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SUSTAINABLE PRODUCTION OF POSTBIOTICS FOR FOOD APPLICATIONS

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Food Engineering

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Resumo

Os alimentos são uma necessidade essencial da vida humana e possuem nutrientes que apoiam o crescimento e a saúde. Nestas últimas décadas, os alimentos funcionais com ingredientes como probióticos, prebióticos e pósbióticos têm vindo a ganhar muita atenção por parte dos consumidores. Pósbiótico é definido como a "preparação de microrganismos inanimados e / ou dos seus componentes que confere um benefício para a saúde do hospedeiro". Os pósbióticos são compostos bioativos funcionais gerados através da fermentação de bactérias do ácido lático, como o *Lactobacillus plantarum* e *fermentum* e / ou a levedura *Saccharomyces cerevisiae*. Os metabolitos mais estudados que derivam dos pósbióticos são os ácidos gordos de cadeia curta, polissacarídeos extracelulares, lisados celulares, ácido teicoico e vitaminas. Têm também diversas propriedades biológicas como anti-inflamatório, imunomodelatório, anti-hipertensivo, anti-proliferativo e antioxidante. O objetivo deste trabalho foi utilizar os subprodutos da cana-de-açúcar (bagaço e palha) como fonte de açúcar seguido de fermentação com ou sem *L. plantarum* e *S. cerevisiae* como alternativa sustentável para aplicações alimentares. Para que os subprodutos produzissem extratos pósbióticos foi necessário realizar uma sacarificação e fermentação. As condições de sacarificação foram otimizadas testando as enzimas celluclast e xilanase, diferentes tempos de reação (24 e 48 h) e o impacto da biomassa. Foi realizada uma sacarificação e fermentação simultânea com diferentes microrganismos (*L. plantarum, fermentum* e *S. cerevisiae*). Numa segunda fase, foi testada uma sacarificação e fermentação sequencial com *L. plantarum* durante 48 h e *S. cerevisiae* durante 72 h, com remoção da biomassa após sacarificação e das células intactas no final da fermentação. Os extratos foram caracterizados numa fase inicial no que diz respeito às atividades antioxidantes e antimicrobianas. Os extratos finais obtidos pela melhoria das condições do processo foram caracterizados quanto ao potencial prébiotico e antidiabético, proteína total, conteúdo e compostos fenólicos totais, o perfil de ácidos orgânicos e monossacarídeos e o efeito imunomodelatório e citocompatibilidade. Os principais resultados obtidos na fase inicial mostram que os extratos não fermentados têm maior atividade antioxidante que os extratos fermentados com as estirpes de *Lactobacillus*. Já com a levedura *Saccharomyces* os extratos fermentados têm melhor atividade antioxidante que os não fermentados. Em relação à atividade antimicrobiana, os extratos fermentados não inibem o crescimento das bactérias patogénicas alimentares. Como seria de esperar os não fermentados ainda continham monossacarídeos e o ácido acético foi identificado em todos os extratos analisados, fermentados e não fermentados. O mesmo conteúdo de proteína total 32-38%, foi encontrado em todos os extratos. O extrato de palha fermentado com a levedura *Saccharomyces* foi o que teve o maior conteúdo em compostos fenólicos (16.15 mg GA/ g dw), sendo identificados 6 como o ácido p-cumárico e o 4-hidroxibenzaldeído e o ácido acético como o ácido orgânico mais predominante. Os extratos fermentados reduziram os mediadores pró-inflamatórios nas células Caco-2. Já o potencial prebiótico dos dois extratos não mostraram grande impacto em *Lactobacillus* e em *Bifidobacterium* apesar de terem aumentado ao longo do tempo os ácidos orgânicos de cadeia curta (SCFA) com maior incidência no acetato, butirato e propionato. Assim, os pós-bióticos produzidos pela levedura e bactéria demonstram potencial nas propriedades químicas e biológicas para utilizar na área alimentar e nutracêutica.

Palavras-chave: Pós-bióticos; Cana-de-açúcar; *L. plantarum*; *S. cerevisiae*; fermentação; subprodutos

Abstract

Food is an essential need of human life and has nutrients that support growth and health. In recent decades, functional foods with ingredients such as probiotics, prebiotics and postbiotics have gained much attention among consumers. Postbiotics are defined as "a preparation of inanimate micro-organisms and/or their components that confers a health benefit to the host". Postbiotics are functional bioactive compounds generated through the fermentation of lactic acid bacteria, such as *Lactobacillus plantarum* and *fermentum* and/or the yeast *Saccharomyces cerevisiae*. The most studied metabolites derived from postbiotics are short chain fatty acids, extracellular polysaccharides, cell lysates, theicoic acid and vitamins. They also have several biological properties such as anti-inflammatory, immunomodulatory, antihypertensive, anti-proliferative and antioxidant. The objective of this work was the use of sugar cane by-products (bagasse and straw) as a source of sugar after fermentation with L. *plantarum* and *S. cerevisiae* as a sustainable alternative for food applications. For the by-products to produce post-biotic extracts it was necessary to perform saccharification and fermentation. The saccharification conditions were optimised by testing the enzymes celluclast and xylanase, different reaction times (24 and 48 h) and the impact of milling the biomass. A simultaneous saccharification and fermentation with different microorganisms (*L. plantarum, fermentum* and *S. cerevisiae*) was performed. In a second phase, sequential saccharification and fermentation was tested with *L. plantarum* for 48 h and *S. cerevisiae* for 72 h, with removal of biomass after saccharification and of intact cells at the end of the fermentation. The extracts were characterised at an initial stage for potential screening with respect to their antioxidant and antimicrobial activities, and the final extracts obtained by improving the process conditions were characterised for prebiotic and antidiabetic potential, total protein, total phenolic content and compounds, the profile of organic acids and monosaccharides and the immunomodulatory effect and cytocompatibility. The main results obtained in the initial phase show that the non-fermented extracts have greater antioxidant activity than the fermented extracts with the *Lactobacillus* strains, while with the *Saccharomyces* yeast the fermented extracts have better antioxidant activity than the non-fermented ones. Regarding the antimicrobial activity, the fermented extracts did not inhibit the growth of food pathogenic bacteria. Monosaccharides were identified in the final extracts in the unfermented extracts and acetic acid was identified in all the extracts analysed. Total protein is similar in all extracts ranging from 32-38%. The straw extract fermented with *Saccharomyces* yeast had the highest phenolic content (16.15 mg GA/ g dw). Six phenolic compounds were identified and the predominant ones were p-coumaric acid and 4-hydroxybenzaldehyde and the most predominant organic acid was acetic acid. The fermented extracts showed a good capacity to reduce the pro-inflammatory mediators in Caco-2 cells. The prebiotic potential of the two extracts did not show great impact in *Lactobacillus* and *Bifidobacterium*, although they increased over time the short chain organic acids (SCFA) with greater incidence in acetate, butyrate and propionate. Thus, the postbiotics produced by yeast and bacteria show potential in chemical and biological properties for use in the food and nutraceutical area.

Keywords: Postbiotics; Sugarcane; *L. plantarum*; *S. cerevisiae*; fermentation; by-products

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List of Abbreviation

A&A	Antibiotic and Antimiotic
AAPH	2,2'-azobis(2-amidinopropane) Dihydrocloride
ABTS	2.2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic Acid)
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
BC	Bagasse with *S*. *Cerevisiae*
BCt	Bagasse Control
BP	Bagasse with *L*. *Plantarum*
BF	Bagasse with *L*. *Fermentum*
BSM	Bifidos Selective Medium
CBA	Colombia Blood Agar
CFU	Colony Forming Units
CMC	Culture Media Supplemented
Ct	Control
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxid
DNA	Deoxyribonocleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polysaccharides
FBS	Fetal Bovine Serum
FOS	Fructooligosaccharides
FSR	Full-Sensitivity Resolution
GA	Galic Acid
HPLC-RID	High Performance Liquid Chromatography-Refractive Index Detector
IC	Inoculum Control
ISSAPP	International Scientific Association of Probiotics and Prebiotics
LAB	Lactic Acid Bacteria
LPB	*Lactobacillus Plantarum* with Bagasse
MBC	Minimum Bactericidal Concentration
MCA	MacConkey Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRS	Man, Rogosa and Sharpe
MS	Mass Spectrometry
NAD+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NEAA	Non-Essential Amino Acid
OD	Optical Density

OH	Hydroxyl Groups
ORAC	Oxygen Radical Absorption Capacity
PBS	Phosphate-Buffered Saline
PDA	Potato Dextrose Agar
qPCR	Real Time Polymerase Chain Reactor
RCF	Relative Centrifugal Force
RF	Radio Frequency
SA	*Staphylococcus Aureus*
SC	Straw with *S. Cerevisiae*
SCFA	Short-Chain Fatty Acids
SCt	Straw Control
SF	Straw with *L. Fermentum*
SHF	Separate Hydrolysis and Fermentation
SP	Straw with *L. Plantarum*
SQSF	Sequential Enzyme Saccharification and Fermentation
SSF	Saccharification and Simultaneous Fermentation
TOF	Time of Flight
TSB	Trypticase Soy Broth

1. Introduction

In recent years consumers have shown a great interest in buying healthy foods as well as started paying more attention to nutritional values and therefore there was an increase in the consumption of functional foods containing such as probiotics, prebiotics, symbiotics and more recently postbiotics **(Thorakkattu, P., et al., 2022)**. Definitions of these concepts have been published by the International Scientific Association of Probiotics and Prebiotics (ISAPP). Probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit to the host", while a prebiotic is a "substrate that is selectively used by host micro-organisms conferring a health benefit". A symbiotic is "a mixture comprising live micro-organisms and substrate(s) selectively used by host micro-organisms conferring a health benefit to the host". Postbiotic is a "preparation of inanimate microorganisms and/or their components that confers a health benefit to the host" **(Salminen *et al*, 2021)**. These host benefits can be immunomodulatory function, anti-inflammatory, antihypertensive, anti-proliferative and antioxidant effects **(Aguilar-Toalá *et al.*, 2018)**.

By-products of sugarcane lignocellulosic biomass, such as bagasse and straw, have been increasingly used not only to produce second generation (2G) bioethanol, but also to produce extracts for fermentation processes **(de Oliveira Nascimento *et al.,* 2022)**. Sugarcane (*Saccharum officinarum*) is a grass of the Poaceae family, of the genus *Saccharum*, native to the tropical regions of South Asia with rapid expansion to South-Central and Northeast Brazil. Its production in this country reached a total of approximately 650 million tonnes in the 2020/2021 harvest and is currently the world's largest producer of sugar and ethanol **(ÚNICA – União da Indústria de Cana-de-Açúcar, 2022)**. It is estimated that each hectare of land produces 88 tons of sugarcane, 1 ton of which produces 80 litres of ethanol and results in 28 tons of bagasse. To obtain these products, the by-products undergo several stages, such as milling, clarification and cogeneration. Normally the straw is used in the production of biogas through anaerobic digestion and the bagasse in the supply of heat and energy in boilers. The straw is composed of 40% cellulose, 30% hemicellulose and 25% lignin, while the bagasse is composed of 42% cellulose, 27% hemicellulose and 21% lignin. Both are also composed of 10% minority compounds such as minerals and ash **(Gomez *et al*., 2010) (Paes, *et al*., 2005)**.

The use of by-products as substrate of fermentation lays down in the presumption that these materials may undergo hydrolysis by enzymes where there is the release of monosaccharides, such as glucose that can then be used by various microorganisms in fermentation. *Lactobacillus plantarum* and *Lactobacillus fermentum* after performing fermentation can have as final products organic acids, bacteriocins and hydrogen peroxide, while the final products of the yeast *Saccharomyces cerevisiae* are oligosaccharides, organic acids, amino acids and peptides **(Valerio *et al.,* 2008)**.

The present work intends to demonstrate the use of the major sugarcane by-products as substrate in the production of postbiotics using the as microorganism *Lactobacillus plantarum, fermentum* and the yeast *Saccharomyces cerevisiae*.

2. Postbiotics production

2.1. Bagasse and Straw

Sugarcane (*Saccharum officinarum*) is native to the tropical regions of Asia but quickly expanded to the South Central and Northeast of Brazil (**Figure 2.1.**) with a large annual production and with sugar and ethanol as major end products. To produce sugar and ethanol, the raw material has to be processed and consequently by-products such as bagasse and straw are produced **(UNICA - União da Indústria de Cana-de-Açúcar, 2022)**.

