, and the mixture was autoclaved for 30 min at 121 °C, followed by vacuum filtration through a sintered glass filter. The liquid fraction was used to quantifyglucose, xylose, arabinose, and acetic acid by high performance liquid chromatography (HPLC) (Item 2.6). The acid-soluble lignin content was determined by ultraviolet-visible (UV-Vis) spectrophotometry at wavelengths of 215 and 280 nm, using 4% sulfuric acid as a reaction blank. The solid fraction was dried at 105 ºC (until constant mass) to determine the insoluble lignin content. Total lignin was obtained by adding

soluble and insoluble lignin. All tests were performed in triplicate.

4.2.3 Alkaline pretreatment

The biomass of guava seed cake and banana leaf and pseudostem were pretreated with a solution of 100 mL of potassium hydroxide 20% (w/w) and autoclaved for 30 min at 121 ºC. The solid phase was separated by filtration, washed with distilled water until pH was close to 7.0, and dried at 45°C (BRIENZO el al., 2016; FORSAN; DE FREITAS; BRIENZO, 2023). The material was stored in hermetically sealed bagsfor further characterization and enzymatic hydrolysis.

4.2.4 Enzymatic activity of endoglucanase and endoxylanase

Endoglucanase activity was measured by adding 0.1 mL of enzyme cocktail (Celluclast – Novonesis, cocktail rich in endoglucanase) to 0.9 mL of 0.44% carboxymethylcellulose (CMC) substrate in sodium acetate buffer with pH 5.2, with the reaction performed at 50 ºC. After 5 min of reaction 1.5 mL of 3,5-dinitrosalicylic acid (DNS) was added, and the solution was boiled in water for 5 min. Absorbance was measured at 540 nm using a spectrophotometer, with distilled water as the blank. For the control, 0.9 mL substrate was subjected to the same reaction conditions, with the enzyme solution added after the DNS. A glucose standard curve was constructed at concentrations of 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 $g.L^{-1}$ for the quantification of reducing sugars. The endoglucanase activity was expressed in international units (IU) per mL.

The endoxylanase was produced by *Aspergillus versicolor* growing in wheat bran, and purified in Sephadex G-75 column (DE FREITAS et al., 2021). Enzyme activity was performed according to the Bailey, Biely, Poutanen (1992), with modifications. In a test tube, Birchwood xylan (Sigma) 1% (w/v), sodium phosphate buffer pH 6, and a volume of endoxylanase between 10 and 250 µL were added, ensuring the absorbance did not exceed. The tube was heated to 55°C for 5 min and approximately 250 µL of the reaction mixture was transferred to another tube containing 250 µL of 3,5-dinitrosalicylic acid (DNS) to stop the reaction. To determine the concentration of reducing sugars, a standard curve was made using xylose, and the absorbance was measured with a spectrophotometer at 540 nm. The activity was expressed in units per volume ($U.mL^{-1}$) of enzyme.

4.2.5 COS and XOS production by enzymatic hydrolysis

About 0.1 g of the biomasses pretreated with KOH were hydrolyzed with *Apergillus*

versicolor endoxylanase with 5 mL of sodium phosphate buffer, pH 6.0, enzyme load of 30 $IU.g^{-1}$, for 24 h and stirring in a Shaker at 170 rpm at 55 ºC. The mixture was boiled for 5 min and centrifuged. The liquid fraction was removed and 5 mL of sodium acetate buffer pH 5.2 and endoglucanase 20 $IU.g^{-1}$ were added to the solid fraction. The mixture was stirred for 6 h at 170 rpm at 50 ºC, boiled and centrifuged and the liquid fractions were filtered through a 0.22 µm syringe filter, and characterized in HPLC according to item 4.2.7. The conversion of cellulose to COS and xylan to XOS was calculated (FORSAN et al., 2023).

4.2.6 Determination of monosaccharides and acetic acid

The contents of glucose, xylose, arabinose, and acetic acid from the chemical composition characterization were determined by HPLC using a Bio-Rad Aminex HPX87H (300 × 7.8 mm) column maintained at 50 °C, Waters 2414 refractive index detector at 40 ºC. The mobile phase consisted of sulfuric acid 0.005 mol L^{-1} at a flow rate of 0.6 mL min^{-1}, and sample injection volumes of 20 µL. The samples were filtered using a syringe filter with 0.22 µm pore size. The contents of glucan, xylan, arabinan, and acetyl groups were calculated by multiplying the percentages of glucose, xylose, arabinose, and acetic acid by their hydrolysis factors (0.9, 0.88, 0.88, and 0.72, respectively). The hemicellulose content was reported as the sum of xylan, arabinan, and acetyl groups.

4.2.7 Determination of COS and XOS

COS and XOS concentrations were determined by HPLC using Waters equipment, under the following conditions: Aminex HPX-87C BIO-RAD column (300 × 7.8 mm); temperature: 80 °C; eluent: ultrapure water with 0.6 mL min^{-1} flow; sample volume: 20 µL; detector: refractive index at 40 °C, analysis time: 15 min. Glucose (C1) (Sigma), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5), and cellohexaose (C6) (Megazyme) solutions were used as standards. Samples were filtered using a syringe filter with 0.22-µm pore size. Xylose (X1) (Sigma), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) (Megazyme) solutions were used as standards (FORSAN et al., 2021b; DE FREITAS; BRIENZO, 2022).

4.2.8 DPPH free-radical scavenging assay

The analysis was performed according to Lalhminghlui; Jagetia (2018), with modifications. The COS and XOS samples were divided into two groups: unfiltered and filtered with a Sep-Pak C_{18} cartridges to remove pigments and phenolic compounds, with the advantage of not retaining carbohydrates (DADIC; BELLEAU, 1980; ROUTRAY; ORSAT, 2013). Approximately 0.5 mL of glucose, xylose, cellobiose (Sigma-Aldrich), XOS and COS samples at concentrations of: 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 8 and 16 $g.L^{-1}$ (high concentrations were obtained by freeze-drying) were mixed with 1 mL of 0.1 $mmol.L^{-1}$ DPPH solution in ethanol. The mixture was incubated in the dark for 30 min, and absorbance was measured using a UV-VIS Spectrophotometer at 523 nm. To prepare the negative control, 0.5 mL of ethanol and 1 mL of DPPH solution were used. Ethanol was used as the blank (Figure 4.1). The scavenging activity was calculated using the following equation:

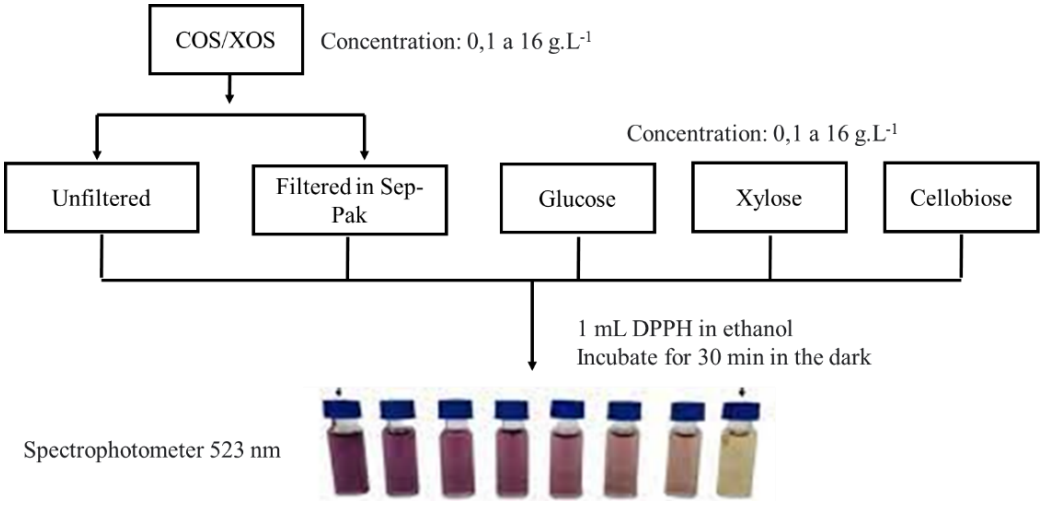
$$\text{DPPH Scavenging (\%)} = ((A_{control} - A_{sample})/A_{control})) \times 100,$$

Where:

Acontrol: absorbance of the control.

Asample: absorbance of the sample

Figure 4.1 - Flowchart of DPPH free-radical scavenging assay



4.2.9 Determination of phenolic compounds by the Folin-Ciocalteu method

To determine the content of phenolic compounds in the samples, the Folin-Ciocalteu method for microplates, with modifications was used (SINGLETON; ROSSI, 1965). A volume of 25 µL of each sample (COS and XOS) was added to each well of the microplate, followed by 200 µL of ultrapure water and 25 µL of Folin-Ciocalteu reagent (diluted 1:3). After 5 min, 25 µL of 10% w/v sodium carbonate was added, and the mixture was keptin the dark for 60 min. After this period, the plate was read using a Tecan Sunrise™ absorbance microplate reader at 760 nm. A calibration curve was prepared with gallic acid at concentrations ranging from 20 to 200 $mg \cdot L^{-1}$, and the results were expressed as µg gallic acid equivalents (GAE) per mg of XOS. Reactions were carried out in triplicate.

4.2.10 Determination of aromaticcompounds by scanning spectrophotometer

Aliquots of the COS and XOS samples were analyzed in a UV–vis scanning spectrophotometer (Global Analyzer, software UV/VIS Analyst) covering the ultraviolet (200–380 nm) and visible (380–800 nm) ranges, with 5 nm steps. For comparison with the samples, the following solutions were also analyzed: 1% (w/v) cellobiose, sodium acetate buffer with Celluclast enzyme, sodium phosphate buffer with endoxylanase from *Aspergillus versicolor* lignin soluble in sulfuric acid (obtained from chemical characterization), extractives removed from banana pseudostem biomass, and 1% (w/v) gallic acid.

4.2.11 Statistical Analysis

Statistical analyzes were performed in the Design expert 6.0 software using the Tukey test to compare the averages obtained in the quantification of total phenolic compounds (TPC) in COS and XOS solutions. Statistical analysis considered the confidence level of 95%.

4.3 Results and discussion

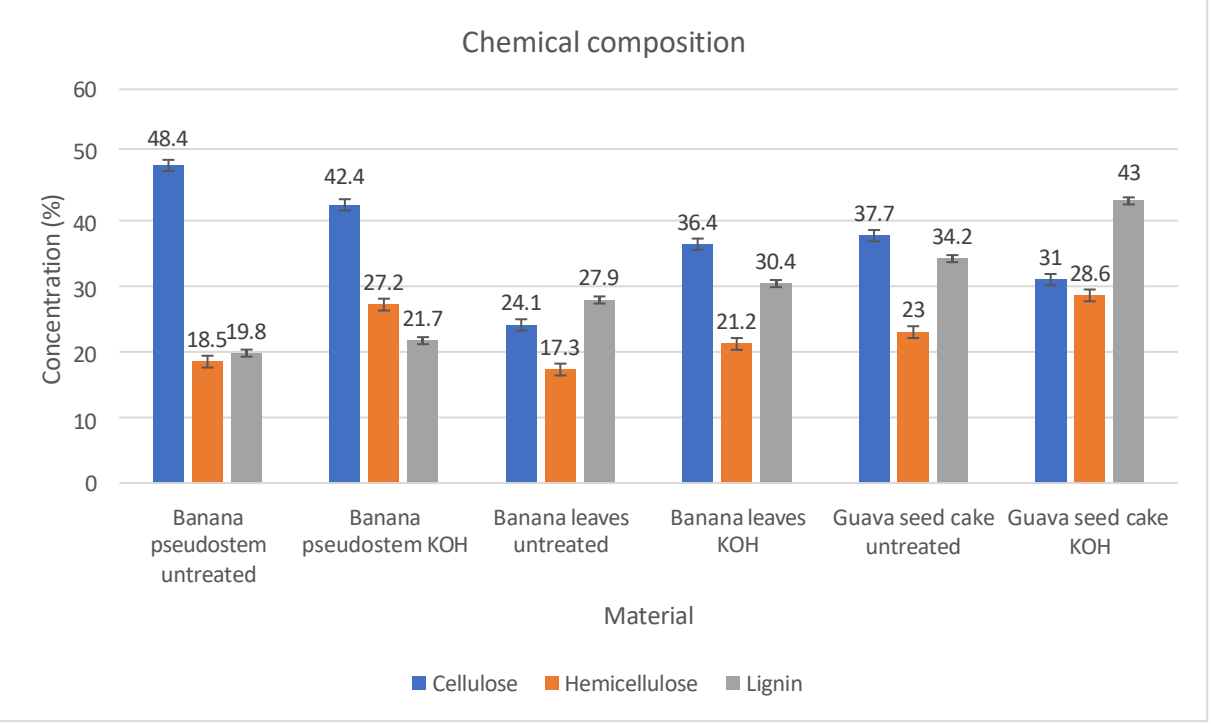
4.3.1 Chemical composition

The chemical composition of untreated and pretreated materials is reported in figure 4.2. The biomass that presented the highest concentration of cellulose was the banana pseudostem

(48.41%), followed by Guava seed cake (37.74%) and banana leaves (24.09). The chemical characterization of banana pseudostem and banana leaves from the works of Freitas; Brienzo, 2022 and Richard, 2024, the values were around of 41.9% and 28.71% for cellulose, 14.8% and 26.24% for hemicellulose and 14.02% and 19.79% for lignin. In comparison to the present study, cellulose values were similar, lignin values were lower and hemicellulose values varied.

It was possible to observe that the percentage of cellulose increased around of 12% only when the banana leaf was pretreated with KOH while the percentage of hemicellulose and lignin increased when all biomasses were pretreated with KOH. In alkaline pretreatment, swelling and reduction in the degree of cellulose polymerization occur, removal of acetyl groups and uronic acids from hemicellulose, breaking the bond between lignin and carbohydrates (improving carbohydrate reactivity), and decomposition of lignin into low molecular weight compounds (SINGH et al., 2022).