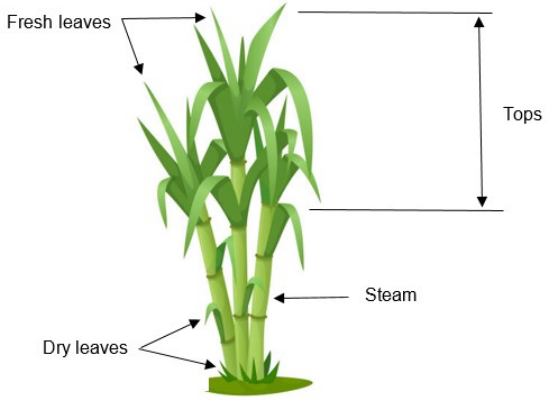

Figure 2.1. Morphology of sugarcane plant.

The main constituents of bagasse and straw are cellulose, hemicellulose and lignin, although in different quantities (**Figure 2.2.**) In bagasse, cellulose, which is the main component of the cell wall, represents 42%, hemicellulose 27%, lignin 21% and the remaining 10% are minority components, such as minerals and ash. These ash are essentially silica and the presence of these polymers makes the bagasse rich in hydroxyl and phenolics. In the straw, cellulose represents 40%, hemicellulose 30%, lignin 25% and the rest are minority components such as ashes and minerals. These minority components are two to four times higher than those of bagasse although it varies depending on the site of harvest, climatic conditions and vegetative development stages **(Gomez *et al.,* 2010; Paes *et al.,* 2005)**.

Sugarcane has several phenolic compounds in its constitution, which have increasingly attracted the attention of researchers due to its various activities, clinical potential and beneficial effect as a functional food **(Takara *et al.,* 2007)**. The most common compounds present in the sugarcane are naringenin, apigenin, tricin and lutolin derivates. These compounds have health benefits associated to antioxidant capacity and potential to protect cells from degenerative process and reduce the development of cancer and cardiovascular disease **(Alves *et al.,* 2016)**.

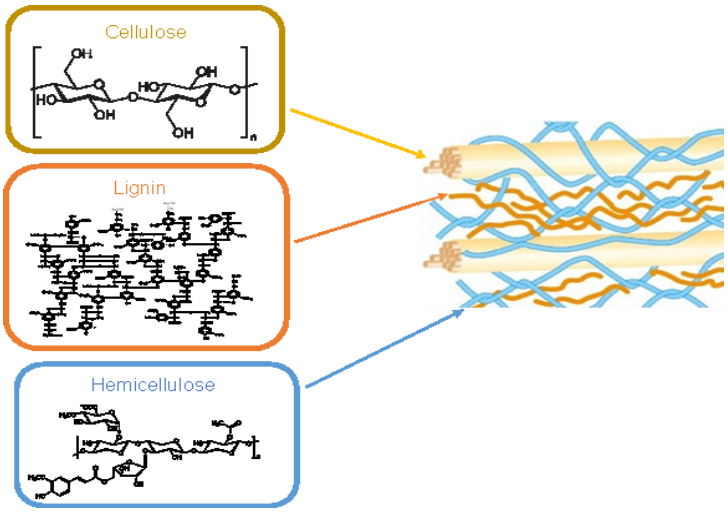

Figure 2.2. Structure of lignocellulosic biomass with cellulose, hemicellulose and lignin represented.

Cellulose is composed of β-glucose molecules that are joined through β-1,4 glycosidic bonds and result in a polymer of high molecular weight and a degree of polymerisation based on glucose units between 1,000 and 15,000 **(Hasan *et al.,* 2018)**. These molecules are linear, fibrous, and moist, with high tensile strength, insoluble in most solvents and form inter- and intramolecular hydrogen bonds so they are the structural basis of vegetative cells and are found in abundance in nature **(Seddiqui *et al.,* 2021)**. Hemicellulose, present in fibrous vegetal, is a non-cellulosic polysaccharide composed of several sugar units: β-D-xylose, β-D-mannose, β-D-glucose, α-L-arabinose, α-D-galactose and with small, branched molecular chains. The hemicelluloses are subdivided into four categories that depend on the number of linked monosaccharides found in the main chain: arabinans, xylans, mannans and xyloglucans. Its main function is to reinforce the plant cell wall by associating it with cellulose and lignin molecules **(Ebringerová *et al.,* 2005)**.

Finally, lignin is in the class of organic polymers and is found in the vascular tissues of plants with a very important role in the formation of the cell wall which makes it rigid, facilitates the transport of water and prevents the degradation of the polysaccharide wall. This polymer is aromatic, branched amorphous and heterogeneous, consisting of three phenylpropane repeat monomers: p-coumaryl alcohol, sinapyl alcohol and coniferous alcohol. Depending on its plant origin, which can be long-fibre wood (conifers), short-fibre wood (hardwoods) and grasses, its composition and basic structure may vary **(Welker *et al.,* 2015)**. Sugarcane is a grass and therefore the formation of lignin involves the polymerisation of the three monomers.

2.2. Fermentation

2.2.1. Type of Industrial Fermentation

There are different types of fermentation processes established in the industry which vary according to the type of product that which fermentation is intended to. The three most used fermentation

processes at industrial scale are: Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) and Sequential Saccharification and Fermentation (SQSF) **(Devarapallim *et al*., 2015)**.

Separate Hydrolysis and Fermentation (SHF) is carried out in two steps: an enzymatic saccharification at temperatures between 45-50 ºC and a pH of 4.8, followed by a fermentation under optimal conditions (temperature between 30-35 ºC and pH between 4.5-5) to obtain the highest number of monosaccharides. This process is individually good and can handle a large solid load through intermittent feeding **(Taharzadeh *et al*., 2007)**.

Simultaneous Saccharification and Fermentation (SSF) combines the two steps in a single operating unit, with good productivity and ethanol yield. It is carried out at constant conditions, with a temperature of 37 ºC and a pH of 5. Its main advantages are the reduction in the number of vessels required which leads to a reduction in investment costs, the use of monosaccharides that are released in saccharification, the non-formation of inhibitory components in saccharification and therefore an increase in the overall performance of the process and there is no need to separate the glucose from the lignin fraction which leads to avoid sugar losses **(Taharzadeh *et al*., 2007)**. This process normally works with the same optimal parameters but has as a major disadvantage, the difficulty to recycle the enzyme and microorganism in the fermentation due to its difficulty to separate from the lignin, and because of this yield loss occurs. To use the SSF process, the microorganisms must have tolerance to inhibitors and temperature and the ability to utilize multiple sugars **(Olofsson *et al*, 2008)**.

The Saccharification and Sequential Fermentation (SQSF) begins with pre-hydrolysis for 24 h and it follows the SSF process. This process is more advantageous than the others due to its higher productivity, yield and ethanol concentration.

2.2.2. Fermentative probiotic bacteria and yeast

Lactic acid Bacteria (LAB) are known to ferment carbohydrates and produce lactic acid, acetic acid and ethanol as main end products. Due to their morphological, metabolic and physiological characteristics these bacteria have more than 20 different genera, although for the food industry only 11 are of interest, these being: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. Orla-Jensen classifies LABs according to their morphology (cocci or rods, tetrad formation), mode of glucose fermentation (homo- or hetero-fermentation), growth at certain "cardinal" temperatures (e.g. 10 °C and 45 °C) and range of sugar utilisation **(De Vuyst *et al*., 2012)**. They can be found in nutrient-rich environments such as feed and food, animal and human gastrointestinal tract and saliva.

Lactobacilli are widely used because they are a very heterogeneous species, thus encompassing a wide range of phenotypic, biochemical and physiological properties. They are thus divided into three major groups: I - obligate homofermentative; II - facultative heterofermentative; III - facultative heterofermentative. These groups are distinguished by the presence or absence of homo- and heterofermentative sugar metabolism, fructose-1,6-diphosphate aldolase, phosphoketolase, CO2 from glucose and CO2 from gluconate **(Salminen *et al.,* 1993)**.

L. plantarum belongs to group II - facultatively heterofermentative, which means that there is the presence of pentoses in fermentation, FDP adolase, and when fermented there is the presence of CO_2 from gluconate and phosphoketolase. Only glucose CO_2 is absent. This strain can grow at temperatures between 15-45 ºC and pH values as low as 3.2. It has a great ability to ferment a huge variety of carbohydrates and is therefore used as a probiotic culture in fermentations of vegetable and animal raw materials. In human food, it can be found in pickles, olives and fermented pasta and has several applications in this area, such as improving the sensory characteristics of food and prolonging the shelf life and safety of fermented products. Already in human health it is used in areas such as immunology, gastroenterology, urology and the gut-brain axis **(Todorov *et al.*, 2010)**.

L. fermentum belongs to group III - obligately heterofermentative, which means that there is the presence of CO_2 from glucose, phosphoketolase and when fermented there is the presence of CO_2 from gluconate. Pentose fermentation and FDP adolase is absent. This strain can grow at temperatures above 15 ºC, but its optimum temperature is 45 ºC and a pH value between 5-7. This strain is commonly found in dairy preparations such as yoghurt, cheese and fermented milk, as well as in probiotic supplements. In health, it helps fight obesity, harmful bacteria and infections, reduce cholesterol, boost immunity and improve memory and learning deficits **(Naghmouchi *et al.*, 2020)**.

Saccharomyces cerevisiae is a robust yeast that can withstand stressful conditions and has a high fermentation efficiency. It grows rapidly and therefore has efficient sugar utilization, a high capacity to produce and consume ethanol, a tolerance to high ethanol concentrations (100 g/L) and low oxygen levels. This yeast, under optimal conditions, has an ethanol production yield higher than 0.45 g/g and a specific rate up to 1.3 g/ g cell mass h-1. For optimum growth the optimum temperature is between 30-35 ºC. It is classified as a top fermenting strain and by replication produces biologically active compounds such as oligosaccharides, organic acids, amino acids and peptides **(Lodolo *et al.*, 2008)**.

2.2.3. Saccharification process

Sugarcane's main component, cellulose, can be hydrolysed to produce glucose, expanding the substrate's potential applications. This hydrolysis is a big challenge due to the difficult digestibility of the cellulose structure **(Kumar *et al.*, 2021)**. Biomass can be hydrolysed by enzymes that act synergistically in the process of converting cellulose into glucose. To improve the process, it is necessary for the biomass to go through a pre-treatment process to increase the accessibility of the enzyme to the cellulose and to improve the hydrolysis ability of the fibre. This process aims to degrade the lignin structure and disturb the crystalline structure of the cellulose to obtain potentially fermentable sugars during saccharification stage. Several pre-treatments have been developed such as physical (grinding and milling), physicochemical (steam pre-treatment), chemical (alkali, dilute acid, ionic liquid, oxidising agents and organic solvents), biological (microbial delignification), or a combination of these (e.g., thermos-chemical, treatments) **(Kumar *et al,* 2009)**. For each biomass feedstock the best pre-treatment technology should be adopted to cover all properties.

Saccharification of lignocellulosic biomass often requires a cocktail of cellulolytic enzymes in a simultaneous one-step hydrolysis of the substrate. However, variables like pH, temperature, and the enzyme-substrate ratio have varying effects on the activity of different enzymes. Process optimization

is therefore necessary in a complex multi-enzyme process, such as the saccharification of lignocellulosic biomass, to reduce the impact of interactions between enzymes and process factors **(Dorleku *et al,* 2022)**.

Saccharification can take place in two different ways: through acid saccharification, which uses pasteurization reactors at high temperatures, or through enzymatic saccharification, which uses cellulases and is less polluting because it takes place at low temperatures without pasteurization. In the saccharification process, the activity of the hydrolytic enzymes is measured by the amount of enzyme requires to produce 1 µmol of reducing sugar (D-glucose) per min under optimum condition.

Cellulases are enzymes that break down cellulose (β-1,4-D-glucan linkages) and procedure as primary products glucose, cellobiose and cello oligosaccharides and are used in various industries, such as food. Cellulases can be of 3 types: endoglucanases, exoglucanases and beta-glucosidases. Endoglucanases act on the inside of the cellulose fibre, releasing oligosaccharides (small sugars), exoglucanases act at the ends of the cellulose fibre, releasing cellobiose, and beta-glycosidases break the chemical bonds which form cellobiose, releasing glucose units (**Figure 2.3.**) **(Canilha *et al.,* 2012)**.

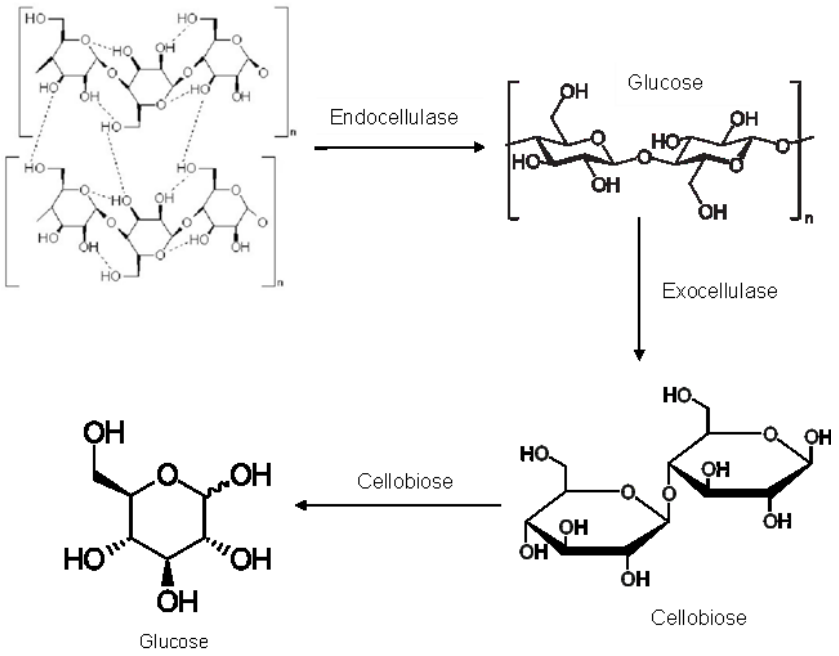

Figure 2.3. Example of the three stages of the cellulose hydrolysis by cellulose enzyme complex.

Hemicellulases are enzymes that break down hemicelluloses from plant biomass, including xylans, xyloglucans, arabinoxylans and glucomannans. The enzyme used was xylanase, which degrades the linear polysaccharide beta-1,4-xylan into xylose and consequently decomposes the hemicellulose. It has several commercial applications, such as increasing the digestibility of silage for animal feed, improving baking and the production of alcoholic beverages (**Figure 2.4.**) **(Canilha *et al.,* 2012)**.

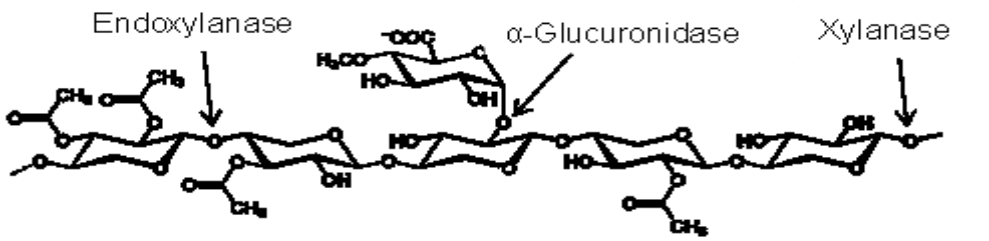

Figure 2.4. Xylan degrading and accessory hemicellulases.

2.3. Fermented foods

Fermented foods have been part of community culture for many centuries, even before any direct health benefits were truly understood, and this is due to the constant development of methods to pre-serve perishable foods such as milk, meat and vegetables. In the last few years, the mechanisms by which these fermentations can benefit human health have begun to be elucidated and so there have been more recommendations for their consumption through dietary guidelines. Fermented foods make up on average 30% of the world's diet and so the industry is bringing these products increasingly to the market. The growth in market demand for fermented foods is due to the interest consumers have in healthy foods and the good sensory attributes of these products **(Hugenholtz 2013)**.

Food products undergo a substantial and distinctive transformation due to microbial metabolism during fermentation in terms of their sensory, physical, and nutritional. It would take a complex set of physicochemical processes to achieve an equivalent result as microbial fermentation (Steinkraus 2004). Fermented foods promote health and well-being, and this is due to the presence of probiotic microorganisms, bioactive components and micronutrients **(Sachchan *et al.,* 2022)**.

Initially, fermented products were yoghurt, cheese, fermented sausages, more recently fermented drinks such as kefir and kombuchá have appeared **(Netsanet Shiferaw Terefe, 2016)**. In **Table 2.1** we can see some fermented products that have appeared over time and we can see that more and more new products will appear on the market due to a greater exploration of microorganisms and fermentations.

Table 2.1. Products for sale that use fermentation and bacteria.

Commercial name	Microorganism	Target food for use	Manufacturer
Lyofast LPR A	*L. plantarum* and *L. rhamnosus*	Dairy products	Italy
Kefir	*Lactobacillus* and *Bifidobacterium*	Milk	Russia
Actimel	*L. bulgaricus* and *Streptococcus thermophiles*	Yogurt	France
Camembert	Lactic acid bacteria	Chesse	South Africa
Alheiras	Lactic acid bacteria	Fermented sausages	Portugal
FreshQ®	*L. rhamnosus* and *L. paracasei*	Yogurt and cheese	Denmark
Befresh™	A group of bacterial strains including *Lactobacillus* spp. and *Propionibacterium freudenreichii* subsp. *Shermanii*	Fresh and fermented dairy and fruits	Belgium

Balanced nutrition and a healthy intestine are important for the well-being of human beings. The intestinal mucosa is a large surface area of 200 m^2 and is responsible for most of the absorption of nutrients and for getting the most out of everything we eat. This entire function is carried out with the help of resident commensal micro-organisms (microbiota) which participate in the degradation of complex macromolecules. The microbiota and the host co-exist harmoniously and offer benefits to each other. These benefits include stimulating the immune system, improving digestion, food absorption, reducing the growth of pathogenic flora and the integral maintenance of the intestinal barrier. These effects can be observed locally but also in the different organs of the human body due to the systemic distribution of substances and cells produced in the intestine. When the composition of the microbiota is dysregulated for various reasons, it can lead to allergic or autoimmune diseases (inflammatory bowel disease, type 1 diabetes), cancer and psychiatric disorders, particularly in susceptible individuals. As a result, we have begun to study strategies and therapies to keep the microbiota as healthy as the human being **(Tsilingiri *et al.,* 2012)**.

Thus, there are four ways of modulating the microbiota, using prebiotics, probiotics, symbiotics and more recently the use of postbiotics. Definitions of these concepts have been published by International Scientific Association of Probiotics and Prebiotics (ISAPP). Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”, whereas a prebiotic is a “substrate that is selectively utilized by host microorganisms conferring a health benefit”. A symbiotic are “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host”. Postbiotic is a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” **(Salminen *et al.*, 2021)**.

Postbiotics are differentiated in their elemental composition as they contain lipids (e.g. butyrate, propionate), proteins (e.g. lactocepin), carbohydrates (e.g. galactose-rich polysaccharides and teichoic acids), vitamins (B group), organic acids (e.g. propionic acid), complex molecules (e.g. peptidoglycan-derived muropeptides, lipoteichoic acids), metabolites, short-chain fatty acids (SCFAs), microbial cell fraction, extracellular polysaccharides (EPS) and cell lysate. It also has various physiological functions (**Figure 2.5.**) such as immunomodulating, anti-inflammatory, hypocholesterolemic, anti-obesogenic, anti-hypertensive, anti-proliferative and antioxidant effects. **(Aguilar-Toalá *et al.*, 2018)**.

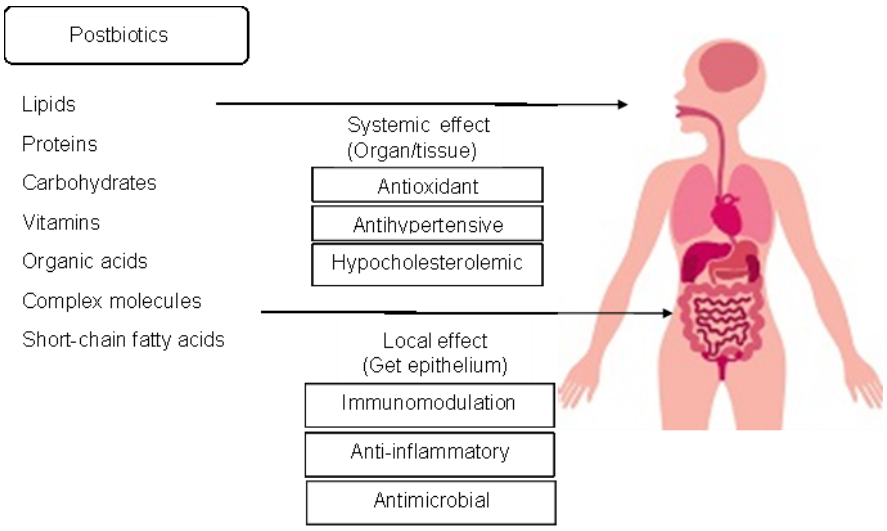

Figure 2.5. Examples of postbiotics and their potential local systemic positive effects in the host.

The application of probiotic and prebiotic is not 100% beneficial, as resistance to antibiotics may occur, the viability of the bacteria may not be maintained during the manufacture and storage of the product, because there may be interaction with other microbial species, acidity, water activity and temperature, among others. On the other hand, postbiotics are more stable than live bacteria, the peptides have antimicrobial properties and are therefore water soluble and active over a wide pH range, which makes their application permitted in a wide range of food products (**Table 2.2.**). A major advantage of postbiotics is their favourable safety profile because they do not have to absorb millions of live microbes. Although several foods are naturally abundant in postbiotics (e.g yogurt, kefir, pickled vegetables and kombucha) or their precursors **(Chaluvadi, Hotchkiss, & Yam, 2016)**, some postbiotics have been intentionally applied to certain foods rather than considering its *in situ* production by the producer strains.

Table 2.2. *In vitro* and *in vivo* studies of postbiotics and their bioactivity and/ or effects.

Bacteria	Components	Type of study	Bioactivity or effect	References
L. plantarum K8 (KTCT10887BP)	Lipoteichoic acids	Human monocyte THP-1 cells	Immunomodulation	Kim et al. (2011)
L. paracasei B2106o	Cell-free supernatants	Human mucosa explant of colon	Anti-inflammatory	Tsilingiri et al. (2012)
L. plantarum RG11, RG14, RI 11, UL4, TL1 and RS5	Cell-free supernatants	*In vitro*	Antimicrobial	Kareem et al. (2014)
L. fermentum BGHV110	Cell lysate suspension	Human hepatoma HepG2 cells	Hepatoprotective	Dinié et al. (2017)
L. casei ATCC 393	Sonicated-cell suspension	Murine CT26 and human HT29 colon cancer cells line	Antiproliferative	Tiptiri-Kourpeti et al. (2016)
7 *Bifidobacterium,* 11 *Lactobacillus*, 6 *Lactococcus*, and *10 Strep. Thermophiles* strains	Intracellular content	*In vitro*	Antioxidant	Amaretti et al. (2016)

2.4. Objectives

Therefore, the aim of this research work was to produce postbiotic extracts for food application through sustainable fermentation of sugarcane by-products and to validate their nutraceutical potential.

The specific objectives were the following:

- Optimize the production process of postbiotics using sugarcane straw and bagasse as main substrate and three different microorganisms (*Lactobacillus plantarum, Lactobacillus fermentum* and *Saccharomyces cerevisiae*) in fermentation;
- Characterized the obtained extracts for its chemical and biological properties targeting food application, like the antioxidant, antimicrobial, prebiotic and antidiabetic potential, cytocompatibility, total protein, phenolic content and compound and their profile of organic acid and monosaccharides;
- Evaluate the potential of the final postbiotic extracts as modulator of human intestinal microbiota and immune system.

3. Materials and Methods

3.1. Optimization of the process of production of postbiotics

3.1.1. Saccharification conditions

The sugarcane bagasse and straw (*Saccharum officinarum,* L.) was collected from Paraíso and Bonfim provided by Raízen (São Paulo, Brazil). Samples were transported to the CBQF-UCP laboratory (Porto, Portugal) and immediately dried in an oven (Nabertherm, Porto Salvo, Portugal) at 40 ºC and grinded (knife mill SM100, Retsch, Vila Nova de Gaia) to a particle size < 4mm. After that, the sugarcane bagasse and straw were sieved for 10 min, with an amplitude of 100 and on a sieve 900 µm.

To verify the best saccharification conditions, milled and unmilled sugarcane bagasse and straw were used. The biomass was milled in a kitchen robot until it became powdered, while the unmilled biomass was only sieve as previously described. For the fermentation the unmilled biomass was used.

Five grams of each sample was weighted into 50 mL flask, and supplement with 10 g/L peptone (Sigma-Aldrich; Missouri, USA), 5 g/L yeast extract (Sigma-Aldrich; Missouri, USA), 2 g/L tri-Ammonium citrate ($C_6H_{14}N_2O_7$; VWR International; Pennsylvania, USA), 2 g/L potassium phosphate dibasic (K_2HPO_4; Honeywell; North Carolina, USA), 5 g/L sodium acetate (Nao_2CCH_3; Merk KGaA; Darmstadt, Germany), 0.1 g/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$; Sigma-Aldrich; Missouri, USA) and 0.05 g/L manganese (II) sulfate tetrahydrate ($MNSO_4 \cdot 4H_2O$; Merk KGaA; Darmstadt, Germany). These supplements were added to the sodium citrate buffer solution which contains 4.31 g/L of citric acid ($C_6H_8O_7$; AppliChem GmbH) and 8.68 g/L sodium citrate ($NA_3C_6H_5O_7$; Sigma-Aldrich; Missouri, USA). Following this, the flasks are autoclaved at 121 ºC for 10 min.