Figure 4.2 - Chemical composition of banana pseudostem and leaves and guava seed cake untreated and pretreated

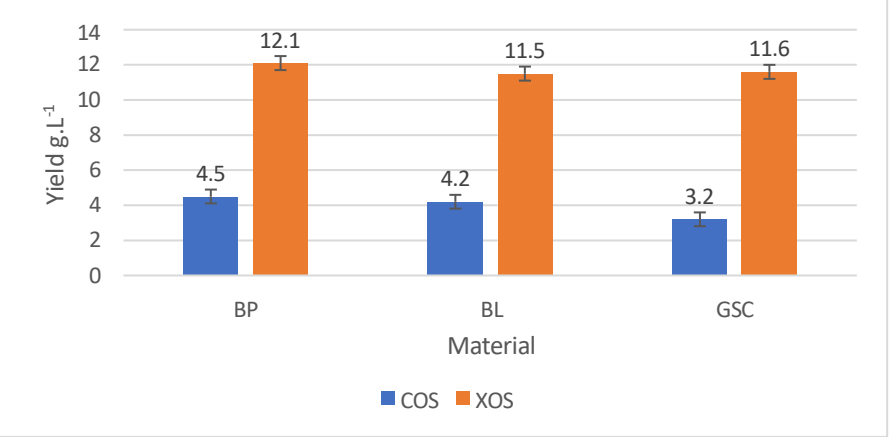


Source: Author

4.3.2 XOS and COS yield.

The XOS yield was 12.1, 11.5 and 11.6 $g.L^{-1}$ for banana pseudostem, banana leaves and guava seed cake, respectively, while the COS yield was 4.5, 4.2 and 3.2 $g.L^{-1}$ for banana pseudostem, banana leaves and guava seed cake, respectively (Figure 4.3).

Figure 4.3 - Yield of COS and XOS after enzymatic hydrolysis with xylanase and endoglucanase of the pretreated banana pseudostem and leaf and guava seed cake bagasse. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

During alkaline pretreatment, polysaccharides are not degraded into furfural and hydroxymethylfurfural, however, lignin residues can remain linked to xylan, harming the enzyme activity (UÇKUN KIRAN; AKPINAR; BAKIR, 2013). Hydrolysis was carried with xylanase before endoglucanase to reduce the recalcitrance of cellulose and make it more accessible to the enzyme's action.

When XOS were produced with rice bran pretreated with NaOH 1% for 1 h at 120 °C and hydrolyzed with xylanase from *Orpinomyces* sp. at 50 °C for 96 h, it was possible to obtain a concentration of 2.2 $g.L^{-1}$, approximately 5 times smaller than the present study (FERNANDES et al., 2024).

Another study produced XOS using xylan extracted from sugarcane bagasse with potassium hydroxide 12 % w/v, at 121 ºC for 45 min and hydrolyzed with endo-1,4-xylanase cocktail 40 $U.g^{-1}$ of xylan, for 24 h (Novonesis). The concentration obtained was approximately 3.5 $g.L^{-1}$, about 3 times lower compared to the present study (PASCHOA et al., 2024).

In the production of XOS using banana pseudostem delignified with 20% hydrogen peroxide (w/w), ground in a ball mill for 30 min, and hydrolyzed with endoxylanase from *Aspergillus versicolor* 50 $IU.g^{-1}$ for 24 h, It was possible to obtain a concentration equivalent to 2.85 $g.L^{-1}$. In this study, COS was also produced, with the same raw material, under the same delignification conditions. The material was hydrolyzed with Celluclast endoglucanase (Novonesis) 50 $IU.g^{-1}$ for 24 h and the concentration obtained was 1.26 $g.L^{-1}$ (FREITAS; BRIENZO, 2022). In the present study, the yield of XOS and COS was approximately 4 and 3.5 times higher than the previously mentioned study. Probably the use of potassium hydroxide in the

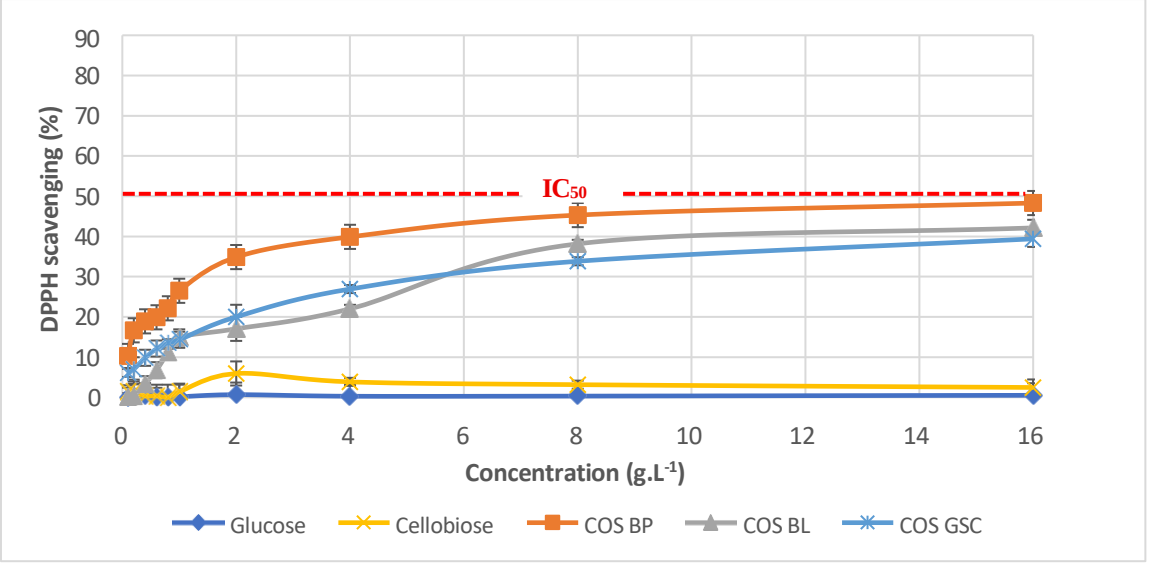
pretreatment of banana pseudostem was more effective in relation to hydrogen peroxide, to make the material more accessible to the action of enzymes. In the case of COS, the addition of endoxylanase before endoglucanase was positive in reducing cellulose recalcitrance.

4.3.3 DPPH scavenging assay

The antioxidant capacity was evaluated by the IC_{50}, which is the concentration of the sample capable of eliminating 50% of the initial DPPH radical. Unfiltered COS and XOS exhibited antioxidant activity, unlike cellobiose and its monomers glucose and xylose (Figure 4.4 and 4.5) and COS and XOS filtered in Sep-Pak cartridges (Figure 4.6),

In figure 4.3 it is possible to observe the values of the reduction of the DPPH radical in percentage, with the use of commercial glucose (Synth), the maximum value was approximately 0.7%, with 2 $g.L^{-1}$. For cellobiose the maximum value was 5.9% with 2 $g.L^{-1}$. Regarding unfiltered COS, it was not possible to find the IC_{50} even with high concentrations (16 $g.L^{-1}$). The highest values of DPPH radical scavenging were 48.3%, 42.1% and 39.4% for COS from banana pseudostem, banana leaves and guava seed cake, respectively.

Figure 4.4 - DPPH radical scavenging activity of the glucose, cellobiose and unfiltered cellooligosaccharides from banana pseudostem and leaves and guava seed cake. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake, IC_{50}: inhibitory concentration

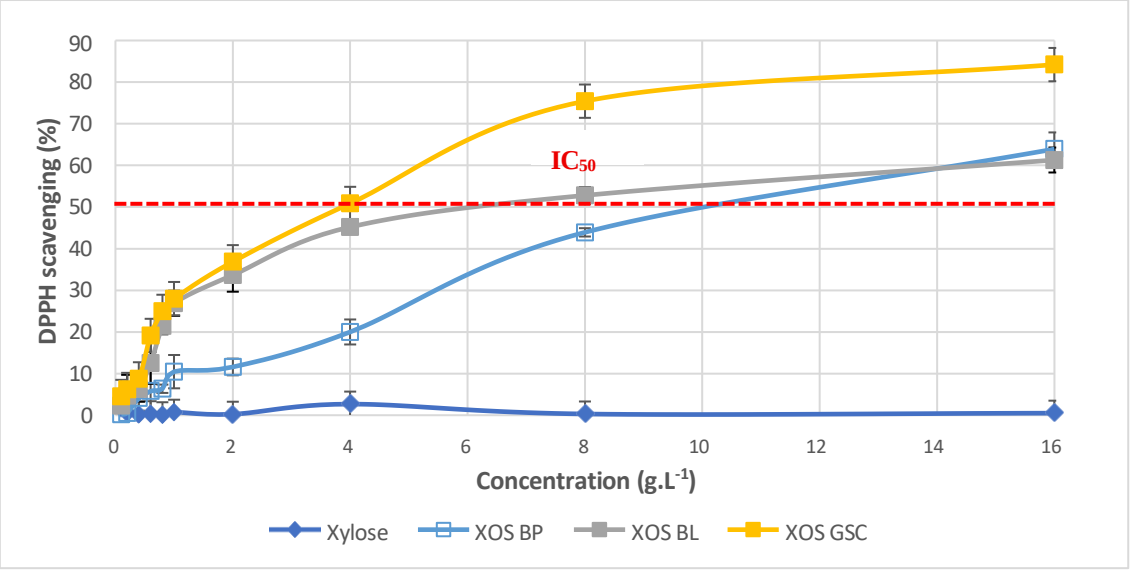


Source: Author

The maximum reduction of the DPPH radical with xylose solution (company) was approximately 2.7%, with 4 $g.L^{-1}$. It was possible to obtain the IC_{50} with all XOS unfiltered, with

concentrations of approximately 8 $g.L^{-1}$ and 4 $g.L^{-1}$ for banana pseudostem and guava seed cake, respectively (Figure 4.5).

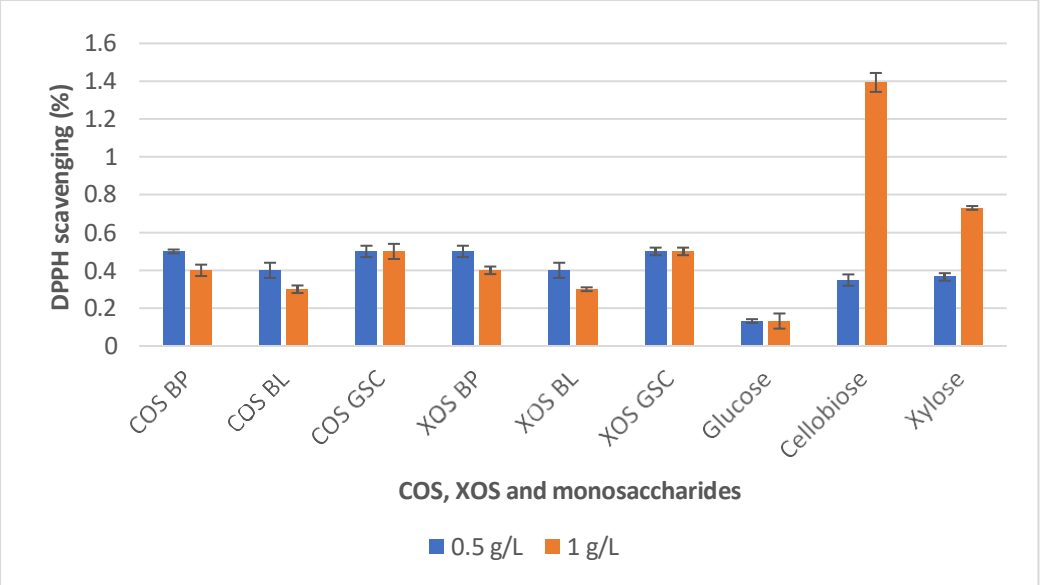
Figure 4.5 - DPPH radical scavenging activity of the xylose and unfiltered xylooligosaccharides from banana leaves and pseudostem and guava seed cake**.** BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake, IC_{50}: inhibitory concentration



Source: Author

In the present study, no antioxidant activity was observed with industrial-grade cellobiose with a high degree of purity (98%) (Sigma-Aldrich) (Figure 4.4). This data, compared to the literature, indicates that purified oligosaccharides do not have antioxidant activity, except when they are in a solution containing other compounds linked to their structure or in the mixture obtained from hydrolysis (VICTORIA GAUTÉRIO et al., 2022). The source of the XOS solution could be responsible for components that act as antioxidants. To confirm this hypothesis, it was carried out an antioxidant analysis of COS and XOS solutions filtered through a Sep-Pak cartridges to remove these compounds, at concentrations of 0.5 and 1 $g.L^{-1}$. More concentrated solutions were not made because the DPPH reduction was very low. The filtration with this C_{18} resin filter can remove phenolic compounds, aromatic molecules, lignin derivatives, and degradation products from carbohydrates. As result, a minimal reduction in the DPPH radical was observed (Figure 4.6), confirming that the components responsible for antioxidant activities were removed from the COS and XOS solution. The DPPH scavenging with filtered COS and XOS solution did not exceed 0.5% in both cases, against 48.3% and 84.2%, with não filtered solution of COS from banana pseudostem and XOS from guava seed cake, respectively. This reduction in the scavenging property represents 83.7% less.

Figure 4.6 - DPPH radical scavenging activity of the glucose, cellobiose, xylose, cellooligosaccharides and xylooligosaccharides filtered in Sep-Pak cartridgesfrom banana pseudostem and leaves and guava seed cake.BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

There are few studies on the antioxidant activity of COS, unlike XOS. This lack of data is possibly due to the difficulty in producing COS. Through this study it was intend to obtain data that will contribute to future research. The influence of XOS chemical structure on antioxidant properties remains unclear.

In the study by Fuso et al., 2023, the antioxidant activity of XOS and xylose against the free radical DPPH was tested. Similarly to the present study, pure commercial xylose did not show antioxidant activity. Industrialized XOS with degree polimerization between 2 and 6 (Megazyme and Fluka Chemicals), with purity between 90 and 95%, either showed no reduction or only minimal reduction of the DPPH radical. However, XOS obtained through enzymatic hydrolysis of xylan demonstrated high activity with a degree of polymerization between 6 and 9 xylose units, with IC_{50} equal to 0,06 $mg.mL^{-1}$. With degree of polymerization between 2 and 6 they showed low activity, not exceeding 38% reduction of the DPPH radical.

Huang et al. (2019) evaluated the DPPH scavenging activity with purified XOS and soluble lignin (SL) obtained from *Moso* bamboo. The increase in activity was directly proportional to the concentration and reached 85.7% and 80.7% with a concentration of 2.5 $g.L^{-1}$ and 1.2 $g.L^{-1}$ for XOS and SL, respectively. Above these values the activity was stable. IC_{50} values were 0.46 $g.L^{-1}$ and 1.1 $g.L^{-1}$ for SL and XOS, respectively. These results indicate that SL performs better in

scavenging free radicals than XOS, due to the presence of phenolic and aliphatic hydroxyl groups. With XOS extracted from corn cob with concentrations ranging from 0.2 to 3 $mg.mL^{-1}$, the percentage of DPPH radical scavenging ranged from 9.7 to 74.2%. The IC_{50} was 1 $mg.mL^{-1}$ (GOWDHAMAN; PONNUSAMI, 2015). The IC_{50} values obtained by Huang et al., 2019 and Gowdhaman; Ponnusami, 2015 were similar and superior to the present study, which had the highest IC_{50} equivalent to 4 $g.L^{-1}$ with guava seed cake.

XOS from sugarcane straw and xylan from coffee husks, at a concentration of 2 $g.L^{-1}$, were able to reduce the DPPH radical by 71 and 78%, respectively. (ÁVILA et al., 2020). With XOS from sugarcane bagasse at the same concentration, the reduction of the DPPH radical reached 84.5%, and the IC_{50} was reached with a concentration of 0.62 $g.L^{-1}$ and with XOS from millet (ragi) the reduction was 70% at a concentration of 60 $\mu g.mL^{-1}$ (BIAN et al., 2013; VEENASHRI; MURALIKRISHNA, 2011). In the present study, it was possible to obtain a reduction in the DPPH radical of 84.2% with XOS from guava seed cake, but with a concentration of 16 $g.L^{-1}$.

The antioxidant activity of oligosaccharides was probably due to the release of soluble or insoluble phenolic compounds, like ferulic, vanillic, gallic, acid, ρ-hydroxybenzoic, syringic, ρ-coumaric, uronic and methyl glucuronic acid, as well as the content of reducing sugars, which can donateelectrons to free radicals. Additionally, the antioxidant activity of oligosaccharides can be enhanced by modifying their structure through acetylation and carboxymethylation reactions. (VICTORIA GAUTÉRIO et al., 2022; GOWDHAMAN; PONNUSAMI, 2015; VALLS et al., 2018; ÁVILA et al., 2020).

4.3.4 Antioxidant compounds identification

The Folin-Ciocalteu method was used to determine the presence of total phenolic compounds in the COS and XOS solution, possibly contributing for the reduction of the DPPH radical. In the XOS solution, a higher concentration of total phenolic compounds (TPC) was found in relation to the COS solutions (Table 4.1). XOS from guava seed cake showed the highest reduction in the DPPH radical and the mixture with the highest amount of TPC, followed by the XOS from banana leaves and banana pseudostem, with the pseudostem showing the highest TPC value among these two. Regarding COS, although the IC_{50} was not reached in any case, it was possible to observe a greater reduction in the DPPH radical obtained with COS from pseudostem, leaf and guava, respectively. The TPC value was similar for banana biomass and lower for guava (Table 4.1).

Table 4.1 - Total phenolic compounds (TPC) in the cellooligosaccharides (COS) and xylooligosaccharides (XOS) solutions and DPPH IC_{50} values. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake

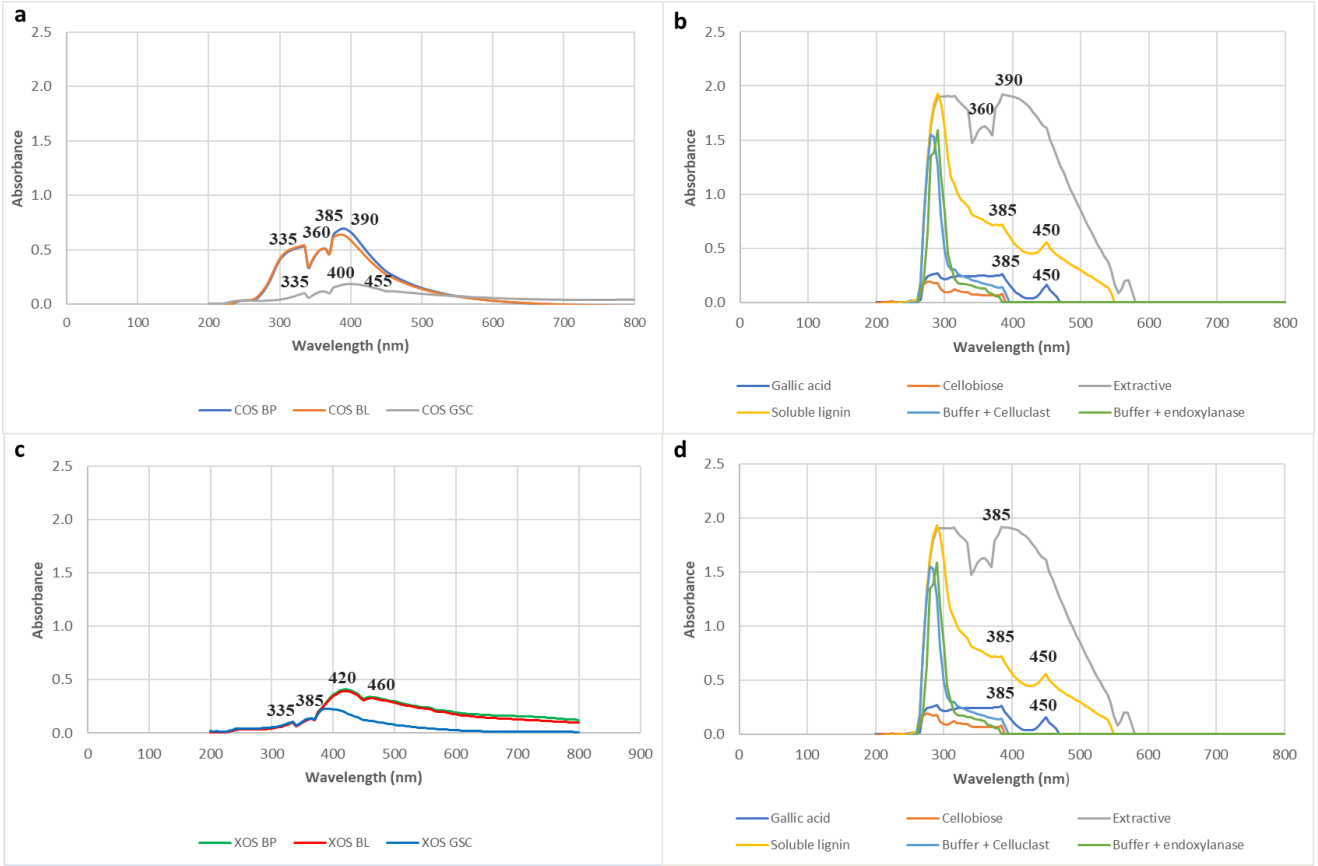
Material	*TPC (µg GAE/mg COS or XOS)*	*DPPH IC_{50} Values ($g.L^{-1}$)*
COS BP	$3.47^{a} \pm 0.21$	-
COS BL	$3.70^{b} \pm 0.39$	-
COS GSC	$2.61^{c} \pm 0.27$	-
XOS BP	$4.97^{d} \pm 0.48$	8
XOS BL	$3.86^{e} \pm 0.14$	6
XOS GSC	$5.55^{f} \pm 0.58$	4

Source: Author

(-): did not reach IC_{50}. Experiments performed in triplicate. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake, GAE: gallic acid equivalentes, DPPH: 2,2-diphenyl-1-picrylhydrazyl.

A UV-vis spectroscopic analysis was performed with COS and XOS solution. The profiles of the COS and XOS that before 335 nm and from 460 nm, no spectral signals were found (Figure 4.7, Table 4.2).

Figure 4.7 - UV-vis spectroscopic profiles (200-800 nm) of (a) cellooligosaccharides, (b) solutions for comparison, (c) xylooligosaccharides and (d) solutions for comparison. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake



Source: Author

Scanning spectrophotometry was carried out on compounds present in the biomass and which may have been transferred to the COS and XOS solutions during the production stages (since no data was found in the literature for comparison). This includes soluble lignin (extracted from banana pseudostem), extractives, and carboxylic acids (represented by gallic acid), which may contribute to antioxidant activity. Additionally, sodium acetate buffer solutions with the Celluclast enzyme and sodium phosphate buffer with *Aspergillus versicolor* endoxylanase, which were used in the enzymatic hydrolysis step, and cellobiose solution, an oligosaccharide formed in greater quantities, were also analyzed (Table 4.2). The analysis revealed similar spectral features in samples of soluble lignin, gallic acid and extractives solution, possibly due to the presence of carboxylic acids in all solutions.

In the COS solutions from pseudostem and banana leaf, and the XOS solutions from guava seed cake there is probably the presence of extractives (360 and 385 nm) and lignin (385 nm) or acid groups (represented for gallic acid in the assay) (385 nm). In the solution of COS from guava seed cake and XOS from banana pseudostem there may be the presence of lignin (450 nm) or acid groups (gallic acid) (450 nm). It was not possible to identify/suggest the compounds present in the banana leaf XOS solution using this method (Table 4.2).

Table 4.2 - Spectral signals were found in samples. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake

Samples	Spectral signals (nm)
COS BP	335, 360, 390
COS BL	335, 360, 385
COS GSC	335, 400, 455
XOS BP	335, 420, 455
XOS BL	335, 420
XOS GSC	335, 385
Soluble lignin from banana pseudostem	290, 385, 450, 570, 605
Extractives	315, 360, 385, 570
Galic acid	290, 385, 450
Sodium acetate buffer + Celluclast	280
Sodium phosphate buffer + *Aspergillus versicolor* endoxylanase	270
Cellobiose	290, 315

Source: Author

4.4 Conclusion

COS and XOS solutions produced by enzymatic hydrolysis of the banana pseudostem and leaf and guava seed cake and purified have a low capacity to reduce the DPPH radical. Unfiltered XOS solution has presented greater antioxidant activity than COS, it was possible to obtain IC_{50}

values ranging between 4 and 8 $g.L^{-1}$. When the analysis was carried out with COS and XOS solutions filtered with a Sep-Pak cartridgesthere was no antioxidant activity. Using the Folin-Ciocalteau and scanning spectrophotometry methods, it was possible to suggest the presence of soluble lignin, extractives and carboxylic acids in unfiltered samples of COS and XOS, possibly responsible for the antioxidant activity. The solutions used as standard in scanning spectrophotometry were produced due to the lack of data for comparison in the literature, and may assist future work. It is possible to conclude that the production method with endoxylanase and endoglucanase was advantageous for producing COS and XOS simultaneously, and the fact that there is antioxidant activity only with unpurified solutions, reduces the need for complex and expensive processes to obtain oligosaccharides with high-purity.

CHAPTER V: POTENTIAL PREBIOTIC ACTIVITY OF CELLOOLIGOSACHARIDES AND XYLOOLIGOSACCHARIDES FROM BANANA AND GUAVA WASTE IN *LACTIPLANTIBACILLUS PLANTARUM* AND *BIFIDOBACTERIUM BREVE*

Abstract

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) have presented the potential to promote the growth of probiotics and prevent the proliferation of some pathogenic bacteria. In this study, COS and XOS were produced with banana pseudostem and leaf and guava seed cake pretreated with potassium hydroxide, and hydrolyzed with cellulases (Celluclast) and endoxylanase from *Aspergillus versicolor*. COS and XOS (isolated or mixed) and commercial xylose and cellobiose were used as carbon sources for the growth of *Lactiplantibacillus plantarum* 1462 and *Bifidobacterium breve* BB-03. Microbial growth, carbon source consumption and short-chain fatty acids were mensured. There was a higher release of cellobiose and xylobiose in the hydrolysis of banana pseudostem, corresponding to 4.5 $g.L^{-1}$ and 12.06 $g.L^{-1}$, respectively. There was formation of cellotriose only in the enzymatic hydrolysis of guava seed cake. Both microorganisms were able to grow efficiently in the presence of isolated or mixed COS and XOS, without carbon source preference, athougth, in some cases the growth has presented similar or higher than that obtained with glucose (control). This enables the simultaneous production of oligosaccharides, without the need to separate the compounds, for use as a prebiotic. It was possible to conclude that *L. plantarum* and *B. breve* are capable of fermenting COS and XOS and producing lactic acid and short-chain fatty acids, mainly acetic acid, responsible for the beneficial effects on the host's health.

Keywords: carbon source; oligosaccharides; probiotics; short-chain fatty acids; banana pseudostem; guva seed cake.

5.1 Introduction

Prebiotics are non-digestible food ingredients that prevent the growth of some pathogenic bacteria and serve as a substrate to increase and sustain probiotics, that is, the beneficial microbiota in the intestine of humans and animals, mainly composed of bacteria such as *Lactobacillus, Bacillus* and *Bifidobacterium*, and yeasts of the *Saccharomyces* genus (BAMIGBADE et al., 2022; DAHIYA; NIGAM, 2022). Intestine microorganisms can assist in the general health of the host,

metabolizing food components and providing vitamins, amino acids, minerals such as calcium and other substances of interest and exerting influence on brain processes. However, when an imbalance occurs in the microbiota due to factors such as diet, environment and health status of the host, the number of pathogenic bacteria increases, and causes a decrease in the absorption of nutrients, and production of substances such as cadaverine, histamine, putrescine, ammonia and gases that harm the intestinal mucosa (TOFALO; COCCHI; SUZZI, 2019; SILVA; BERNARDI; FROZZA, 2020).

Prebiotics must be resistant to gastrointestinal absorption, gastric acid and digestive enzymes. It is necessary for significant quantities to be available, especially in the large intestine, to serve as a substrate for intestinal microbiota (KARNAOURI et al., 2019). Several compounds act as prebiotics such as inulin, lactitol, lactulose, polydextrose, fructooligosaccharides (FOS), galactooligosaccharides (GOS), mannanoligosaccharides (MOS), trans-galacto-oligosaccharides (TOS), isomaltooligosaccharides (IMOS), xylooligosaccharides (XOS), cellooligosaccharides (COS), among others (MEGUR et al., 2022; DAVANI-DAVARI et al., 2019). Prebiotics can be obtained through the ingestion of fruits, vegetables, roots, tubers, whole grains, seeds and nuts. However, it is not always possible to obtain sufficient amounts from a natural diet alone, making it interesting to use supplements obtained industrially (FERREIRA et al., 2023; DAHIYA; NIGAM, 2022).

COS are made up of 2 to 6 glucose units linked by glycosidic bonds, which can be produced through chemical and enzymatic processes from materials such as lignocellulosic biomass. In the last years, studies on COS production have been increasingly in focus evidence, due to the abundance of cellulose in plants waste, which makes large-scale production possible but material recalcitrance is still a challenge (CHEN; SHROTRI; FUKUOKA, 2021). Studies suggest that COS stimulate the growth of probiotics such as *Clostridium butyricum, Lactococcus lactis, Lactobacillus rhamnosus, Lactiplantibcaillus plantarum, Lactobacillus gasseri,* and *Lactobacillus paracasei* (FORSAN; DE FREITAS; BRIENZO, 2023). XOS are compounds formed by 2 to 10 xylose units, and present the potential to stimulate the growth of *Bifidobacterium* spp. and several species of *Lactobacillus*, such as *Lactiplantibacillus plantarum*, *Levilactobacillus brevi*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Limosilactobacillus fermentum, Lactococcus lactis*, *Lacticaseibacillus rhamnosus,* among others (FORSAN et al., 2021b; DAHIYA; NIGAM, 2022; DE FREITAS; CARMONA; BRIENZO, 2019).