The saccharification was performed in the two raw material (sugarcane bagasse and straw). Each raw material was in two different states (milled and unmilled) and three different saccharification processes were performed for each state at two different times (24 h and 48 h), without enzyme, with the enzyme Celluclast (for bagasse 46 FPU/mL and for straw 36.2 FPU/mL) (Novozyme, Denmark), and with a mixture of Celluclast and shearzyme plus (Novozyme, Denmark).

The biomass and enzymes were added to both biomass in three different conditions 1) only biomass without enzymes, 2) biomass and Celluclast enzyme and 3) biomass and both of enzymes (Celluclast and sherzyme plus). All the conditions were performed in duplicate.

During saccharification process, samples (4 mL) were collected at times 0, 24 and 48 h, being 3 mL stored at -4 ºC for further analysis and 1 mL was directly used to measure total sugars presents in the sample.

3.2. Fermentation conditions

3.2.1. Microorganisms

Bacteria strains were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DMS; Germany) and the National Collection of Industrial Food and Marine Bacteria (NCFB; United Kingdom). The strains used for fermentation were *Lactobacillus plantarum* 299v (DSM 9843) and *Lactobacillus fermentum* (NCFB 1750). Overnight inoculums were prepared in Man-Rogosa-Sharpe broth medium (MRS broth; Biokar Diagnostics; Allonne, France) and incubated at 37 ºC.

Yeast strain *Saccharomyces cerevisiae* (DSM 70449) was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DMS; Germany), overnight inoculum prepared in Yeast Malt broth (YM; Biokar Diagnostics; Allonne, France) incubated at 30 ºC.

3.2.2. Simultaneous Saccharification and Fermentation (SSF)

At the end of saccharification, *L. plantarum, L. fermentum* and *S. cerevisiae* were inoculated in the sugar rich media at different final cellular concentration. For *L. plantarum,* final cellular concentrations ranged from 10^4-10^7, for *L. fermentum*, final cellular concentrations ranged from 10^4-10^5 and for *S. cerevisiae*, final cellular concentrations ranged from 10^4-10^6. Fermentations were carried out for 0, 24, 48 and 72 h at 30 ºC, in duplicate. The entire sample content was used for the preparation of the cell free extract.

At 0, 24, 48 and 72 h, samples were taken to evaluated cellular concentration, pH measurements and total sugar content. The remaining non used sample was stored at 20ºC for further analysis.

3.2.3. Sequential Saccharification and Fermentation (SQSF)

For SQSF, the extracts that resulted best in SSF were chosen. At the end of saccharification, the raw material is removed by centrifugation and then flame filtration using a 0.22 µn filter and placed only the liquid in other Erlenmeyer previously autoclaved at 121ºC for 10 minutes. The liquid goes to fermentation and the biomass was discarded. After that, *L. plantarum* and *S. cerevisiae* were inoculated in the sugar rich media at different final cellular concentration. For *L. plantarum,* final cellular concentration was 10^5 CFU/ mL and for *S. cerevisiae*, final cellular concentration was10^6 CFU/ mL. Fermentations were carried out for 0, 24, 48 and 72 h at 30 ºC, in duplicate. For the preparation of the cell free extract only the liquid part where the fermentation occurred was use.

At 0, 24, 48 and 72 h, samples were taken to evaluated cellular concentration, pH measurements and total sugar content. The remaining non used sample were stored at 20ºC for further analysis.

3.3. Preparation of cell free extracts

At the end of all fermentations, mechanical cell lysis took place using a sonicator CY-500, Optic Ivymen System (Comecta; Barcelona, Spain). Sample were sonicated during 10 min with pulses of 15 s on and 45 s off, with a temperature of 20 ºC and an amplitude of 70 %.

Following sonication, in extracts that performed an SSF, physical centrifugation occurred for 15 min at 4480 Relative Centrifugal Field (RCF). This centrifugation took place so that all insoluble material is removed. As for the extracts that performed SQSF, there was a differential centrifugation for 30 min at 769 RCF. This centrifugation occurs so that only the cells that are intact are removed and all cell fragments are maintained.

Lastly, the supernatant was removed and store at -80 ºC. Samples were freeze-dried to obtain a powder (gamma 2-16 LSCplus, Martin Christ, Osterode am Harz, Germany) and then were used for all the chemical and biological analyses

3.4. Analytical methodology

3.4.1. Chemical characterization

3.4.1.1. Total sugar content by Phenol-sulphuric method

Total sugars were determined at 0, 24, 48 h of saccharification and at 0, 24, 48 and 72h of fermentation using the phenol-sulphuric acid method, described by **Dubois *et al.*, 1951**.

Glucose was used for the calibration curve and the concentrations used were between 0.31–5.00 mg/mL.

Samples (1 mL) were centrifuged for 10 min at 4480 RCF and diluted to half by mixing with deionised water.

In a test tube, 80 µL of the sample, 150 µL of phenol solution (5% v:v) and 1 mL of sulphuric acid were added. The tubes were kept in the oven at 100 °C for 10 min. Finally, the sample absorbance was measured in a spectrophotometer (Shimadzy, Kyoto, Japan) at 490 nm. All assays were done in duplicate and results presented in mg/ mL.

3.4.1.2. Cellular concentrations and pH measurement

Viable counts were determined at 0, 24, 48 and 72 h performing decimal dilutions in peptone water, plated using the drop method described by Miles and Misra (1938) on MRS agar (Biokar Diagnostics; Allonne, France), incubated at 37 ºC for 24 h for lactobacilli species while for *S. cerevisiae,* samples were plated in PDA (potato dextrose agar) using spread technique, and incubated at 30 ºC during 24 h. All assays were performed in duplicated and total count results were plotted as a plot of log CFU versus time.

The pH values were measured with the Seven Compact pH meter with a InLab Expert Pro-ISM pH electrode (Mettler, Toledo; USA), at the end of fermentation for each sample in duplicate.

3.4.1.3. Determination of Antioxidant Capacity

3.4.1.3.1. ABTS radical cation

The total antioxidant capacity was determined with the sample diluted in 3 mL of deionized water using 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) radical cation assay as described by **Gião *et al.*, (2007)**. The solution of $ABTS^{\bullet+}$ was obtained by adding 0.03834 g of ABTS (Sigma-Aldrich; Missouri, USA) in 10 mL of deionized water and 0.0066 g of potassium persulfate ($K_2O_8S_2$, Sigma-Aldrich, Sintra, Portugal) in 10 mL of deionized water. The two solutions were combined and allowed to stir overnight in dark.

The concentrated solution was diluted in deionized water to obtain an initial absorbance of 0.700 ± 0.020 at 734 nm. The extracts were initially prepared at 30 mg/mL and the subsequent dilutions 0.35-15.0 mg/mL were analysed. In a 96 well microplate, 15 µL of each sample was added and 15µL of deionized water was used as the blank.

For the calibration curve it was used trolox (0.0075 mg/ mL to 0.0750 mg /mL). In all wells it was added 200 µL of diluted $ABTS^{\bullet+}$ solution and the optical density was read in a microplate reader (Synergy H1; BioTek, Vermont, USA) at 30 ºC for 5 min. Results were expressed by the EC_{50} (mg/g dw).

3.4.1.3.2. DPPH

The 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical assay was performed as described by **Brand-Williams *et al.,* (1995)**. The DPPH˙ solution was obtained by adding 0.024 g of DPPH (Alfa Aesar, Thermo Fisher Scientific; Massachusetts, USA) in 100 mL of methanol. This stock solution can be stored for a maximum of one month at -20 ºC protected from light.

The concentrated solution was diluted in methanol to obtain an initial absorbance of 0.600 ± 0.100 at 515 nm. The extracts were initially prepared at 30 mg/mL and the subsequent dilutions 5.62-15.0 mg/mL were tested. In a 96 well microplate, 25 µL of the sample, and 25 µL of deionized water was used as the blank. For the calibration curve it was used trolox (0.0075 mg/ mL to 0.0750 mg /mL).

In all wells 175 µL of $DPPH^{\cdot+}$ solution was added, and the optical density was read in a micro-plate reader (Synergy H1; BioTek, Vermont, USA) at 30 ºC for 30 min. Results were expressed by the EC_{50} (mg/g dw).

3.4.1.3.3. Oxygen Radical Absorption Capacity (ORAC)

The antioxidant capacity was determined using the oxygen radical absorption radical assay which measures the fluorescence degradation with of 2,2'-azobis(2-amidinopropane) dihydrocloride (AAPH) as described by **Contreras *et al.,* (2011)**.

For the PBS buffer, 9 g of monosodium phosphate (NaH_2PO_4; Sigma-Aldrich, Sintra, Portugal) was dissolved in a litre of deionized water and pH value adjusted to 7.44 at 20 ºC. For the fluorescein stock solution, 0.01097 g of fluorescein disodium salt ($C_{20}H_{10}Na_2O_5$; Sigma-Aldrich, Sintra, Portugal) was dissolved in 25 mL of PBS and stored at 4 ºC protected from light.

For the fluorescein solution, 100 µL of the stock solution is diluted in 10 mL of PBS, and then 250 µL of the previous solution was placed and completed with 25 mL of PBS (116.66 nM).

For the trolox solution, 0.0125 g of trolox was weighed and dissolved in one mL of methanol and completed with 50 mL of PBS. For the working solution, 1 mL of the previous solution was removed and completed with 10 mL of PBS (T0 solution) and the dilutions were made for the calibration curve (10-80 µM). For the AAPH solution, 0.13018 g were weighed and dissolved in 10 mL of PBS.

In a 96 well microplate, 20 µL of the sample was added (initial concentration of 16.67 mg/mL), 20 µL of each trolox concentration, 20 µL of PBS (blank), 80 µL of PBS (control) and in all wells was added 120 µL of fluorescein solution. The mixture was incubated for 10 min at 37 ºC in a microplate reader (Synergy H1; BioTek, Vermont, USA). Finally, 60 µL of AAPH were added except for the control and incubated for 120 min in the same microplate reader and fluorescence measured (Ex=485, Em=580).

Results were expressed by the µM Trolox equivalents per g dw.

3.4.1.4. Identification and quantification of organic acids and monosaccharides – HPLC-RID

To characterize the profile of organic acids and monosaccharides, the sample was prepared to concentration 25 mg/mL and filtered with 0.45 µm filter (Minisart; Sartorius stedium, Gottingen, Germany). Chromatographic analysis was performed with high performance liquid chromatography, Agilent 1200 HPLC system with a refractive index (RI) detector (1260 Infinity II, Agilent Technologies; California, USA) operating at 50 ºC. Separation was performed in the Aminex HPX87Pb column (BioRad, Hercules, CA) with a mobile phase of 0.005 M of sulphuric acid (H_2SO_4) at a flow rate of 0.6 mL/min.

The different peaks were analysed by comparing the retention times with the standards of organic acids (malic, lactic, acetic, butyric and ethanol) and sugars (xylose, mannose, galactose, glucose, arabinose fructose) (Sigma-Aldrich, Sintra, Portugal). Quantification was performed by interpolation of samples area into the calibration curve of each standard according to the **equation 3.4.1.4.1**. Samples were analysed in triplicates and results expressed in µg/g dw.

$$Conc.(g/L) = (Area \pm Intersection\ at\ origin)/slope \qquad \textbf{Equation (3.4.1.4.1)}$$

3.4.1.5. Characterization of total protein

Total protein was determined with 0.1 g of sample using a Velp NDA 701 Dumas Nitrogen/Protein analyser (PLT Scientific Sdn Bhd, Selangor, Malaysia). This method starts with a combustion furnace to burn the sample, obtaining elemental compounds. Water was removed by a first physical trap and a second chemical one. Between the two, the elemental substances pass through a reduction furnace. The auto-regenerative CO_2 absorbers let only the elemental nitrogen pass, which is detected by Thermal Conductivity Detector (Innovative TCD autocalibrating; PLT Scientific Sdn Bhd, Selangor, Malaysia).

3.4.1.6. Total Phenolic Content – Folin Ciocalteu's assay

The Folin Cicalteu's assay was used to quantify the total phenolic compounds by absorbance, according to the method described by **Singleton *et al.,* (1965)**. For the analysis samples were previously prepared at 25 mg/mL in deionised water.

In a test tube 50 µL of sample was mixed with 50 µL of Folin Cicocalteu's reagent at 1 N (Sigma-Aldrich, Sintra, Portugal), 1000 µL of sodium carbonate (Na_2CO_3; Sigma-Aldrich, Sintra, Portugal) at 7.5% (w/v) and 1400 µL of deionized water. The mixture was incubated for 1 h at room temperature and in the dark and then the absorbance read at a wavelength of 750 nm.

The total phenolic were calculated according to the calibration curve prepared with gallic acid (Sigma-Aldrich, Sintra, Portugal) (0.493 to 0.062 mg/mL) and the results are given in mg of gallic acid equivalent to g of dw extract media according to the **equation 3.4.1.6.1**.

$$Total\ phenolic\ compound\ \left(\frac{g}{L}\right) = \frac{Abs\ of\ the\ sample - b}{m} \qquad \textbf{Equation (3.4.1.6.1)}$$

b – Intersection of the gallic acid calibration curve

m – Slope of the gallic acid calibration curve

3.4.1.7. Individual Phenolic Compounds Identification by LC-ESI-QqTOF-HRMS

The extracts were analysed by LC-ESI-UHR-QqTOF-MS [27] by previously dissolving 50 mg dry extract in 1 mL of ultra-pure water. The separation was performed in a UHPLC UltiMate 3000 Dionex (Thermo Scientific), coupled to an ultrahigh resolution. Qq-time-of-flight (UHR-QqTOF) mass spectrometer with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany). Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100 mm × 2.1 mm, 2.2 µm) (Dionex). Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Separation was carried out over 24.5 min under the following gradient conditions: 0 min, 0% B; 10 min, 21.0% B; 14 min, 27% B; 18.30 min, 58%; 20.0 min, 100%; 24.0 min, 100%; 24.10 min, 0%; 26.0 min, 0% at a flow rate of 0.25 mL/min. The injection volume was 5 µL. An ultrahigh-resolution quadrupole−quadrupole time-of-flight (UHR−QqTOF) mass spectrometers used.·Parameters for MS analysis are set using negative ionization mode with spectra acquired over a range from m/z 20 to 1000in an Auto MS scan mode. The selected parameters are as follows: End plate off set voltage, 500 V; capillary voltage, 3.0kV; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision radio frequency (RF), from 250 to 1000Vpp; transfer time, from 25 to 70 µs; collision cell energy, 5 eV; and pre-pulse storage,6µs.·Post-acquisition internal mass calibration used sodium formate clusters, with sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

The elemental composition for the compound was confirmed according to accurate mass and isotope rate calculations designated mSigma (Bruker Daltonics). The accurate mass measurement was within the lowest elemental composition, and mSigma values provided confirmation. Compounds were identified based on its accurate mass $[M\text{-}H]^-$.

Quantification (mg/mL) was performed with the values of the areas by interpolation to the calibration curves (citric, malic, azelaic, ferulic, neochlorogenic, ρ-coumaric acid, schaftoside, 4-Hydroxybenzaldehyde and 3,4-Dihydroxybenzaaldehyde) (Sigma-Aldrich, Sintra, Portugal). The final concentration (mg/g dw) of each identified compound was achieved according to **equation 3.4.1.7.2**.

$$C\left(\frac{g}{g}dw\right) = \frac{C\left(\frac{mg}{mL}\right) * V_{water}\,(mL)}{m_{dry\ extract}\,(g)} * 1000 \qquad \textbf{Equation (3.4.1.7.2)}$$

3.4.2. Biological activity

3.4.2.1. Antimicrobial activity

The pathogenic bacteria strain was obtained from the American Type Culture Collection (ATCC; Manassas, USA). The strain used was *Staphylococcus aureus* (ATCC 25923), and it was reactive and prepared in Trypticase Soy Broth (TSB; Biokar Diagnostics; Allonne, France) and incubated at 37 ºC overnight. The pathogenic bacteria inoculum was reactivated in Muller Hinton Broth (MHB;Biokar Diagnostics; Allonne, France) medium at 37 ºC overnight and then adjusted in the spectrophotometer to an OD between 0.8 to 1.0, for a wavelength of 660 nm.

The lyophilized sample was diluted to 5 mL in MHB. The initial concentration was 50 mg/mL and the subsequent dilutions were 40, 30, 20 and 10 mg/mL.

To a 96 well plate was added 200 µL of positive control (inoculum with MHB) negative control (MHB) and sample. Finally, the samples absorbance was read in a microplate reader (Epoch 2, Bio-Tek,Vermont, USA) for 24 h. All assays were done in triplicate.

3.4.2.2. Antidiabetic activity

The antidiabetic activity was evaluated by the α-glucosidase inhibition activity. The α-glucosidase inhibition was calculated using a colorimetric based quantitative method with α-glucosidase from *L.plantarum* and *S. cerevisiae* determined by a reaction where sample hydrolyses ρ-nitrophenyl – α – D-Glucopyranoside resulting in a colorimetric product that absorbs at 405 nm which is proportional to the α-glucosidade activity present in the sample.

It was prepared one litter of a 0.1 M phosphate buffer solution with a pH value of 6.9. For that, it was weighted 15.6 g of dibasic sodium phosphate dihydrate ($HNa_2O_4P{\cdot}2H_2O$; Merk KGaA; Daemstadt, Germany) and 17.8 g of monobasic sodium phosphate dihydrate ($H_2NaO_4P{\cdot}2H_2O$; Merk KGaA; Darmstadt, Germany) in 100 mL of deionized water each one. To make the final volume 800 mL of deionized water was added and the pH to 6.9.

The freeze-dried sample was used and diluted in 4 mL of deionized water. The final extracts obtained from bagasse (1.56 mg/mL) and straw (3.12 mg/mL) were weighed and 5 dilutions in water of each were made in a proportion of 1:2 (v:v).

The α-glucosidase enzyme (1 U/mL), α-glucosidase substrate (5 mM) and the positive control Acarbose (10 mg/mL) were prepared. To prepare the α-Glucosidase 0.1 mg of enzyme was weighed and then added to 10 mL of buffer solution prepared previously. The substrate was weighed (9.05 mg) and then added to 6 mL of buffer solution and the acarbose (50 mg) was added to 10 mL of ultra-pure water and further diluted in a 1:2 (v:v) ratio.

In a 96 well microplate it was added 50 µL of ultra-pure water, 50 µL of acarbose (positive control) between 1-10 mg/mL) and 50 µL of samples at several dilutions, all in triplicate. In all wells 100 µL of the enzyme solution was added and incubated at 25 ºC for 10 min in a microplate reader (Epoch 2, BioTek; Vermont, USA). Finally, 50 µL of the substrate was added to each line and read at 405 nm at time 0 and 5.

The α-glucosidase inhibitory activity was calculated using the formula in **equation 3.4.2.2.1**:

$$\%\ inhibition = \frac{\Delta Abs\ CNT - \Delta Abs\ S}{\Delta Abs\ CNT} \times 100 \qquad \textbf{Equation (3.4.2.2.1)}$$

Δ Abs S is the difference between the sample absorbance measured between 0-5 min.
Δ Abs CNT is the difference between the negative control absorbance measured between 0-5 min.

3.4.2.3. Screening of prebiotic potential

The faecal samples were obtained from healthy donors and maintained under anaerobic conditions, for a maximum of 2-3 h. Faeces are diluted at 10 % (w/w) in 0.1 M PBS (Phosphate-buffered saline; Sigma-Aldrich, St. Louis, Missouri, USA) with a pH value of 7.4 and homogenized in a Mixwell

stomacher (Alliance Bio Expertise; Guipry-Messac, France) for 2 min. The faecal slurry was ready, and the final pH was 7.26.

Basal nutrient medium was comprised of 2 g/L peptone (Sigma-Aldrich; Sintra, Portugal), 2 g/L yeast extract (Sigma-Aldrich; Sintra, Portugal), 0.1g/L sodium chloride (NaCl; AppliChem GmbH), 0.04 g/L dipotassium hydrogen phosphate (K_2HPO_4; Merk KGaA; Darmstadt, Germany), 0.04 g/L potassium dihydrogen phosphate (KH_2PO_4; Merk KGaA; Darmstadt, Germany), 0.01 g/L magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$; Merk KGaA; Darmstadt, Germany), 0.01 g/L calcium chloride hexahydrate ($CaCl_2{\cdot}6H_2O$; Sigma-Aldrich; St. Louis, Missouri, USA), 2 g/L sodium hydrogen carbonate ($NaHCO_3$; Sigma-Aldrich; St. Louis, Missouri, USA), 0.5 g/L L-cysteine HCL (Thermo Fisher Scientific; Waltham, Massachusetts, USA), 0.05 g/L bile salts (Thermo Fisher Scientific; Waltham, Massachusetts, USA), 2 mL tween80 (Sigma-Aldrich; Sintra, Portugal), 0.001 g/L resazurin solution (Thermo Fisher Scientific; Waltham, Massachusetts, USA), 10 µL vitamin K (Sigma-Aldrich; Sintra, Portugal) and the medium final pH was 6.81. Following this, in different tubes it was added fructooligosaccharides (FOS) (Thermo Fisher Scientific; Waltham, Massachusetts, USA) and the two samples lyophilized at a final concentration of 10 g/L.

In the tubes, previously prepared it was added 4.5 mL of medium and inoculated with 1 % (v/v) of faecal inoculum and incubated for 48 h at 37 ºC under anaerobic atmosphere in a Whitley A35 anaerobic workstation (don whitley Scientific; Victoria St, United Kingdom). The samples were collected after 0, 24 and 48 h of incubation and the pH values were measured.

Two controls were prepared, one positive designated as FOS and one negative IC (faecal inoculum), and the two samples freeze-dried, *Lactobacilus plantarum* with bagasse was designated BP and *Saccharomyces cerevisiae* with straw was designated SCcb. All the samples collected were stored at -80 ºC until analysis.

The samples (4 mL) collected in each time were centrifuged at 4480 RCF and 4 ºC for 5 min, and then stored at -80 ºC until analysis.

The two different phases that were obtained, the supernatants and the pellet were used to evaluate organic acid production by HPLC and to extract the genomic DNA by qPCR, respectively.

3.4.2.4. Cell culture assays

3.4.2.4.1. Cell line

The CaCo-2 cell line (ATCC HTB-37) was obtained from American Type Culture Collection (ATCC; Manassas, United States of America) and reactivated in DMEM culture medium (Dulbecco's Modified Eagle Medium), with 1% NEAA (Non Essential Amino Acid), 10 % FBS (Fetal Bovine Serum) and 1% A&A (Antibiotic and Antimitotic) all acquired from Thermo Fisher Scientific; Massachusetts, United States of America were incubated at 37 ºC with an atmosphere of 95 % air and 5 % CO_2.

3.4.2.4.2. Cytotoxicity assay

Cytotoxicity evaluation was performed accordingly to the ISO 10993-5:2009 standard [18] in CaCo-2 cells. Cells were grown to 80-90 % confluence, detached using TrypLE Express (ThermoScientific, Massachusetts, USA) and seeded at $1x10^4$ cells/well in a 96 well microplate (Nunclon Delta,

ThermoScientific, Massachusetts, USA). After 24 h the culture media was carefully removed and replaced with culture media supplemented with CMC at concentrations between 0.31 and 10 mg/mL. DMSO (Sigma, St. Louis, USA) at 10 % (v/v) in culture media was used as a death control and plain culture media was used as growth control. After 24 h of incubation 10 µL of Presto Blue (Thermofisher, Massachusetts, USA) was added to each well and incubated for 2 h. After this period fluorescence (Ex: 560 nm; Em: 590 nm) was measured using a microplate reader (Synergy H1, Biotek Instruments, Winooski, USA). All assays were performed in quadruplicate.

3.4.2.4.3. Immunomodulatory effect on CaCo-2 cells

Caco-2 cells were grown to 80-90% confluence, detached using TrypLE Express, seeded at $2.5x10^5$ cells/well in a 24 wells microplate and incubated for 24 h at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. After 24 h, the culture media was carefully replaced with media supplemented with samples at 15 and 10 mg/mL and the plate re-incubated for another 24 h. As an inflammation control, IL-1β (Invitrogen, USA) was used at 10 ng/mL while for basal activity control plain media was used. At the end of the assay, supernatants were collected, centrifuged to remove debris, and stored at - 80°C for further analysis. The protein content of aliquots of cells extracts was determined using the cb Pierce Assay Kit (ThermoScientific, Massachusetts, USA).

Interleukins 6 (IL-6) and 8 (IL-8) and Tumor Necrosis Factor alpha (TNF-α) detection was performed by enzyme-linked immunosorbent assay (ELISA) using the Human IL-6 Elisa Kit High Sensitivity (Abcam; Cambridge, United Kingdom), the Legend Max Human Elisa Kit IL-8 and the Legend Max Human Elisa Kit TNF-α (BioLegend; San Diego, USA) according to the manufacturer's instructions. Interleukin values were obtained in pg/mL of sample. To diminish the variability associated with any kind of proteomic based assay results were expressed in Relative Percentage of Production relatively to the interleukin levels in the basal (non-stimulated) control. Interleukin content of the basal control was set to 100 %.