Probiotic microoganisms present in the colon and cecum are capable of fermenting soluble fibers, indigestible polysaccharides and proteins under anaerobic conditions, which are not degraded by digestive enzymes in the upper part of the intestine, generating short-chain fatty acids

(SCFAs) as metabolites. These compounds act in the gastrointestinal tract and distant organs, due to their ability to spread into the blood circulation, combating diseases such as type 2 diabetes, obesity, atherosclerosis, arterial stiffness, improving cholesterol metabolism, regulating blood pressure, the immune system and the anti-inflammatory response (NOGAL et al., 2021; DAVANI-DAVARI et al., 2019; THIRUVENGADAM et al., 2023; ZHANG, D. et al., 2023; HOU et al., 2022).

Due to the enormous importance of prebiotics for human and animal health, the economic interest that the global prebiotics market has generated, with the expectation of reaching 7.2 billion dollars in 2024 (CARDOSO et al., 2021). The possibility of reusing fruticulture bioproducts, and the difficulty in obtaining high concentrations of these compounds, research is necessary for large-scale production efficiently. In the present study, banana and guava residues were chosen due to their abundance in Brazil. The present study aimed to evaluate the prebiotic potential of COS and XOS to be provided as carbon source of growth of probiotics *Lactiplantibacillus plantarum* and *Bifidobacterium breve*, naturally found in the intestinal microbiota, monitoring the carbon source consumption, microorganism growth, and short-chain fatty acids production.

5.2 Materials and methods

5.2.1 Sample preparation

The guava was processed by Predilecta (Matão, SP), and the seeds were oils extracted by Condire Company (Dois Corregos, SP), generating the guava seed cake. The banana leaf and pseudostem were obtained from a local plantation, Rio Claro, SP, Brazil. The banana waste was sunlight dried, cut into small pieces, oven-dried at 60 °C, until approximately 10% of moisture (FORSAN; DE FREITAS; BRIENZO, 2023). Both guava seed cake and banana waste were ground in a knife mill with a 20-mesh sieve (0.8 mm) (FERNANDES et al., 2020).

5.2.2 Cellooligosaccharides and Xylooligosaccharides production

Banana pseudostem and leaf and guava seed cake were pretreated with 20% (w/w) potassium hydroxide. About 5 g of each biomass, milled in a knife mill with a 20-mesh sieve (0.8 mm) was treated with 100 mL of potassium hydroxide 20% (w/w), in a Schott flask at 121 °C for 30 min in autoclave (Stermax autoclave). Then the solid fraction was separated from the liquid

fraction by vacuum filtration and washed with water until neutral pH, dried in an oven at 45 °C, ground in a ball mill for 30 min.

The pretreated solids were subjected to hydrolysis with endoxylanase from *Aspergillus versicolor*, with initial activity of the 414.4 $IU.mL^{-1}$, enzyme load of 30 $IU.g^{-1}$ in 5 mL of 50 $mol.L^{-1}$ sodium phosphate buffer, substrate concentration of 2%, pH 6, 24 h reaction time at 55 ºC, with the agitation of 170 rpm. The tubes were boiled for 5 min and centrifuged (Novatecnica, NT815), at 5000 rpm for 20 min. The liquid fraction was removed, filtered with a 0.22 µm syringe filter, and characterized for xylose, and XOS concentrations by HPLC according to item 5.2.4. The solid fraction was hydrolyzed with endoglucanase (Celluclast cocktail from Novonesis, with initial activity of the 2910 $IU.mL^{-1}$) for COS production. About 5 mL acetate buffer pH 5.2, enzyme load 20 $IU.g^{-1}$ was added to the reaction medium.

The mixture was stirred at 170 rpm for 6 h at 50 °C. The tubes were boiled for 5 min and centrifuged (Novatecnica, NT815), at 5000 rpm for 20 min The liquid fraction was removed, filtered with a 0.22 µm syringe filter, and characterized for glucose and COS concentrations by HPLC according to item 5.2.4.

5.2.3 Chemical characterization of waste biomass and pretreated material

Biomass extractives were removed in Soxhlet and about 300 mg of each biomass and pretreated material were mixed with 1.5 mL of 72% (w/w) sulfuric acid and heated at 45 °C for 7 min. 45 mL of distilled water was added and the mixture was heated to 121 ºC for 30 min in autoclave (Stermax autoclave). The mixture was filtered and the liquid fraction was used to determine the acid-soluble lignin content in UV–Vis spectrophotometry at a wavelength of 215 and 280 nm and for quantification of monosaccharides by high-performance liquid chromatography (HPLC), according to item 5.2.4. The solid fraction was used to determine insoluble lignin.

5.2.4 Monosaccharides and oligosaccharides quantification

The chemical characterization liquid samples were analyzed by HPLC, Bio-Rad Aminex HPX87H (300×7.8 mm) column, temperature of 50 ºC, with detector Waters 2414 Refractive Index, 20 µL of sample and sulfuric acid 0.005 $mol.L^{-1}$ as mobile phase with flow of 0.6 $mL.min^{-1}$. The percentages of glucose, xylose, arabinose, and acetic acid were multiplied by 0.9, 0.88, 0.88,

and 0.72, their hydrolysis factors, respectively, to determine the percentages of glucan, xylan, arabinosyl, and acetyl.

COS and XOS were quantified by high performance liquid chromatography (HPLC) using Waters equipment, Aminex HPX-87C BIO-RAD column (300×7.8 mm), temperature of 80 °C; analysis time of 15 min, 20 µL of sample, ultrapure water as eluent with 0.6 $mL.min^{-1}$ flow, detector of refractive index at 40 °C. As standard, solutions of glucose (C1) (Sigma), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5), cellohexaose (C6), xylose (X1) (Sigma), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) (Megazyme).

5.2.5 Bacterial strains

Lactiplantibacillus plantarum 1462 was obtained from the Brazilian collection of environmental and industrial microorganisms (CBMAI), State University of Campinas (UNICAMP), Brazil. *Bifidobacterium breve* BB-03 was obtained from Laboratory of Industrial Biotechnology at São Paulo State University (UNESP), located in Assis, São Paulo, Brazil.

These bacterial strains were chosen due to their probiotic properties. *Bifidobacterium breve* and *Lactiplantibacillus plantarum* were cultivated in MRS medium (de Man, Rogosa, and Sharpe) for 48 h. For *Bifidobacteria* a 0.05% (w/v) cysteine solution filtered in a syringe filter 0.22 µm was introduced into the medium and the culture was incubated at 37 ºC, without shaking. *L. plantarum* were incubated at 30 ºC with shaking. Cell concentration was measured on a flow cytometer (Thermo Fisher), and dilution was carried out with distilled water until the inoculum contained 10^5 cells.

5.2.6 Preparation of culture media and evaluation of the prebiotic activity of COS and XOS and the mixture of both

Modified MRS culture medium was prepared (DE MAN; ROGOSA; SHARPE, 1960) with 25% of proteins and 50% of mineral salts, autoclaved at 121 ºC, for 20 min. For *Bifidobacteria* cultures, a cysteine solution 0.05% (w/v) filtered in a syringe filter 0.22 µm was introduced into the medium. 5 µL of the inoculum of probiotic bacteria *L. plantarum* and *B. breve*, containing 10^5 cells, was added. The carbon sources was each one incorporated isolated into the medium to evaluate prebiotic activity were: glucose (Synth), xylose 99% purity (Êxodo científica),

cellooligosaccharides (COS), xylooligosaccharides (XOS) or cellobiose 98% purity (Sigma-Aldrich), concentration of 10 and 20 $g.L^{-1}$ (concentration obtained by freeze-drying process) and media containing a mixture of COS 20 $g.L^{-1}$ and XOS 20 $g.L^{-1}$ were also prepared, varying the COS/XOS ratio by 80/20%, 60/40%, 40/60% and 20/80%. The culture media with the strains were incubated at 37 ºC for 72 h in a 96-well plate without agitation for *Bifidobacteria* and with agitation for *Lactobacilli*. Tests were performed in quadruplicate. The growth of microorganisms was monitored by optical density (OD) at a wavelength of 630 nm every 15 min, using a Tecan Sunrise™ absorbance microplate reader.

Carbohydrate-free MRS culture medium was used to confirm that growth was not due to other compounds present.

5.2.7 Quantification of lactic acid, short-chain fatty acids and sugar consumption

Lactic acid and short-chain fatty acids (acetic, butyric, propionic, formic acid), generated in the fermentation of carbon sources by *Bifidobacterium breve* and *Lactiplantibacillus plantarum* and sugar consumption were analyzed by high-performance liquid chromatography (HPLC) with column BIO-RAD Aminex HPX-87C, sulfuric acid 0.005 M as mobile phase with flow of 0.6 $mL.min^{-1}$. All assays were performed in replicates.

5.2.8 Statistical Analysis

The results are reported as Mean ± Standard error.

Statistical analysis was performed using the software Design Expert 6.0. Statistical analyses were performed using the Tukey test to compare means obtained in the conversion of cellulose into COS and xylan into XOS and at the maximum OD reached by microorganisms in the fermentation of carbon sources. Statistical analysis considered the confidence level of 95%.

R studio software version 4.3.1 was used to examine T_mid of microbial growth curves.

5.3 Results and discussion

5.3.1 Chemical characterization of biomass and pretreated material

Cellulose, hemicellulose and lignin values found for banana pseudostem in the present study were, respectively, 48.41%, 14.66% and 19.84%. In another study the values found were:

cellulose (30.08%), hemicellulose (27.79%), and lignin (6.08%) (LI et al., 2016). The chemical composition of banana leaves was 24.09% (cellulose), 17.31% (hemicellulose) and 27.9% (lignin). In the study of Zaini et al., 2023 the values found for banana leaves were: cellulose 43.34%, hemicellulose 34.34%, lignin 15%. For guava seed cake, in the present study, the values of cellulose, hemicellulose and lignin were respectively, 37.74%, 23% and 34.15%. In another study the values for guava seed cake were 22.82% (cellulose), 17.49% (hemicellulose), and 41.66% (lignin). The chemical composition of biomass can vary greatly, depen ding on the species, climate, part of the plant analyzed, planting and harvesting method, among others (VIEIRA; GONÇALVES; BRIENZO, 2024) (Table 5.1).

When the biomasses were pretreated with KOH there was an increase in the percentage of cellulose only with banana leaves. The percentage of xylan, arabinosyl and lignin increased with all biomasses (Table 5.1). This result was different from the literature, since alkaline pretreatments generally remove lignin. This chemical treatment has the advantage of not degrading carbohydrates, increasing the porosity of the material and the contact surface, aiding in subsequent enzymatic hydrolysis processes (KIM; LEE; KIM, 2016).

Table 5.1 - Chemical composition of untreated and pretreated material with potassium hydroxide (KOH).

Samples	**Pretreatment**	**Chemical composition (%, dry base)**				
		Glucan	Hemicellulose			Lignin (soluble + insoluble)
			Xylan	Arabinosyl	Acetyl	
Banana pseudostem	Untreated	48.41 ± 0.58	10.48 ± 0.16	3.80 ± 0.11	0.38 ± 0.42	19.84 ± 1.74
	KOH	42.35 ± 2.17	21.46 ± 1.11	5.71 ± 0.92	0	21.72 ± 0.73
Banana Leaves	Untreated	24.09 ± 1.44	10.62 ± 0.59	6.32 ± 0.62	0.37 ± 0.27	27.90 ± 2.43
	KOH	36.39 ± 0.62	13.94 ± 0.28	7.29 ± 0.58	0	30.42 ± 1.53
Guava seed cake	Untreated	37.74 ± 0.06	19.09 ± 0.93	1.89 ± 0.72	2.02 ± 1.53	34.15 ± 1.81
	KOH	31.04 ± 1.97	23.90 ± 1.36	4.43 ± 0.59	0.23 ± 0.03	42.96 ± 0.26

Source: Author
*tests performed in triplicate

5.3.2 Cellooligosaccharides and Xylooligosaccharides production

COS and XOS were produced with a combination of cellulase and endoxylanase, applied successively to the same solid/material. The table 5.2 shows the concentration of XOS and COS produced after enzymatic hydrolysis with endoxylanase of *Aspergillus versicolor* 30 $UI.g^{-1}$ for 24

h followed by hydrolysis with endoglucanase Celluclast 20 UI.g^{-1} for 6 h. There was production mainly of xylobiose and cellobiose, except in the hydrolysis of guava seed cake, where cellotriose was also formed. The highest concentration of cellobiose and xylobiose was obtained in the hydrolysis of banana pseudostem pretreated with KOH, corresponding to 4.5 g.L^{-1} and 12.06 g.L^{-1}, respectively. In the study of Liu; Hu; Haritos, 2024, it was possible to obtain a concentration of COS equivalent to 2.07 g.L^{-1} from grape marc. Regarding XOS production, concentrations of 17.26 g.L^{-1} of grass silage and 3.06 g.L^{-1} of whole rye silage (QUIÑONES et al., 2015) and 8.1 g.L^{-1} of XOS from beechwood xylan were obtained (VALLADARES-DIESTRA; PORTO DE SOUZA VANDENBERGHE; SOCCOL, 2022).

Table 5.2 - Production of COS and XOS by enzymatic hydrolysis of banana pseudostem and leaf and guava seed cake pretreated with potassium hydroxide. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake

Material	**Endoxylanase load (IU.g^{-1})**	**Reaction time (h)**	**X_1 (%)**	**X_2 (%)**	**X_3 (%)**	**X_4 (%)**	**X_5 (%)**	**X_6 (%)**	**XOS (%)**	**XOS (g.L^{-1})**
BP	30		0	59.10	0	0	0	0	59.10^a	12.06
BL	30	**24**	0	57.45	0	0	0	0	57.45^b	11.49
GSC	30		0.04	56.49	0	0	0	0	56.49^c	11.64
Material	**Endoglucanase load (IU.g^{-1})**	**Reaction time (h)**	**C_1 (%)**	**C_2 (%)**	**C_3 (%)**	**C_4 (%)**	**C_5 (%)**	**C_6 (%)**	**COS (%)**	**COS (g.L^{-1})**
BP	20		0.10	53.05	0	0	0	0	53.05^a	4.50
BL	20	6	3.36	58.07	0	0	0	0	58.07^b	4.18
GSC	20		0	16.42	34.39	0	0	0	50.81^c	3.24

Source: Author

C_1 glucose, C_2 cellobiose, C_3 cellotriose, C_4 cellotetraose, C_5 cellopentaose, C_6 cellohexaose, COS celloligosaccharides (C_2+ C_3+ C_4+ C_5+ C_6)

X_1 xylose, X_2 xylobiose, X_3 xylotriose, X_4 xylotetraose, X_5/X_6 xylopentaose/xylohexaose, XOS xylooligosaccharides (X_2+X_3+X_4+X5/X_6)

Equal letters, in the same column, represent the same statistical similarity, less than 0.05 (p<0.05)

5.3.3 Prebiotic activity of cellooligosaccharides and xylooligosaccharides on the growth of *Lactiplantibacillus plantarum* and *Bifidobacterium breve*

In the present study, the prebiotic potential of COS, XOS and the mixture of COS and XOS varying the concentrations, produced by enzymatic hydrolysis using banana pseudostem and leaf and guava seed cake was evaluated, as a carbon source in the growth of probiotic bacteria *Lactiplantibacillus plantarum* and *Bifidobacterium breve*.