4. Statistical analysis

Results were presented as the average ± standard deviation of three independent experiments (n = 3). The normality of data distribution was tested by Shapiro-Wilk test and the null hypothesis that all means are equal was rejected when the difference between means was $p < 0.05$. Following the ANOVA, test multiple comparisons were done at those statistically significant variables using the Tukey's post-hoc test and Bonferroni post-hoc test (homogeneity of variance was assumed) at the $p<0.05$ significance level.

All this statistical analysis was performed using STATISTICA version 13.5.0.17.

5. Results and Discussion

5.1. Saccharification

5.1.1. Effect of time, enzyme and milling on the released sugar content

The first aim of this study was to evaluate the best saccharification conditions in sugarcane bagasse and straw. For this evaluation, the effect of time, enzyme and milling in relation to the sugar content was studied.

The saccharification used the enzyme Celluclast in the extracts to break the bonds in the molecules and transform them from polysaccharides (e.g. cellulose) to monosaccharides (e.g. glucose). The enzyme used provides a reduction in the viscosity of the plant tissue and with this a higher yield in the extraction of plant products. Time is also a factor to be considered because the enzyme can be easily denatured with increasing exposure time at temperatures higher than those the enzyme can withstand **(Aguiar, C. L. D., 1998)**. Biomass comes in two different phases: milled and unmilled and depending on its phase saccharification may become easier **(Sun *et al.*, 2016)**.

5.1.1.1. Bagasse

Sugarcane bagasse is composed of carbohydrate polymers and therefore several strategies have been devised to convert them into fermentable sugars. The cellulose fraction is rich in glucose, whereas the hemicellulose fraction is rich in xylose, glucose and arabinose **(Canilha *et al.,* 2012)**.

Saccharification was tested for 24 and 48 h with Celluclast enzyme and a mixture of Celluclast and xylanase enzymes on un-milled and milled bagasse biomass.

Figure 5.1., shows the total sugar released along saccharification process performed by 48 h. At the end of saccharification of the bagasse without enzyme, the total sugar increased slightly from 1.06 ± 0.05 to 1.33 ± 0.16 mg/mL. With Celluclast the total sugar concentration in the bagasse extract more than doubled from 1.04 ± 1.04 to 3.75 ± 0.27 mg/mL and for bagasse with both enzymes (Celluclast + xylanase) the concentration increased even more from 1.03 ± 0.03 to 4.68 ± 0.15 mg/mL along the time. Concerning the effect of time, in the saccharification without enzyme, there are no significant differences ($p > 0.05$) between time 0, 24 and 48 h. With the enzyme Celluclast there are significant differences ($p < 0.05$) between all times (0, 24 and 48 h) and with both of enzymes (Celluclast + xylanase) there are significant differences ($p < 0.05$) between all times (0, 24 and 48 h). While the effect of enzyme addition along the time, no significant differences ($p > 0.05$) were found between the addition of enzymes, at time 0 h, as expected. At time 24 h, the higher sugar released occurred in the condition with Celluclast ($p > 0.05$), while the addition of celluclast and both Celluclast + xylanase showed to be statically significant from the condition without addition of enzyme ($p < 0.05$). At time 48 h, the addition of enzymes showed to significantly impact the sugar release of all conditions ($p < 0.05$), and as previously mentioned the highest release was showed in conditions with both Celluclast + xylanase.

Thus, with unmilled bagasse, the total sugars release occurred along the saccharification process and the highest sugar release occurred at 48 h with the addition of enzymatic cocktail Celluclast and xylanase.

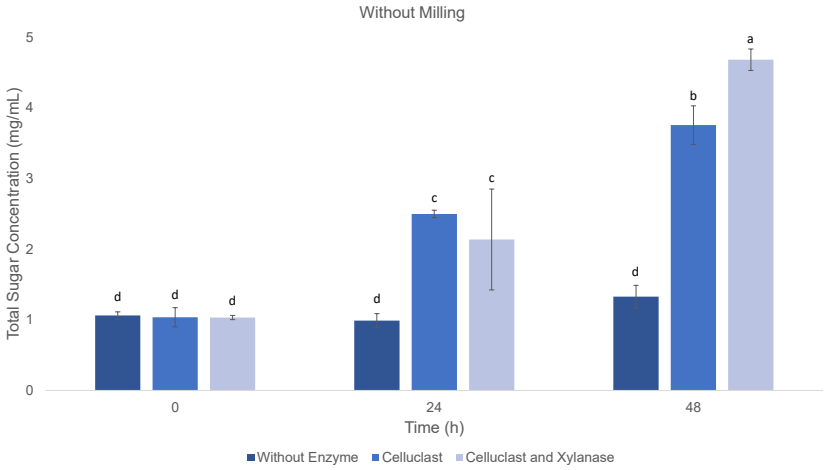

Figure 5.1. Concentration of total sugars (mg/mL) released along saccharification process performed during 48 h in unmilled bagasse without and with addition of enzyme, Celluclast and both Celluclast + xylanase. [a] Different letters mean significant differences ($p < 0.05$) between enzyme type and time.

The milling effect of sugarcane bagasse in the enzymes action along time is shown in **Figure 5.2**. At the end of saccharification of the bagasse without enzyme, total sugar concentration decreased slightly from 0.88 ± 0.41 to 0.46 ± 0.07 mg/mL. With Celluclast the total sugar concentration in the bagasse extract decreased from 1.70 ± 0.30 to 1.42 ± 0.45 mg/mL and for bagasse with both enzymes (Celluclast + xylanase) the concentration increased from 1.34 ± 0.22 to 2.34 ± 1.42 mg/mL along the time. Concerning the effect of time, in the saccharification without enzyme, there are no significant differences ($p > 0.05$) between time 24 and 48 h. With the enzyme celluclast there are significant differences ($p < 0.05$) between initial time and the others time (24 and 48 h). With both of enzymes (Celluclast + xylanase) there are significant differences ($p < 0.05$) between all time (0, 24 and 48 h). While the effect of enzyme addition along the time, significant differences ($p < 0.05$) were found between the no addition of enzyme and addition of enzyme Celluclast and both Celluclast + xylanase, at time 24 h and the higher sugar released occurred in the condition with Celluclast. At time 48 h, the addition of enzymes showed to impact significantly the sugar release of all conditions ($p < 0.05$), and as previously mentioned the highest release was showed in conditions with both Celluclast + xylanase.

Thus, also with milled bagasse, the total sugars release occurred along the saccharification process and the highest sugar release occurred at 48 h with the addition of enzymatic cocktail Celluclast and xylanase.

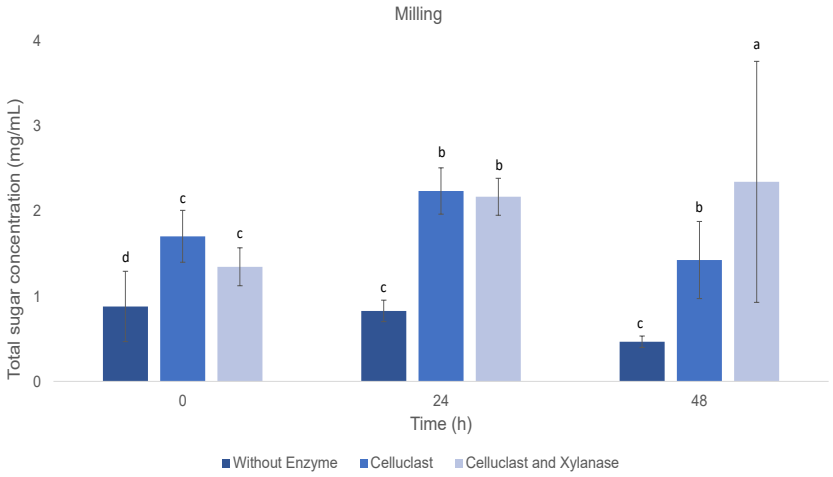

Figure 5.2. Concentration of total sugar (mg/ mL) released along saccharification process performed during 48 h in milled bagasse without and with addition of enzyme Celluclast and both Celluclast + xylanase. [a] Different letters mean significant differences ($p < 0.05$) between enzyme type and time.

For the saccharification of biomass become feasible, the use of enzymes that act synergistically in its depolymerisation is necessary (Velasco, J., et al., 2021). Celluclast is normally used so that there is a high enzymatic convertibility of cellulose and this happens by fragmenting the 1,4-β-D-glycosidic bonds and therefore glucose release occurs **(Ogeda *et al.,* 2010)**. In the milled and un-milled extracts analysed, the best results occurred when the two enzymes (Celluclast + xylanase) were used together.

Xylanase, on the other hand, degrades the most important component of hemicellulose, xylan, by breaking down the backbone of xylan which is made up of xylose residues linked to β-1,4-glycosidic linkages **(Ogeda *et al.*, 2010)**.

With the different enzymes, the biomass was in two different sizes (milled and unmilled) and this difference facilitates saccharification even though the components present in bagasse are entangled in its structure **(Che Kamarlusin *et al.*, 2014)**. Milled of biomass causes the particle size and degree of crystallinity to be reduced **(Sun *et al.,* 2016)**.

The saccharification time points with the different enzymes and different biomass sizes tested show that the best results were with the 48 h time. These results are due to the enzymes because the hydrolysis of cellulose by Celluclast happens in the first 24 h and of hemicellulose by xylanase in the following 24 h **(García-Aparicio *et al.,* 2007)**.

Saccharification of biomass with enzymes is a promising approach which leads to high yields of end products that are economically viable. Usually, the end products that are formed in saccharification are aliphatic acids (e.g. acetic, formic and levilinic acids), furan derivatives (e.g. furfural and 5-hydroxymethylfurfural) and phenolic compounds **(Martín *et al.*, 2002)**. The main sugars in the hydrolysate are glucose which is derived from cellulose and xylose which is derived from hemicellulose **(Collograi *et al.,* 2019)**.

By analysing the **Figure 5.1.** and **5.2.** of the bagasse biomass, we can conclude that the enzymes were an asset for a higher conversion of polysaccharides into sugars. We chose to use only the Celluclast enzyme due to the high price of enzymes and the use of this enzyme alone shows similar

values to those of with both enzymes (Celluclast + xylanase) in the final concentration of sugars. The unmilled biomass has better conversion of sugars over time. The time with better saccharification values was 48 h. The ideal conditions used were: unmilled biomass, with a time of 48 h and the Celluclast enzyme.

5.1.1.2. Straw

Sugarcane straw has the same carbohydrate composition as bagasse and therefore the same strategies for conversion of fermentable sugars were designed. Straw lignin has a molar composition of 4:68:28 with respect to ρ-hydroxyphenyl:guaiacyl:syringyl (H:G:S) **(José, C., *et al.*, 2015)**.

Saccharification was tested during 24 and 48 h with celluclast enzyme and Celluclast and xylanase enzymes together on unmilled and milled straw biomass.

In **Figure 5.3**., show the total sugar released along saccharification process performed by 48 h was higher than with bagasse. At the end of saccharification of the straw without enzyme, total sugar concentration increased from 1.77 ± 0.22 to 3.63 ± 0.31 mg/mL. With Celluclast the total sugar concentration in the straw extract increased from 1.77 ± 0.11 to 7.32 ± 0.14 mg/mL and for straw with both enzymes (Celluclast + xylanase) the concentration increased from 1.57 ± 0.18 to 5.26 ± 1.08 mg/mL along the time. Concerning the effect of time, in the saccharification without enzyme, there are significant differences ($p < 0.05$) between final time (48 h) and other period of time (0 and 24 h). With the enzyme Celluclast there are significant differences ($p < 0.05$) between initial time and the others time (24 and 48 h). With both of enzymes (Celluclast + xylanase) there are significant differences between all times (0, 24 and 48 h). While the effect of enzyme addition along the time, no significant differences ($p > 0.05$) were found between the addition of enzymes, at time 0 h, as expected. At time 24 h, the higher sugar released occurred in the condition with Celluclast + xylanase ($p > 0.05$), and showed to be statically significant from all conditions analysed ($p < 0.05$). At time 48 h, the addition of enzymes showed to significantly impact the sugar release of all conditions ($p < 0.05$), and the highest release was showed in conditions with Celluclast.

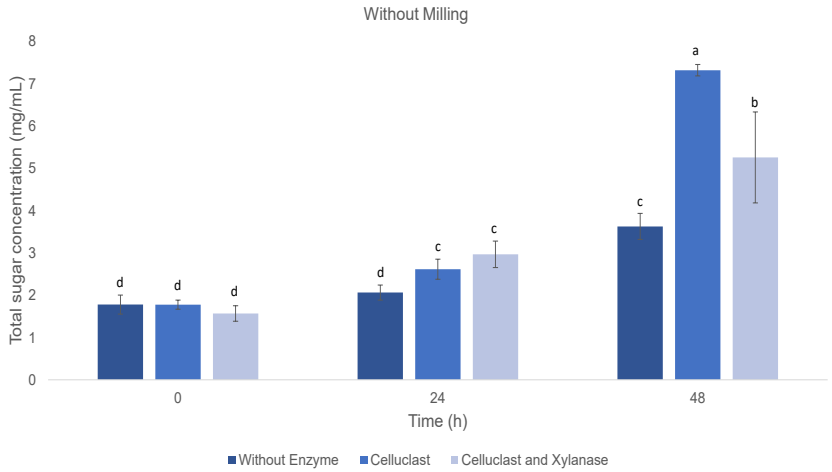

Figure 5.3. Concentration of total sugars (mg/mL) released along saccharification process performed during 48 h in unmilled straw without and with addition of enzyme, Celluclast and both Celluclast + xylanase. [a] Different letters mean significant differences ($p < 0.05$) between enzyme type and time.

The milling effect of sugarcane straw in the enzymes action along time is shown in **Figure 5.4**. At the end of saccharification of the straw without enzyme, total sugar concentration decreased slightly from 2.19 ± 0.15 to 1.58 ± 0.11 mg/mL. With Celluclast the total sugar concentration in the straw extract increased from 2.07 ± 0.58 to 3.88 ± 0.74 mg/mL and for straw with both enzymes (Celluclast + xylanase) the concentration increased from 2.16 ± 0.33 to 4.39 ± 0.67 mg/mL along the time. Concerning the effect of time, in the saccharification without enzyme, there are no significant differences ($p > 0.05$) between time 0, 24 and 48 h. With the enzyme Celluclast there are significant differences ($p < 0.05$) between initial time and the others time (24 and 48 h). With both enzymes (Celluclast + xylanase) there are significant differences ($p < 0.05$) between initial time and the others time (24 and 48 h). While the effect of enzyme addition along the time, significant differences ($p < 0.05$) were found between the no addition of enzyme and addition of enzyme Celluclast and both celluclast + xylanase, at times 24 and 48 h. The highest sugar released occurred in the condition with Celluclast + xylanase, at time 24 and 48 h.

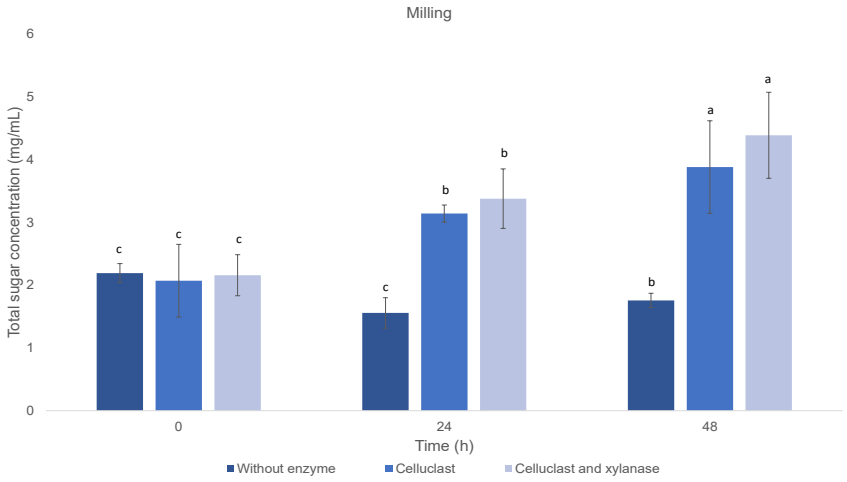

Figure 5.4. Concentration of total sugar (mg/ mL) released along saccharification process performed during 48 h in milled bagasse without and with addition of enzyme Celluclast and both Celluclast + xylanase. [a] Different letters mean significant differences ($p < 0.05$) between enzyme type and time.

Saccharification of straw biomass is performed under the same conditions as bagasse and therefore all mechanisms work under the same conditions. The enzymes act synergistically in the depolymerisation of the biomass **(Velasco *et al.*, 2021)**. In the ground extract the best result occurs when they are used as two enzymes (Celluclast + xylanase), this may be because the straw has a higher fraction of hemicellulose than the bagasse. Whereas in the unmilled extract, the best result occurs when only the Celluclast enzyme is used since hemicellulose is derived from cellulose **(Ogeda *et al.*, 2010)**. Despite the differences in sugar concentration with the enzymes and biomass size, the best saccharification time for all conditions was 48 h.

By analysing the **Figure 5.3.** and **5.4.** it is possible to conclude that the use of the set of enzymes (Celluclast + xylanase) was only advantageous in the milled straw since in the unmilled one the concentration of sugars is much higher when we use the Celluclast enzyme for 48 h. The optimal conditions were: unmilled biomass, with a time of 48 h and the Celluclast enzyme.

5.2. Simultaneous Saccharification and Fermentation

5.2.1. Effect of fermentation time in the sugar consumption

After studying the best saccharification conditions, the fermentation experiments were performed during 48 h with bagasse and straw unmilled with Celluclast and the consumption of sugars by the microorganisms (*Lactobacillus plantarum, Lactobacillus fermentum* and *Saccharomyces cerevisiae*) was evaluated in a 48 h fermentation process. To evaluate the best microorganism that allows to achieve a richer extract with higher bioactive potential, the antioxidant activity (ABTS, DPPH and ORAC) as well as an antimicrobial activity were evaluated.

5.2.1.1. *Lactobacillus plantarum*

The *Lactobacillus plantarum* is a LAB usually used as a starter and adjunct cultures in fermentations of raw materials from plant and animal origin. A few strains are also used as animal or human probiotics **(Mayo *et al.,* 2021)**. This microorganism possesses enzymes that catalyse the production of high added-value compounds, such as organic acid, exopolysaccharides and peptides. LAB can convert sugar into lactic acid which reduces sugar content and pH in fermented products **(Zhou *et al.,* 2020)**.

Figure 5.5 show the fermentation sugar consumption of this microorganism along fermentation using bagasse and straw extracts, respectively. Overall, it is possible to notice that the initial values of sugar in the straw extract are higher than those of the bagasse due to the sugars formed in saccharification.

In **Figure 5.5.a**, is represented the total sugars variation along 48 h fermentation of bagasse with *L. plantarum*, and it was visible a reduction ($p < 0.05$) in sugars at 48 h, however no differences were observed at 24 h. Over the fermentation time, the total sugar concentration decreased from 2.19 ± 0.56 to 1.02 ± 0.51 mg/mL.

The fermentation of sugarcane straw with *L. plantarum* is presented in **Figure 5.5.b**, and it was visible a significant decrease ($p < 0.05$) of total sugars immediately after 24 h fermentation that continued to decrease until 48 h besides not being significantly representative ($p > 0.05$). Over the fermentation time, the total sugar concentration decreased from 4.58 ± 1.52 to 1.40 ± 0.68 mg/mL.

According to **Hashemi *et al.,* (2020)**, the levels of glucose and fructose present in bergamot extracts decreased significantly during fermentation and the consumption of glucose and fructose by the bacterium *L. plantarum* was 25 and 29%. This decrease was also seen in the straw and bagasse extracts analysed, and the consumption of sugars was 69 and 53%, respectively.

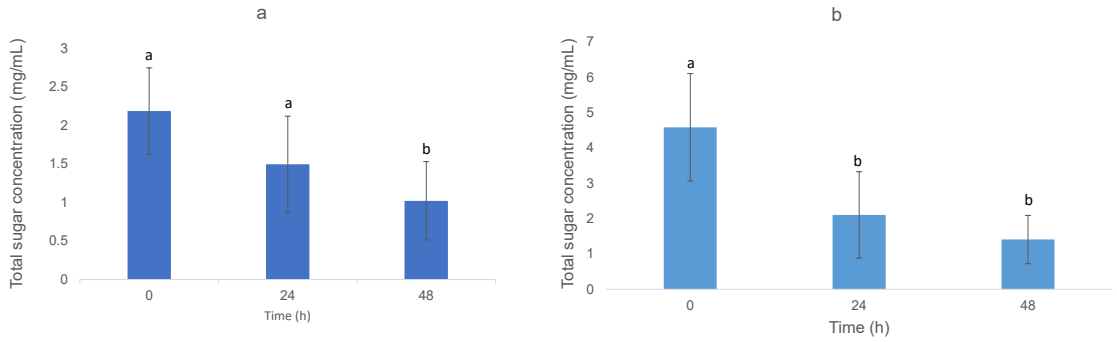

Figure 5.5. Concentration of total sugars (mg/ mL) released along fermentation process performed during 48 h in unmilled (a) bagasse and (b) straw with addition enzyme Celluclast and *Lactobacillus plantarum*. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and time.

5.2.1.2. *Lactobacillus fermentum*

Another microorganism used in a 48 h fermentation with two different biomasses (bagasse and straw) was *L. fermentum.* These bacteria are well adapted to stressful conditions, such as high ethanol concentrations, low pH, low oxygen, and nutrient competition. It also can grow in different carbon source (such glucose, lactose) **(Calazans, 2020)**. *L. fermentum* was able to simultaneously consume glucose and xylose in nutrient rich broth **(Collograi *et al.*, 2019)**.

In **Figure 5.6.a** and **5.6.b**, is represented the total sugars variation along 48 h fermentation with *L. fermentum*, and it was visible a reduction ($p < 0.05$) in sugars after 48 h, however no differences were observed after 24 h ($p > 0.05$). Over the fermentation, the total sugar concentration decreased from 2.37 ± 0.50 to 0.55 ± 0.06 mg/mL in bagasse extract and from 4.28 ± 1.04 to 1.04 ± 0.03 mg/mL in straw extract. We can conclude that the microorganism used the biomasses similarly, with a sugar consumption of 77% in bagasse and 76% in straw.

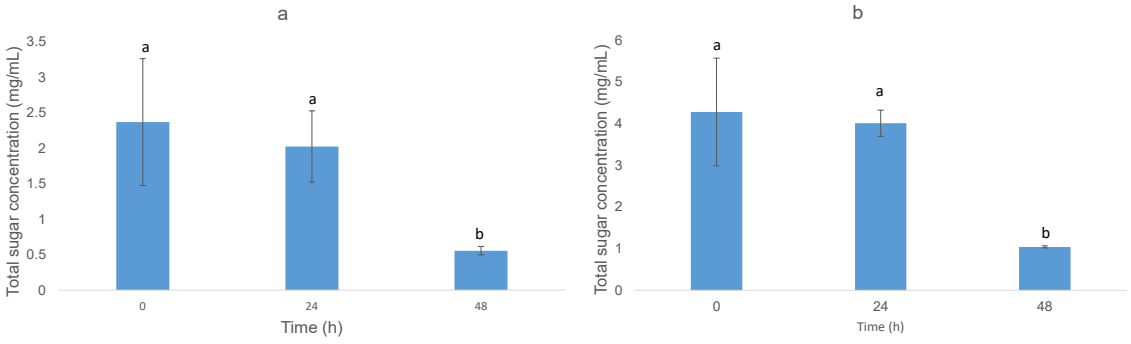

Figure 5.6. Concentration of total sugars (mg/ mL) released along fermentation process performed during 48 h in unmilled (a) bagasse and (b) straw with addition enzyme Celluclast and *Lactobacillus fermentum*. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and time.

5.2.1.3. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was tested in a 72 h fermentation and with the two different biomasses (bagasse and straw). In this experiment the fermentation time was increased from 48 to 72 h since the yeast activity did not finish after 48 h.

In **Figure 5.7.a**, it is represented the total sugars variation along 72 h fermentation of bagasse with *S. cerevisiae* and it was visible a reduction ($p < 0.05$) in sugars at 72 h, however no differences were observed at 24 h ($p > 0.05$). Over the fermentation, the total sugar concentration decreased from 2.37 ± 0.56 to 0.84 ± 0.29 mg/mL.

The fermentation of sugarcane straw with *S. cerevisiae* is presented in **Figure5.7.b**, and it was visible a slight decrease ($p > 0.05$) of total sugars immediately after 24 h fermentation that continued to decrease significantly until 72 h ($p < 0.05$). Over the fermentation, the total sugar concentration decreased from 3.46 ± 1.06 to 1.12 ± 0.36 mg/mL. The results presented in the study by **Gunam *et al.*, (2021)** are similar to those obtained in the experiment performed with the yeast *S. cerevisiae*, where a rapid decrease in sugar reduction occurs up to 48 h of fermentation and an insignificant reduction in the following 24 h. These results are due to the cellular activity of the yeast in the media, which uses the substrate for growth in addition to being converted to metabolites, is what causes the rapid decline in decreasing sugar levels **(Gunam *et al.*, 2021)**.

Sugar concentrations could be lower if the yeast *S. cerevisiae* could ferment pentose sugars and not only glucose **(Thomsen *et al.*, 2009)**.

It can be concluded that the microorganism used the biomasses similarly, with a sugar consumption of 64% in bagasse and 68% in straw.