There was no growth in MRS medium without carbon source. *L. plantarum* and *B. breve* were able to grow in medium containing COS, cellobiose commercial, xylose and XOS. During

COS fermentation by *L. plantarum* the maximum OD obtained was approximately 1.3 with COS from banana pseudostem in the concentration of 10 $g.L^{-1}$ (Figure 5.1b), This value was similar to that obtained during glucose fermentation at the concentration of 20 $g.L^{-1}$ (Figure 5.1a). With COS from other biomasses, the OD was between approximately 1 and 1.2. The COS produced in the present study and used as a carbon source have a predominance of cellobiose, therefore a medium containing commercial cellobiose was prepared as a carbon source, for comparison in microbial fermentation assays. It was possible to observe a smaller growth with commercial cellobiose in relation to COS, with a maximum OD of 0.8 in the concentration of 10 $g.L^{-1}$ (Figure 5.1a).

In xylose fermentation, the maximum OD was 0.6 (Figure 5.1c). When XOS was used as a carbon source, the maximum OD was approximately 1.3 with XOS from banana pseudostem 20 $g.L^{-1}$, with the remainder of XOS, OD varied between 0.7 and 1.1, values lower than those obtained with COS (Figure 5.1d).

When *L. plantarum* grew in medium with glucose and cellobiose, the stationary phase was reached after 50 h (Figure 5.1a), while in medium containing xylose the stationary phase was reached after 30 h at the concentration of 10 $g.L^{-1}$ and after 60 h in the concentration of 20 $g.L^{-1}$ (Figure 5.1c). In medium containing COS and XOS, after the same period, the microorganism was in the exponential phase (Figure 5.1b e 5.1d).

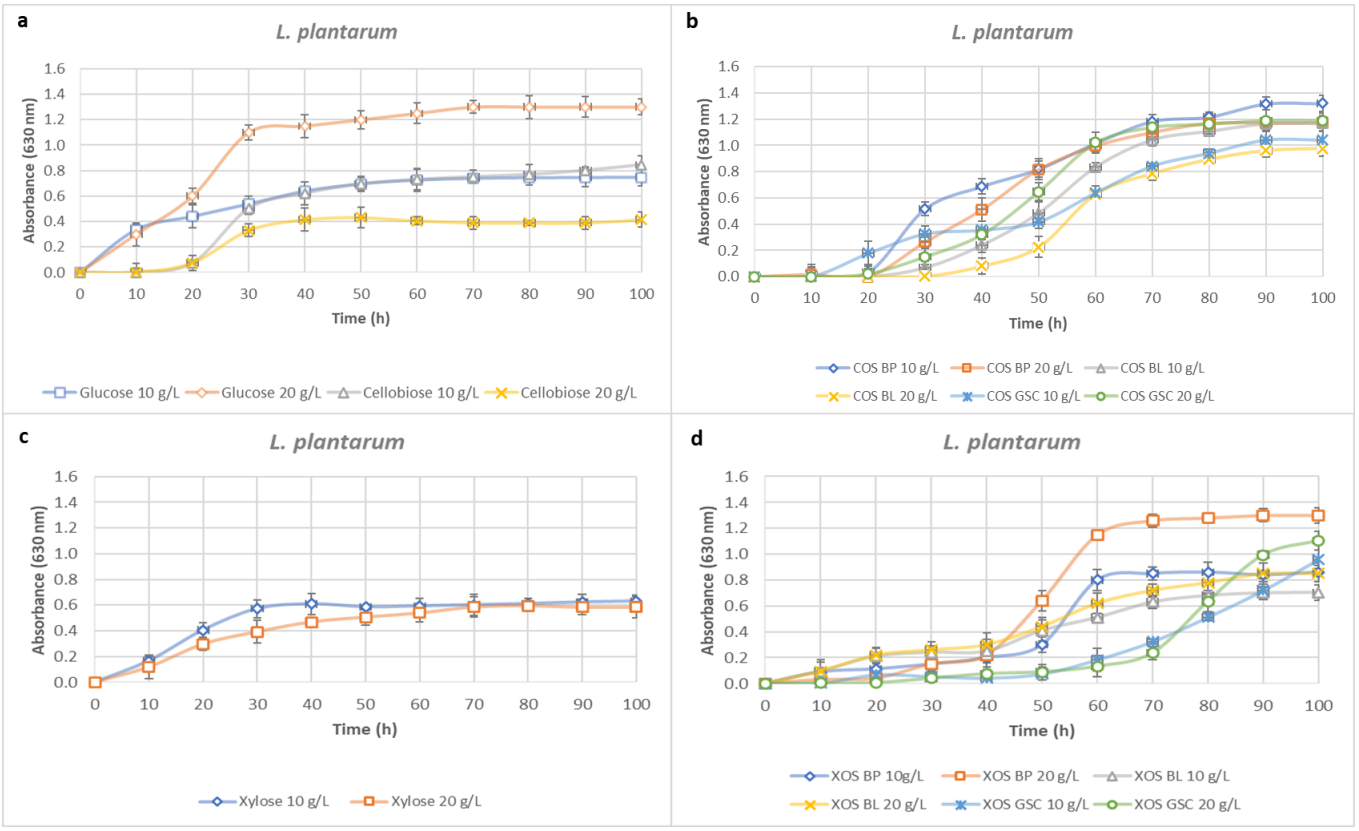
For microorganisms to be able to use COS and XOS, the action of enzymes is necessary: endoglucanase, exoglucanase, β-glucosidase, β-xylosidase, exo-oligoxylanase and α-L-arabinofuranosidase. These enzymes break down oligomers into monomers that can be absorbed and metabolized, facilitating energy production and supporting microbial growth (ALOKIKA et al., 2021; KLANGPETCH et al., 2022).

Some strains of bacteria such as *L. plantarum, L. brevis, L. casei, B. adolescentis* and *B. breve* as well as some yeasts are capable of producing the enzyme β-glucosidase that hydrolyzes cellobiose into glucose (ROKNI et al., 2021; CHEN et al., 2021; POKUSAEVA et al., 2011). Bacteria such as *L. plantarum, L. brevis, L. sakei* and *B. breve* have β-xylosidase in their cells, which breaks down XOS into xylose (ILIEV et al., 2020; SHIN et al., 2003).

L. plantarum has many genes responsible for carbohydrate absorption and metabolism, compared to other lactic acid bacteria (LAB). This high capacity to utilize carbohydrates such as mannose, cellobiose, ribose, galactooligosaccharide and fructooligosaccharides varies between different strains and facilitates their adaptation in different environments (CUI et al., 2021). Bacteria of the genus *Bifidobacterium* are more capable of consuming pentoses, such as xylooligosaccharides, than hexoses, such as galactooligosaccharides and fructooligosaccharides. A daily amount of XOS between 1.4 and 2.8 g is capable of inducing numerous health benefits, a

smaller amount compared to other prebiotics, thus becoming more economically competitive (VALLADARES-DIESTRA et al., 2023).

Figure 5.1 - Growth of *L. plantarum* in medium containing (a) glucose commercial. (b) cellooligosaccharides produced from banana pseudostem and leaves and guava seed cake. (c) xylose commercial and (d) xylooligosaccharides produced from banana pseudostem and leaves and guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



Source: Author

L. plantarum WCFS1 strain grew in MRS medium containing commercial cellobiose (98% purity) and COS hydrolyzed from microcrystalline cellulose, reaching OD equivalent to 7 and 3.1, respectively (ZENG et al., 2024). In the fermentation of birch and spruce COS by *L. plantarum* ATCC 8014, *B. adolescentis* DSM 20083 and *B. longum* DSM 20219 OD values were 5.35, 0.6 and less than 0.6, respectively (KARNAOURI et al., 2019). The values were higher than those obtained in the present study, probably because they were different strains.

Regarding XOS, some studies obtained similar results. When *L. plantarum* was grown in MRS broth with corn cobs XOS in concentrations of 10 $g.L^{-1}$ and 20 $g.L^{-1}$ it was possible to obtain an OD of 0.75 and 0.56, respectively (THAKURIA, 2020). When *L. plantarum* was cultivated in a carbohydrate-free culture medium, the OD 600 did not exceed 0.2 and when it was added to the medium, approximately 0.5 $g.L^{-1}$ of XOS at OD 600 was above 0.6 in 12 h of fermentation. There

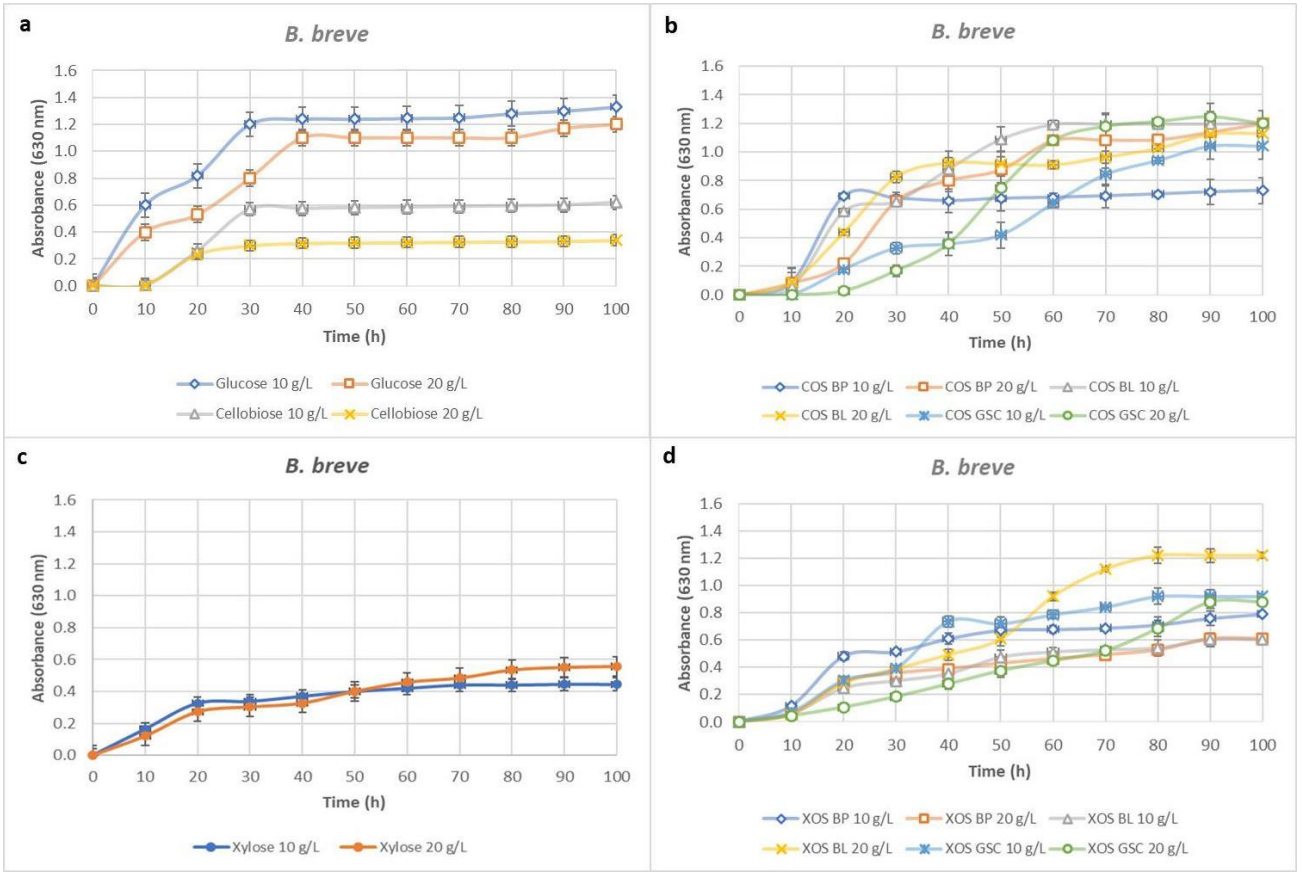
was release of short-chain fatty acids, mainly acetate with concentrations between 1.50 and 1.78 $mg.mL^{-1}$. Furthermore, when a combination of XOS with L. plantarum was consumed by rats, there was an increase in the amount of bifidobacteria and lactobacilli and a decrease in pathogenic bacteria such as *Enterococcus, Enterobacter* and *Clostridia* spp. (YU et al., 2015).

In another study, the impact of XOS consumption on the human intestinal microbiota was analyzed. Participants were divided into two groups: the first consumed 150 g of rice porridge containing 1.2 g of XOS for 6 weeks. The Second just porridge without XOS. An increase in the number of *Lactobacillus* spp. and *Bifidobacterium* spp. was observed. in the group that consumed XOS, demonstrating its prebiotic potential, in addition, there was a decrease in the number of *Clostridium perfringens*, a pathogenic bacteria, which can cause food poisoning and necrotic enteritis (LIN et al., 2016).

When *B. breve* was cultivated in culture medium containing COS, the maximum OD was close to 1.3 with COS from guava seed cake 20 $g.L^{-1}$, value equal to that obtained with glucose. With the rest of the COS the OD was between 0.7 and 1.2 and with cellobiose the maximum OD was 0.6 in the concentration of 10 $g.L^{-1}$ (Figure 5.2a and 5.2b). Similar OD values were found in COS fermentation from grape marc by *B. animalis* spp. *lactis* (LIU; HU; HARITOS, 2024) and in the fermentation of COS from Megazyme by *B. adolescentis* (ZHONG et al., 2020).

In xylose fermentation, the maximum OD was 0.6 (Figure 5.2c). In the case of XOS, the highest OD was obtained with XOS from banana leaves 20 $g.L^{-1}$, equivalent to 1.2. With the remainder of XOS, OD varied between 0.6 and 0.9, values lower than those obtained with COS (Figure 5.2d). These values obtained with *B. breve* were similar to those obtained with *L. plantarum*. In commercial XOS fermentation by *B. breve* the OD achieved was approximately 2.2 (DE FIGUEIREDO; DE BARROS RANKE; DE OLIVA-NETO, 2020).

Figure 5.2 - Growth of *B. breve* in medium containing (a) glucose commercial. (b) cellooligosaccharides produced from banana pseudostem and leaves and guava seed cake. (c) xylose commercial and (d) xylooligosaccharides produced from banana pseudostem and leaves and guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



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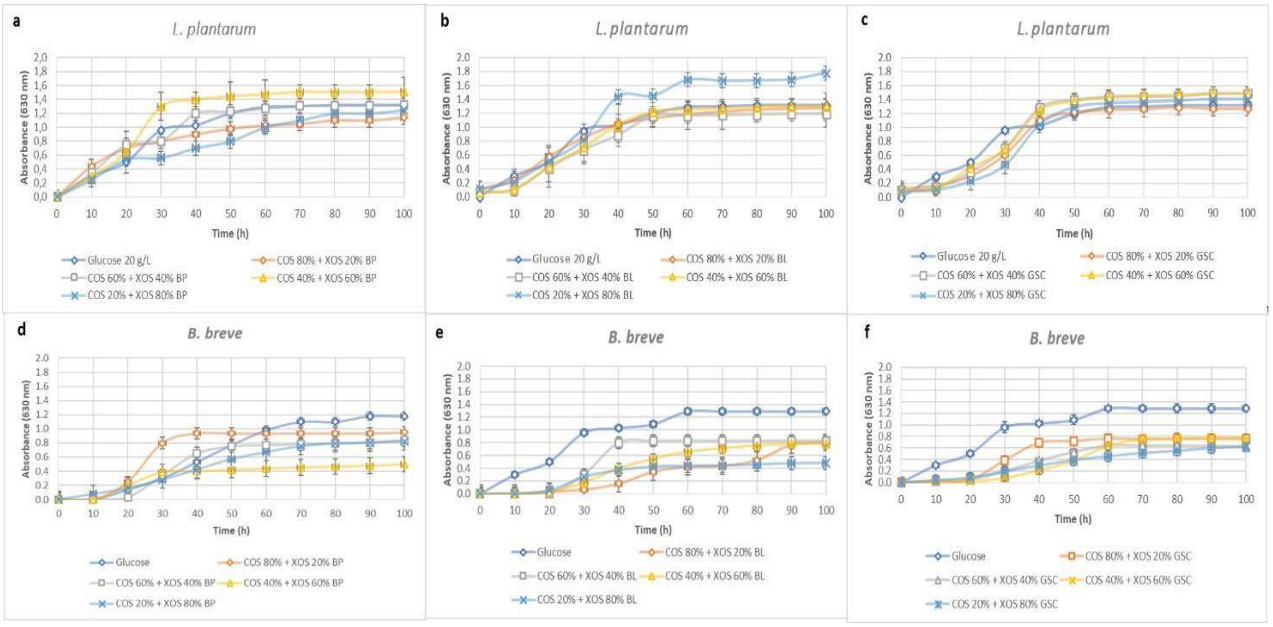
When *L. plantarum* was grown on medium containing a mixture of COS and XOS from banana pseudostem, banana leaf and guava seed cake, the highest OD values were 1.5 (COS 40% and XOS 60%) (Figure 5.3a), 1.8 (COS 20% and XOS 80%) (Figure 5.3b) and 1.5 (COS 40% and XOS 60% and COS 60% and XOS 40%) (Figure 5.3c), respectively.

In the fermentation of culture media containing a mixture of COS and XOS from banana pseudostem, banana leaf and guava seed cake by *B. breve* it was possible to observe an OD of 0.94 (COS 80% + XOS 20%), 0.83 (COS 60% + XOS 40%) and 0.77 (COS 80% + XOS 20%) (Figure 5.3d, 5.3e and 5.3f).

In general, considering the growth with the COS of all biomasses studied, the microorganisms did not show tendency to grow more with COS or XOS, with the OD always above 1.0. In some cases, the OD was higher than that obtained with glucose.

Figure 5.3 - Fermentation of a mixture of COS and XOS in varying proportions by *L. plantarum* from (a) banana pseudostem (b) banana leaves and (c) guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



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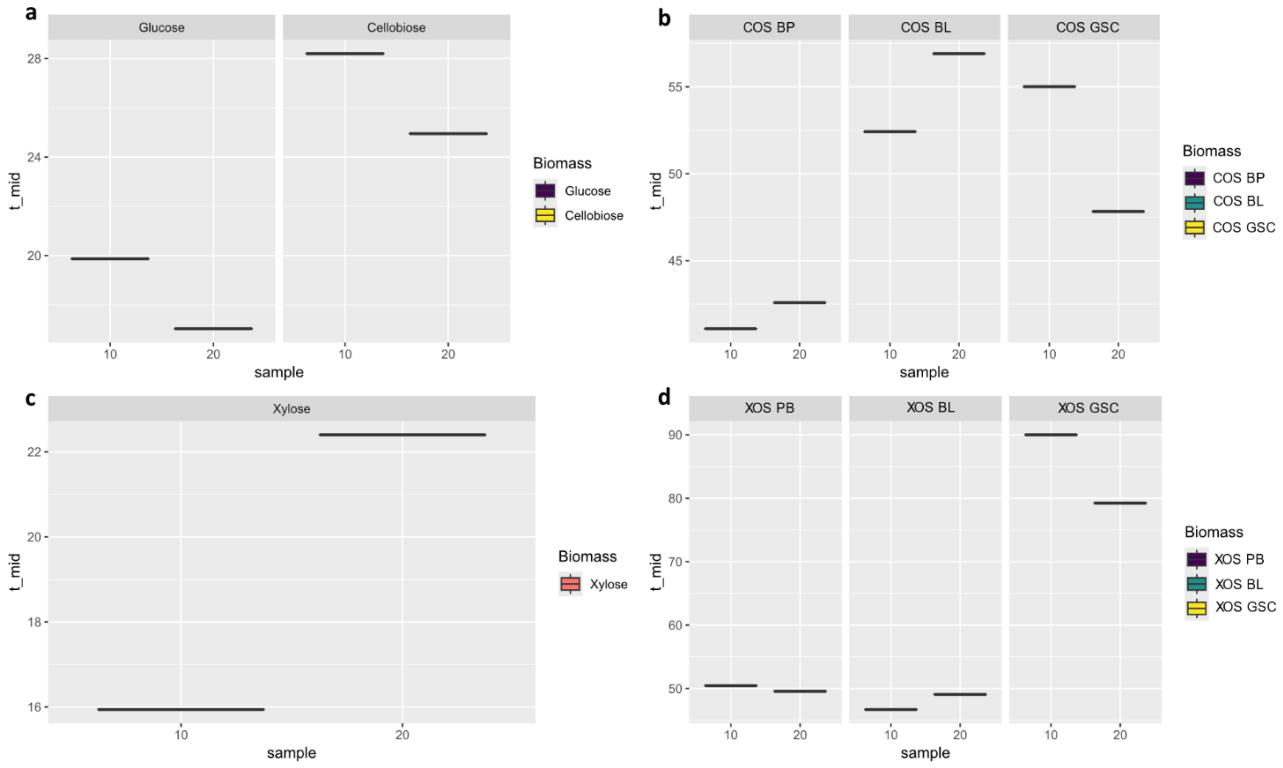
5.3.4 Statistical analysis

The figures 5.4, 5.5 and 5.6 compare the midpoint of growth (or T_mid) of *L. plantarum* and *B. breve* with all carbon sources in this study. T_mid is the time that bacteria need to reach half maximum growth. Lower values indicate a greater ability of the microorganism to utilize the carbon source. *L. plantarum* showed lower T_mid with glucose and xylose solutions, and then with commercial cellobiose and COS and XOS solutions (Figure 5.4).

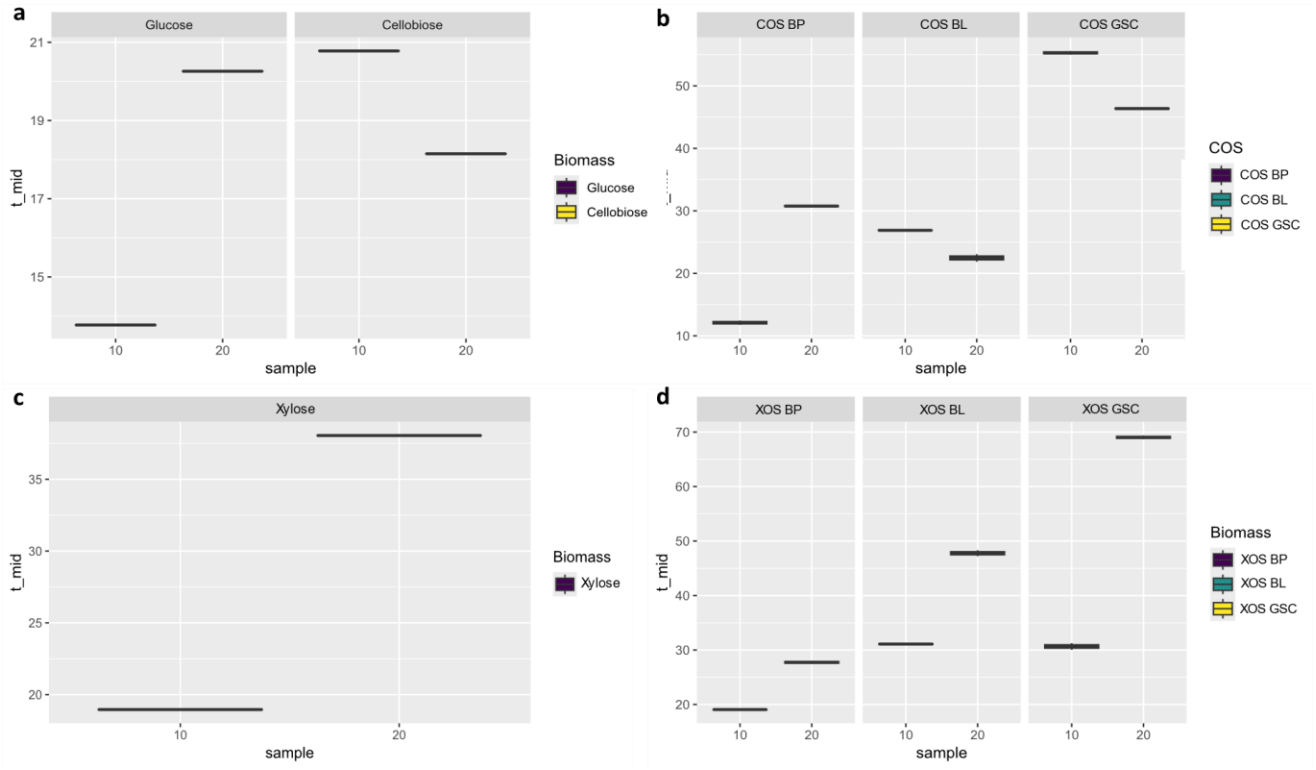
Figure 5.4 - T_mid values obtained with *L. plantarum* during fermentation of (a) glucose and cellobiose, (b) COS of banana pseudostem, banana leaves and guava seed cake, (c) xylose and (d) XOS of banana pseudostem, banana leaves and guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



Source: Author

B. breve showed a variable T_mid pattern. In general, the lowest values were obtained with glucose, cellobiose and xylose, and with COS and XOS solutions, with an exception of COS and XOS from banana pseudostem at a concentration of 10 $g.L^{-1}$, which had a T_mid similar to that of glucose (Figure 5.5).

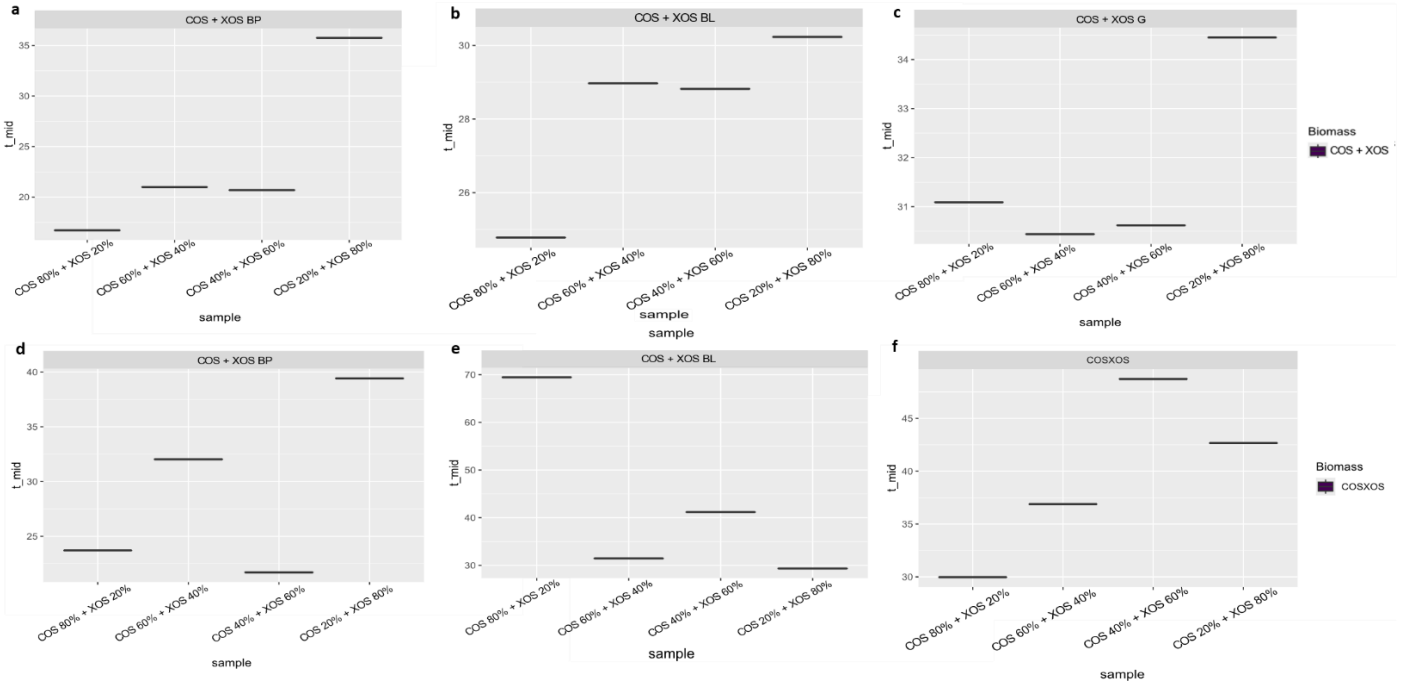
Figure 5.5 - T_mid values obtained with *B. breve* during fermentation of (a) glucose and cellobiose, (b) COS of banana pseudostem, banana leaves and guava seed cake, (c) xylose and (d) XOS of banana pseudostem, banana leaves and guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



Source: Author

In mixed solutions of COS with XOS, the T_mid obtained with *L. plantarum* was between approximately 22 and 35. The growth rate of *L. plantarum* was higher in COS and XOS solutions of banana pseudostem and leaf with higher concentrations of COS. With *B. breve* the T_mid values varied between approximately 22 and 70. In general, the growth rate of *B. breve* was also higher in solutions with a higher concentration of COS (Figure 5.6).

Figure 5.6 - T_mid values obtained with *L. plantarum* during fermentation of (a) COS + XOS of banana pseudostem, (b) COS + XOS of banana leaves, (c) COS + XOS of guava seed cake, and with *B. breve* during fermentation of (d) COS + XOS of banana pseudostem, (e) COS + XOS of banana leaves, (f) COS + XOS of guava seed cake. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



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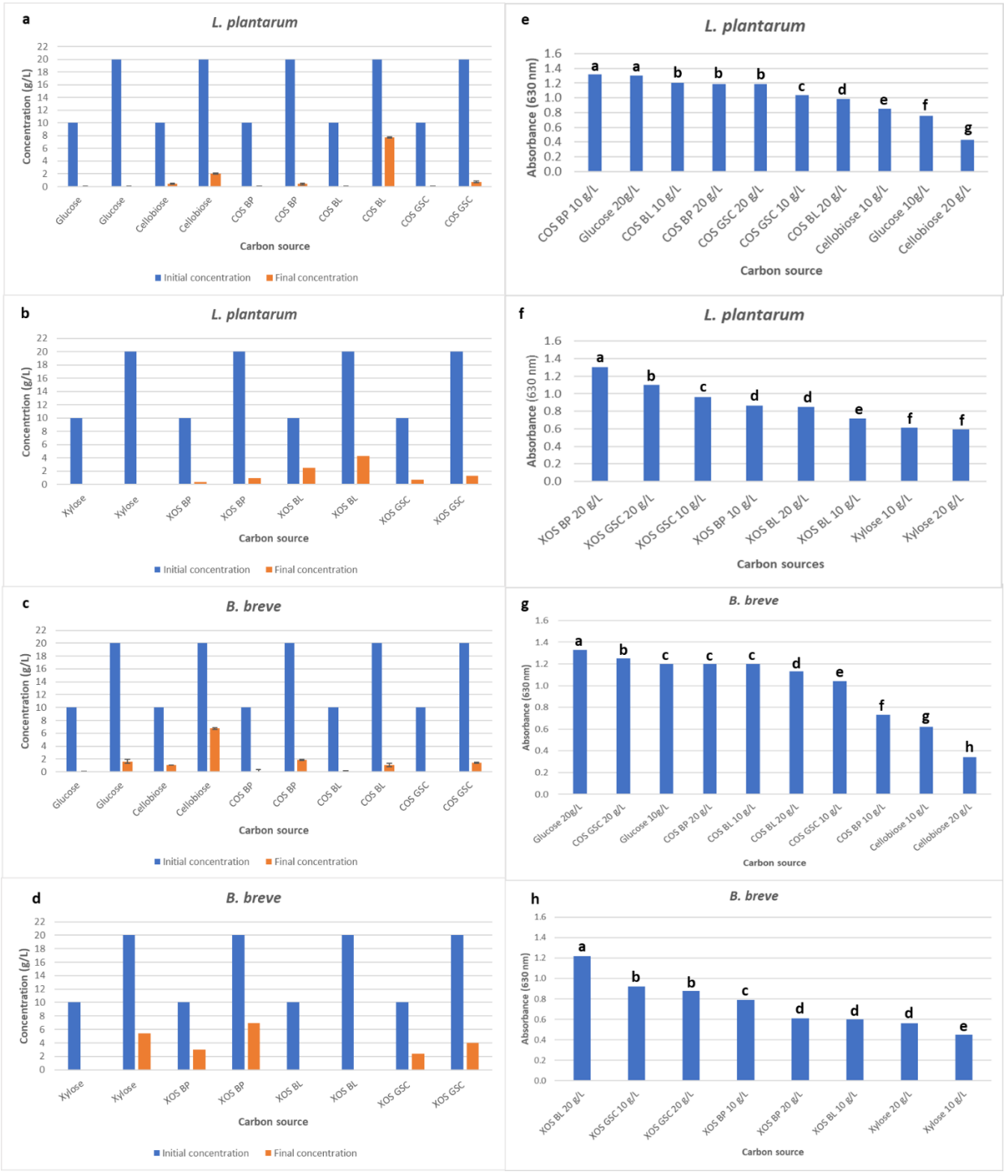
5.3.5 Consumption of carbon sources by microorganisms

Glucose and all COS at concentrations of 10 $g.L^{-1}$ were completely consumed by *L. plantarum* and *B. breve*. The COS of banana leaves in the concentration of 20 $g.L^{-1}$ was the least consumed by *L. plantarum*, remaining 7.8 $g.L^{-1}$ in the culture medium and the substrate that generated the lowest OD among all COS (Figure 5.7a and 5.7e). *B. breve* consumed less cellobiose at the initial concentration of 20 $g.L^{-1}$, leaving 6.7 $g.L^{-1}$ in the medium, in this case the growth of the bacteria was lower when compared to cellobiose 10 $g.L^{-1}$ and all COS.

In the fermentation of xylose and XOS by *L. plantarum*, almost all carbon sources were consumed. Although all xylose was consumed in both concentrations by *L. plantarum*, the OD was the lowest. The growth of microorganisms can be inhibited at high substrate concentrations, due to the metabolic capacity, accumulation of inhibitory compounds, saturation of specific enzymes, among others (EDWARDS, 1970). The least consumed XOS and which promoted the least growth by *L. plantarum* and *B. breve* were XOS from banana leaves and XOS from

banana pseudostem, respectively, both at the initial concentration of 20 $g.L^{-1}$ (Figure 5.7b and 5.7f).

Figure 5.7 - Consumption of carbon sources after 100 h by (a and b) *L. plantarum* and (c and d) *B. breve*. Maximum OD obtained during fermentation. (e) COS by *L. plantarum*. (f) XOS by *L. plantarum*. (g) COS by *B. breve*. (h) XOS by *B. breve.* BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake.

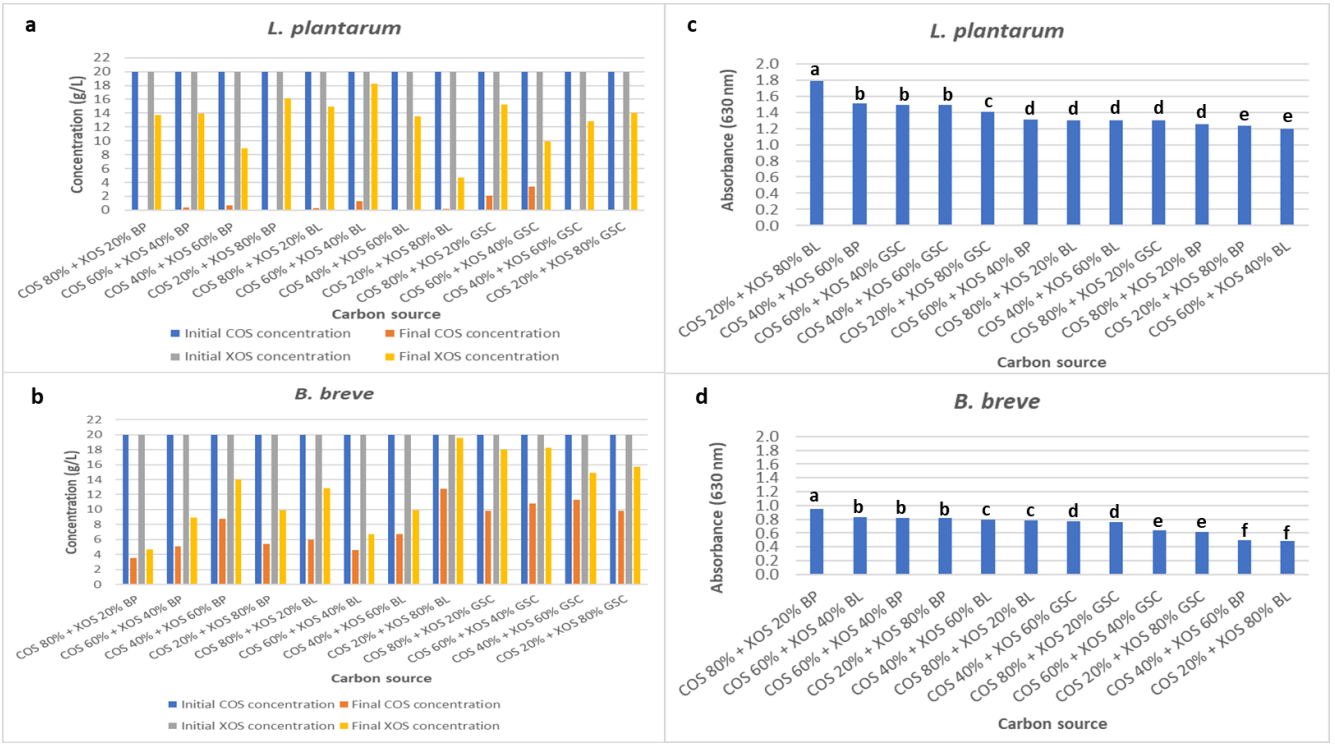


Source: Author

Equal letters represent the statistical similarity, for less than 0.05 ($p < 0.05$)

When mixtures of COS and XOS were used as a carbon source, there was a similar behavior between *L. plantarum* and *B. breve*, with greater consumption of COS in relation to XOS. *L. plantarum* consumed the total concentration of COS, equivalent to 20 $g.L^{-1}$, in most tests, unlike XOS which had the same initial concentration (20 $g.L^{-1}$) but higher final concentrations compared to COS. *L. plantarum* consumed more COS than *B. breve* and obtained higher OD values (Figure 5.8a, 5.8b, 5.8c and 5.8d). Both microorganisms can produce β-glucosidase and β-xylosidase, responsible for cleave cellobiose into glucose and XOS into xylose, respectively (MODRACKOVA et al., 2020; ROKNI et al., 2021; MARIA et al., 2014; SU et al., 2023). Microorganisms generally prefer glucose as a carbon source, but this preference varies between bacterial strains, according to their genotype, responsible for the metabolic pathways (SÁNCHEZ-CLEMENTE et al., 2020; ZHANG, Y. et al., 2023).

Figure 5.8 - Initial concentration of COS and XOS of 20 $g.L^{-1}$ and final concentration after 100 h of fermentation by (a) *L. plantarum* and (b) *B. breve*. Maximum OD obtained during fermentation (c) COS + XOS by *L. plantarum*. (d) COS + XOS by *B. breve*. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



Source: Author

5.3.6 Production of lactic acid and short-chain fatty acids

Lactic acid and short-chain fatty acids (SCFAs) are produced by some bacteria present in the gastrointestinal tract. The main SCFAs are: acetic, butyric and propionic acid. Acetic acid can be produced by bacteria such as *Bifidobacterium spp., Ruminococcus spp., Prevotella spp.* and *Akkermansia muciniphila*, it assists in metabolic homeostasis, in the immune system; and control of body weight due to decreased appetite; in lipid metabolism increasing the uptake of blood cholesterol by the liver and reducing hyperglycemia (SILVA; BERNARDI; FROZZA, 2020; GENEROSO et al., 2021; NOGAL et al., 2021).

Butyric acid is generally produced by bacteria *Faecalibacterium prausnitzii, Ruminococcus bromii, Eubacterium hallii* and some of the genus *Clostridium* and *Roseburia.* It is the main source of energy for colonocytes (colon cells), inhibits the growth of pathogens, improves mucosal integrity, stimulates the production of immunoglobulins, increases the excretion of antimicrobial substances such as defensins, lectins and cathelicidins, protects the intestinal mucosa against the entry of pathogens, preventing inflammation and prevents the proliferation of colorectal cancer cells (CONG; ZHOU; ZHANG, 2022; MOHAMED ELFADIL et al., 2023).

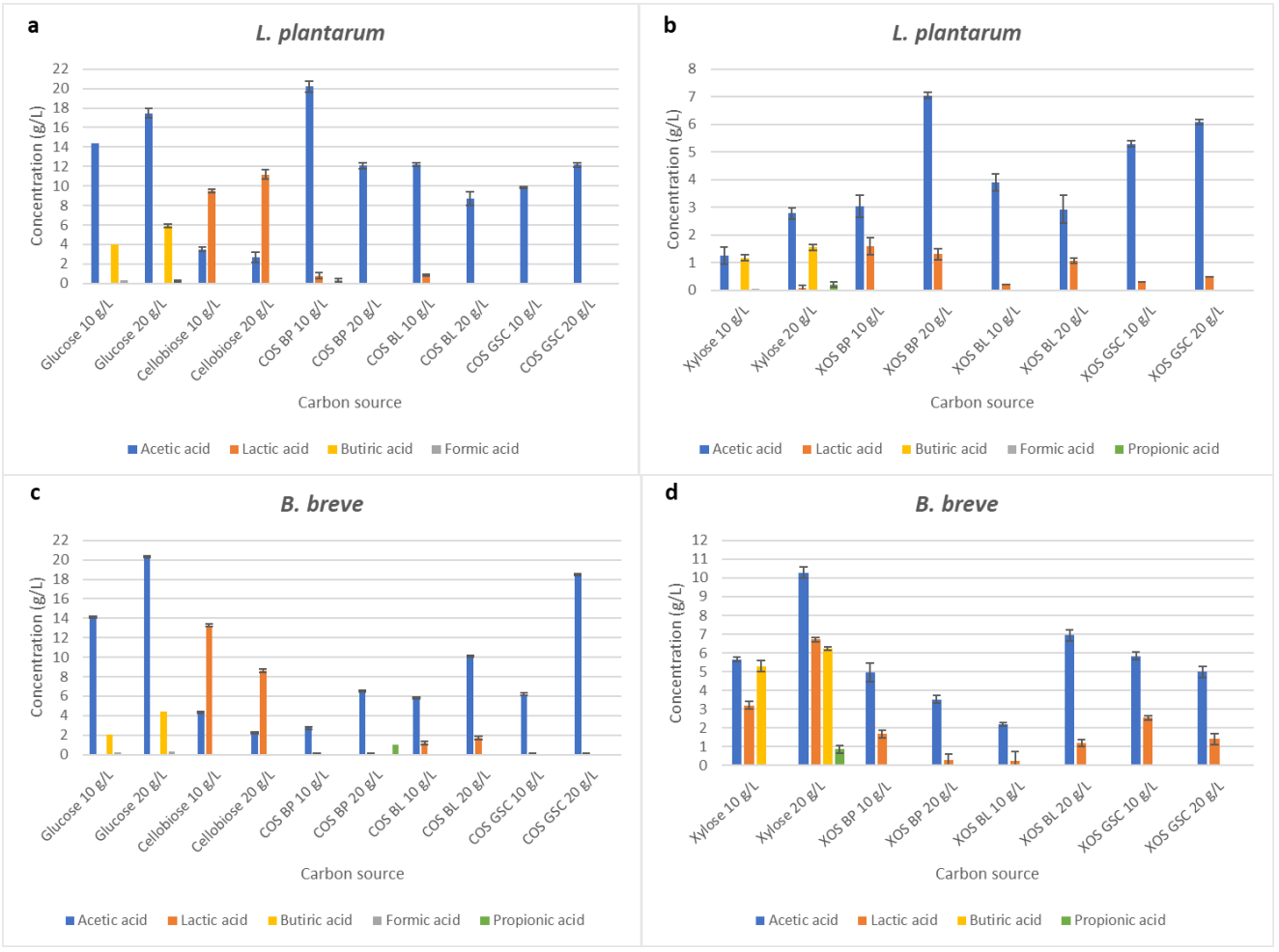
Propionic acid reduces intestinal cholesterol absorption, LDL and cholesterol levels, cell inflammation, vascular calcification, hypertension and attenuates insulin resistance, reducing the incidence of diabetes mellitus (YAN, J. et al., 2022; XIONG et al., 2022). Propionate can be produced by bacteria *Akkermansia muciniphila* and other species such as *Enterobacteria, Bacteroides, Roseburia* and *Blautia* (FROLOVA et al., 2022).

It was possible to observe the formation of acetic acid with all substrates studied when fermented by *L. plantarum* and *B. breve.* In general, the highest concentrations were obtained in the experiments with glucose (17.5 $g.L^{-1}$ with *L. plantarum* and 20.7 5 $g.L^{-1}$ with *B. breve*) and COS (20.2 5 $g.L^{-1}$ with COS from banana pseudostem fermented by *L. plantarum* and 18.7 5 $g.L^{-1}$ with COS from guava seed cake fermented by *B. breve*).

With glucose and xylose in the medium, butyric acid was also formed, in the concentrations of 5.9 5 $g.L^{-1}$ with glicose fermented by *L. plantarum* and 6.2 5 $g.L^{-1}$ com xilose fermented by *B. breve* (Figure 5.9a and 5.9c). Lactic acid was formed in greater concentration with xylose (6.7 $g.L^{-1}$), cellobiose (13.3 $g.L^{-1}$) and XOS from guava seed cake (2.54 $g.L^{-1}$) fermented by *B. breve* (Figure 5.9a, 5.9b, 5.9c and 5.9d).

There was a low formation of lactic acid in the presence of COS from banana pseudostem and leaf at the concentration of 10 g.L^{-1} fermented by *L. plantarum* and COS from the leaf at the concentrations of 10 and 20 g.L^{-1} fermented by *B. breve* (Figure 5.9a and 5.9c).

Figure 5.9 - Production of short-chain fatty acids using as substrate (a) glucose, cellobiose and COS fermented by *L. plantarum*, (b) xylose and XOS fermented by *L. plantarum* (c) glucose, cellobiose and COS fermented by *B. breve* and (d) xylose and XOS fermented by *B. breve*. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake

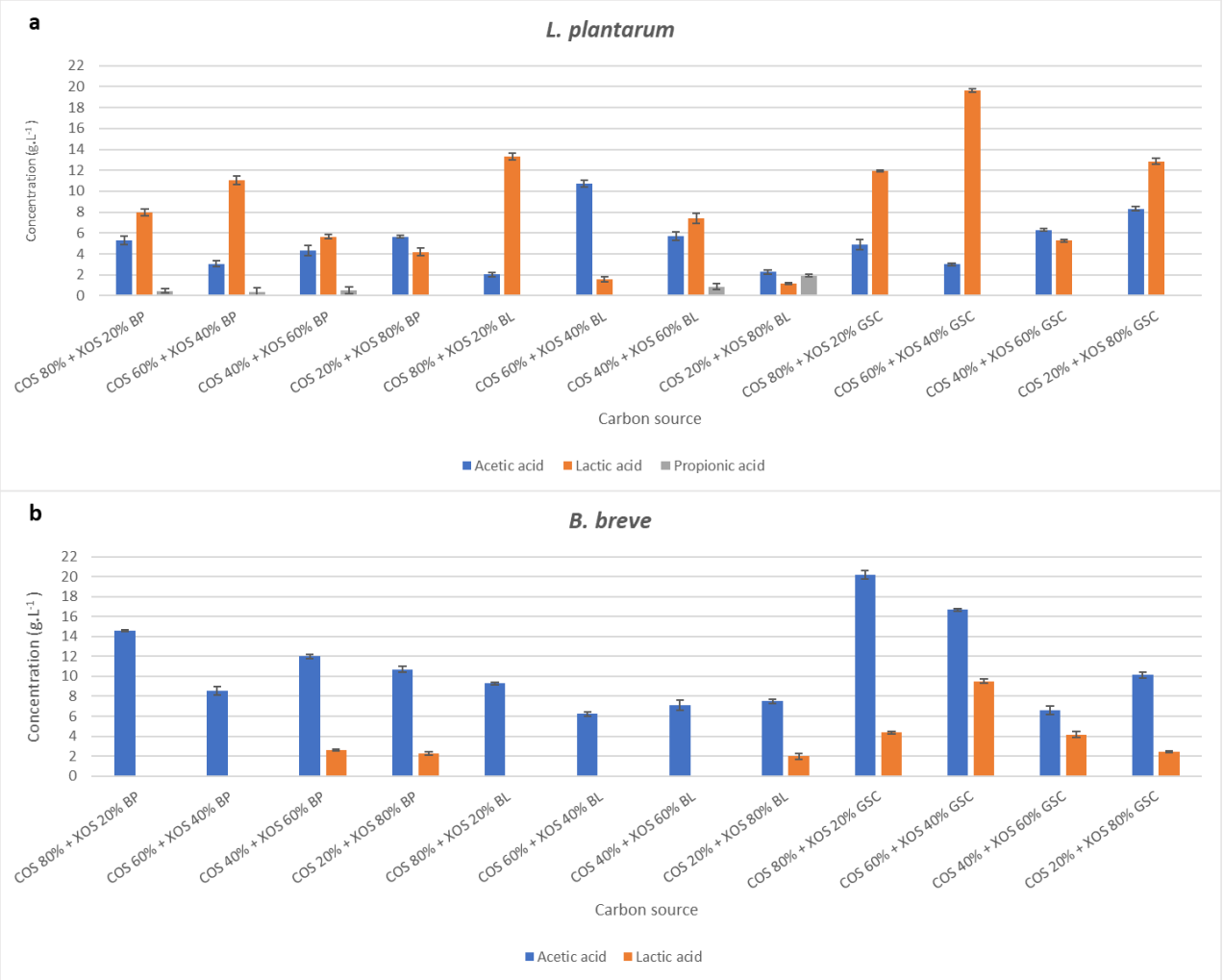


Source: Author

In the fermentation of commercial 2% XOS with 95% purity (Shandong Longlive Biotechnology Co) by *L. plantarum* S26 there was formation of acetic and lactic acid at maximum concentrations of 6.62 and 4 g.L^{-1}. With glucose, concentrations did not exceed 0.09 and 2.43 g.L^{-1} (ILIEV et al., 2020). In the present study, the maximum concentrations of acetic and lactic acid formed during the fermentation of XOS by *L. plantarum* was 7.05 g.L^{-1} with pseudostem XOS 20 g.L^{-1} and 1.6 g.L^{-1} with pseudostem XOS 10 g.L^{-1}.

When mixtures of COS and XOS were added to the culture medium containing *L. plantarum*, acetic and lactic acid were formed, and in the medium containing *B. breve* there was formation of acetic acid in all tests and lactic acid only in some and in smaller quantities. When only COS was fermented by *L. plantarum* and *B. breve*, there was practically no formation of lactic acid, unlike when XOS was fermented alone or in association with COS (Figure 5.10a, 5.10b).

Figure 5.10 - Production of short-chain fatty acids in medium containing a mixture of COS and XOS in varying proportions: COS 80% and XOS 20%, COS 60% and XOS 40%, COS 40% and XOS 60% and COS 20% and XOS 80% fermented by (a) *L. plantarum* (b) *B. breve*. BP: banana pseudostem, BL: banana leaves, and GSC: guava seed cake



Source: Author

5.4 Conclusion

It was possible to produce COS and XOS with a low degree of polymerization using endoxylanase from *Aspergillus versicolor* and commercial Celluclast endoglucanase. COS and XOS, isolated or mixed, proved to have prebiotic activity, stimulating the growth of *L. plantarum* and *B. breve*, bacteria present in large numbers in human and animal microbiota, capable of fermenting sugars, producing mainly lactic acid and acetic acid (short-chain fatty acid). The microorganisms grew similarly in COS and XOS, in general, with slightly higher growth with COS. *L. plantarum* grew more with COS and XOS of banana pseudostem and *B. breve* with COS and XOS of banana leaf. When the solution containing mixed COS and XOS was used as carbon source, there was an inversion, with *L. plantarum* growing more with COS and XOS of banana leaf and *B. breve* with COS and XOS of banana pseudostem. *L. plantarum* and *B. breve* produced acetic acid in all fermentation tests with COS and XOS alone or mixed. Lactic acid was produced by both bacteria only in some trials with XOS and in very small quantities with COS. In trials with COS mixed with XOS, lactic acid was produced by *L. plantarum* in all cases and by *B. breve* only in some cases. The fact that both bacteria grow on COS and XOS alone or mixed is advantageous, as it enables the production of both oligosaccharides during the same enzymatic hydrolysis process.

CHAPTER VI: CONCLUSION

COS and XOS have enormous potential for application in various industries due to their numerous benefits for human and animal health with emphasis on their prebiotic effect. Its large-scale production is still a challenge, although they can be produced with biomass, an abundant and cheap raw material, it is very difficult to separate their main components—cellulose, hemicellulose, and lignin—and break down the polysaccharides into the desired oligomers. It is important that research advances so that new techniques are developed, making these products increasingly accessible to the entire population.

In this study, it was possible to observe that the pretreatment of banana and guava biomasses with alkaline reagents, and the hydrolysis with *Aspergillus versicolor* endoxylanase followed by hydrolysis with commercial Celluclast endoglucanase were conditions that increased the yield of cellulose conversion into COS, probably due to the fact that the removal of xylan, leaves the cellulose more exposed to the action of endoglucanase. Reducing particle size in a ball mill was not effective in all cases. The use of endoxylanase also had the advantage of producing XOS, another oligosaccharide of great industrial interest, which is already commercialized in several countries and has its anntioxidante e prebiotic effect confirmed by several studies.

Enzymatic hydrolysis was performed with variable endoglucanase loads (20, 50 e 100 $IU.g^{-1}$). A load of 20 $IU.g^{-1}$ was defined as the best condition due to the greater conversion of cellulose into COS in most tests and because it is more economical. Under the best conditions for COS production, (unground material, pretreated with KOH, hydrolyzed with endoxylanase 30 $IU.g^{-1}$ followed by endoglucanase 20 $IU.g^{-}1$) it was possible to obtain glucan to COS conversion yield of approximately 59%, 70% and 55% for banana pseudostem, banana leaf and guava bagasse, respectively. There was a prevalence of cellobiose and xylobiose formation with all biomasses.

The COS and XOS solutions filtered in Sep-Pak cartridgeswere not able to reduce the DPPH radical, as well as glucose, xylose and cellobiose solutions. Unfiltered XOS solutions showed higher antioxidant activity than COS solutions. It was possible to achieve IC_{50} with concentrations between 4 and 8 $g.L^{-1}$. With COS the IC_{50} was not reached up to concentrations of 16 $g.L^{-1}$. Antioxidant activity must be related to the presence of compounds present in solutions.

Using the Folin-Ciocalteau method, it was possible to determine the presence of total polyphenols and using the scanning spectrophotometry method it was possible to determine the

presence of soluble lignin, extractives and carboxylic acids, in all unfiltered COS and XOS samples. These compounds are probably responsible for reducing the DPPH radical.

The microorganisms *L. plantarum* and *B. breve* were able to grow in culture medium with COS and XOS as a carbon source. In general, growth was slightly greater with COS compared to XOS. *L. plantarum* consumed more COS and XOS from banana pseudostem and *B. breve*, grew more with COS and XOS from banana leaves.

When mixed COS and XOS solutions were used as carbon source, there was growth. With the mixture of COS and XOS from banana leaves, in the proportion 20/80 %, fermented by *L. plantarum*, the OD was approximately 1.8, the highest of all. In the fermentation of COS and XOS from pseudostem by *B. Breve*, the maximum OD (1.0) was reached, but it was the lowest obtained in the present study.

COS and XOS produced in the present study did not demonstrate high antioxidant capacity, but are capable of reducing the DPPH radical when not purified. COS and XOS have potential to be used as prebiotics, as they were able to stimulate the growth of *L. plantarum* and *B. breve*, generating mainly lactate and acetate, a short-chain fatty acid, both with the potential to help in the prevention and treatment of various diseases.

FINAL CONSIDERATIONS

Oligosaccharides have the potential to be applied in the food, pharmaceutical and chemical industries, and can be produced with lignocellulosic biomass, coming from agricultural and industrial waste, an abundant and cheap source.

There are still many challenges for their large-scale production. The process tends to be complex and expensive, requiring the use of advanced equipment and technologies, chemical reagents and enzymes. Production efficiency is usually low, due to the recalcitrance of biomass. Characterization and purification methods are not standardized, making their application in products such as food and medicine difficult.

For oligosaccharides to become economically viable, it is necessary to reduce production costs, improve production methods, develop strains of microorganisms that produce more specific and effective enzymes, immobilization of enzymes for reuse, optimization of the hydrolysis process, with suitable conditions such as ideal enzyme load, to avoid waste, appropriate temperature and pH, and standardize techniques to ensure quality.

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