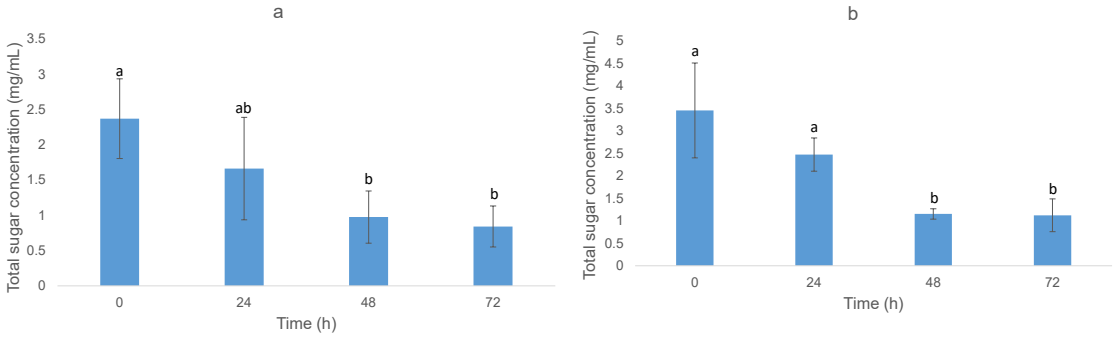

Figure 5.7. Concentration of total sugars (mg/ mL) released along fermentation process performed during 48 h in unmilled (a) bagasse and (b) straw with addition enzyme Celluclast and *Saccharomyces cerevisiae*. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and time.

5.2.2. Initial screening of SSF extracts

5.2.2.1. Antioxidant Capacity by ABTS and DPPH

The antioxidant capacity was measured at the end of fermentation to verify the capacity of *L. plantarum, L. fermentum* and *S. cerevisiae* to generate compounds with capacity to neutralize free radicals and if that was dependent on the biomass used and on the fermentation time.

The extracts obtained presented higher antioxidant activity according to ABTS and DPPH for *L. plantarum, L. fermentum* and *S. cerevisiae* can be seen in **Figure 5.8., 5.9.** and **5.10.**, respectively.

The solvent effect is one of the most important parameters to evaluate the chemical behaviour of the different antioxidant compounds **(Çelik *et al.,* 2010)**. The differences that are found may be related to the presence of a greater amount of hydrophilic compounds in the extracts since the ABTS method was performed in aqueous conditions as opposed to DPPH where methanol was used as the dispersion solvent. Hydrogen bonds in polar solvents such as water used in the ABTS method cause large changes in the H-atom phenolic antioxidant donor activities and consequently affects the reductive antioxidant capacity of the samples **(Çelik *et al.,* 2010)**. The compounds present in the extracts have higher free radical scavenging capacity through electron transfer than protons.

In ABTS method before fermentation of *L. plantarum* (**Figure 5.8.**), no differences were observed between bagasse and straw (BCt and SCt) and after fermentation a significant decrease in antioxidant capacity was observed for bagasse (BP) since the EC increased ($p < 0.05$). In the DPPH method, the extract that was significantly different ($p < 0.05$) was the one obtained from sugarcane straw before fermentation exhibiting higher antioxidant capacity with lower EC50 than the remaining samples. The results of this experience show that the extracts with the best antioxidant capacity by ABTS were straw control (SCt), bagasse control (BCt) and straw extract (SP) with a EC50 of 8.27 ± 0.34, 9.29 ± 0.58 and 10.38 ± 0.96 mg/mL, respectively. In DPPH method, the extract with the best antioxidant capacity was straw control (SCt) with a EC50 of 13.88 ± 0.01 mg/mL.

Phenolic compounds presence natively in sugarcane, which increased the antioxidant activities in DPPH and ABTS methods **(Zhou *et al.*, 2020)**. Also, Brian *et al.* reported that the xylo-oligosaccharides extracted from sugarcane also exhibit antioxidant activity, like phenolic compounds **(Sanarat *et al.*, 2021)**.

This result agrees with Li et al., who state that the fermented apple juice using *L. plantarum* exhibited a significant increase in percentage of DPPH and ABTS inhibition after 24, 48 and 72 h fermentation **(Myo *et al.*, 2021)**.

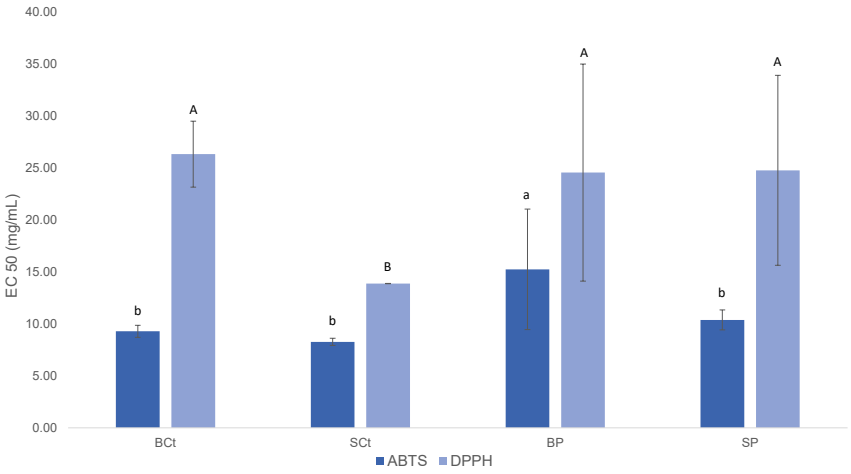

Figure 5.8. Total antioxidant capacity at 48 h for bagasse control (BCt), straw control (SCt), bagasse and straw extracts after fermentation with *Lactobacillus plantarum* (BP and SP). [a] Different letters

mean significant differences ($p < 0.05$) between biomass type and microorganism. [a] Different lowercase (a) and uppercase (A) letters represent significant differences between samples measured for ABTS and DPPH methods respectively.

In ABTS method before the fermentation of *L. fermentum* (**Figure 5.9.**), no differences were observed between bagasse and straw (BCt and SCt) and after fermentation a significant decrease in antioxidant capacity was observed for bagasse and straw (BF and SF) since the EC increase ($p < 0.05$). In DPPH method, the extracts that were different ($p < 0.05$) were obtained from sugarcane bagasse before and after fermentation and exhibited lower antioxidant activity with higher EC50 than the others samples (SCt and SF). The results of this experience show that the extracts with best antioxidant capacity by ABTS were straw and bagasse control (SCt and BCt) with a EC50 of 8.27 ± 0.34 and 9.29 ± 0.58 mg/mL, respectively. In DPPH method, the extracts with the best antioxidant activity were straw control and straw with *L. fermentum* (SCt and SF) with a EC50 of 13.88 ± 0.01 and 16.89 ± 2.72 mg/mL, respectively. The increases in the EC50 value compared to the controls are due to the degradation of the phenolic compounds present in the sugarcane extracts and the hydrolysis of these bioactive compounds. The subsequent enzymes produced by the fermenting bacterium may have depolymerized the phenolic compound as a result of the polyphenols' oxidation and condensation processes, which result in the formation of other chemicals **(Adebo *et al.*, 2018)**.

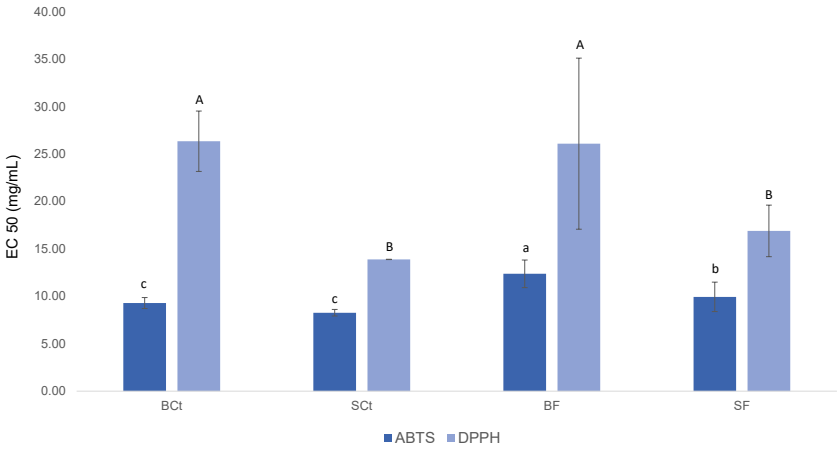

Figure 5.9. Total antioxidant capacity at 48 h for bagasse control (BCt), straw control (SCt), bagasse and straw extracts after fermentation with *Lactobacillus fermentum* (BF and SF). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism. [a] Different lowercase (a) and uppercase (A) letters represent significant differences between samples measured for ABTS and DPPH methods respectively.

In ABTS method before fermentation with *S. cerevisiae* (**Figure 5.10.**), no differences were observed between bagasse and straw control (BCt and SCt) and after fermentation a significant increase in antioxidant capacity was observed for straw (SC) since the EC decrease ($p < 0.05$). In the DPPH method, the extracts that were different ($p < 0.05$) were obtained from sugarcane bagasse before and after fermentation (BCt and BC) and sugarcane straw (SCt) before fermentation and exhibited lower

antioxidant activity with higher EC50 than the remaining samples. These results show that the extract with the best antioxidant capacity by ABTS was straw with *S. cerevisiae* (SC) with a EC50 of 5.13 ± 2.62 mg/mL. In DPPH method, the extract with the best antioxidant capacity was straw control (SCt) with a EC50 of 13.88 ± 0.01 mg/mL. Fermentation showed an enhancement of the number of antioxidants present in sugarcane extract.

The high antioxidant activity in this study was mainly due to the yeast *S. cerevisiae* since most fungi can produce antioxidants, such as gallic and ferulic acid. This happens during biochemical reactions involving fermentation, the fungi release enzymes that can break the glycosidic bonds of hydroxyl groups of phenols that are attached to sugar compounds and this degradation in turn increase the number of free phenols, thus increasing the bioactivity of food material **(Ejuama *et al.*, 2021)**.

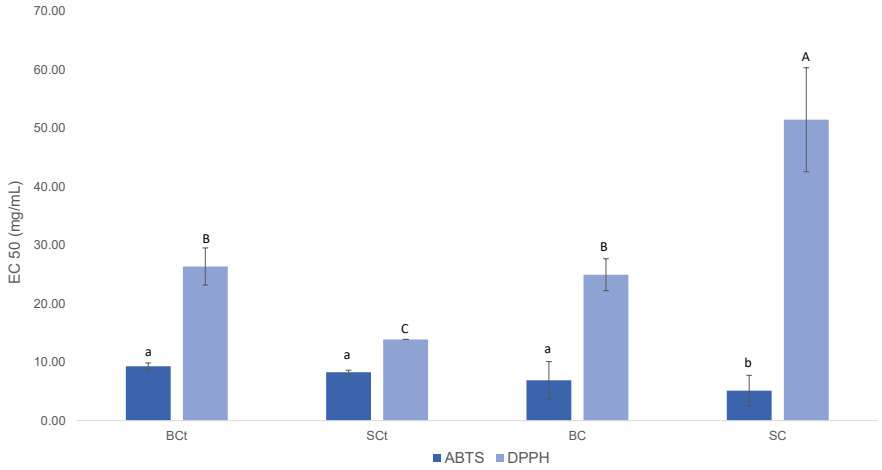

Figure 5.10. Total antioxidant capacity at 48 h for bagasse control (BCt), straw control (SCt), bagasse and straw extracts after fermentation with *Saccharomyces cerevisiae* (BC and SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism. [a] Different lowercase (a) and uppercase (A) letters represent significant differences between samples measured for ABTS and DPPH methods respectively.

It can be concluded from the analyses carried out that the extracts of controls have greater antioxidant activity than the extracts fermented with the microorganisms. In the non-fermented and fermented extracts, the straw has a lower EC50 than the bagasse and for the same extracts, the ABTS method is substantially better than the DPPH method. The straw extract fermented with *S. cerevisiae* has the best value in ABTS method and the straw extract fermented with *L. fermentum* has the best value in DPPH method.

5.2.2.2. Oxygen Radical Absorbance Capacity (ORAC)

The extracts obtained presented higher antioxidant activity according to ORAC for *L. plantarum, L. fermentum* and *S. cerevisiae* can be seen in **Figure 5.11.**

In ORAC method before fermentation with *L. plantarum*, *L. fermentum* and *S. cerevisiae* (**Figure 5.11a, 5.11b** and **5.11c**), no differences were observed between bagasse and straw control (BCt and SCt) and after fermentation a significant decrease in antioxidant capacity were observed for bagasse and straw (BP and SP) since the value decrease ($p < 0.05$). The results of the experiences show that the extract with the highest antioxidant capacity by ORAC were straw and bagasse control (SCt and BCt) with a value of 1.78 ± 0.01 and 1.51 ± 0.27 µmol TE/ g sample.

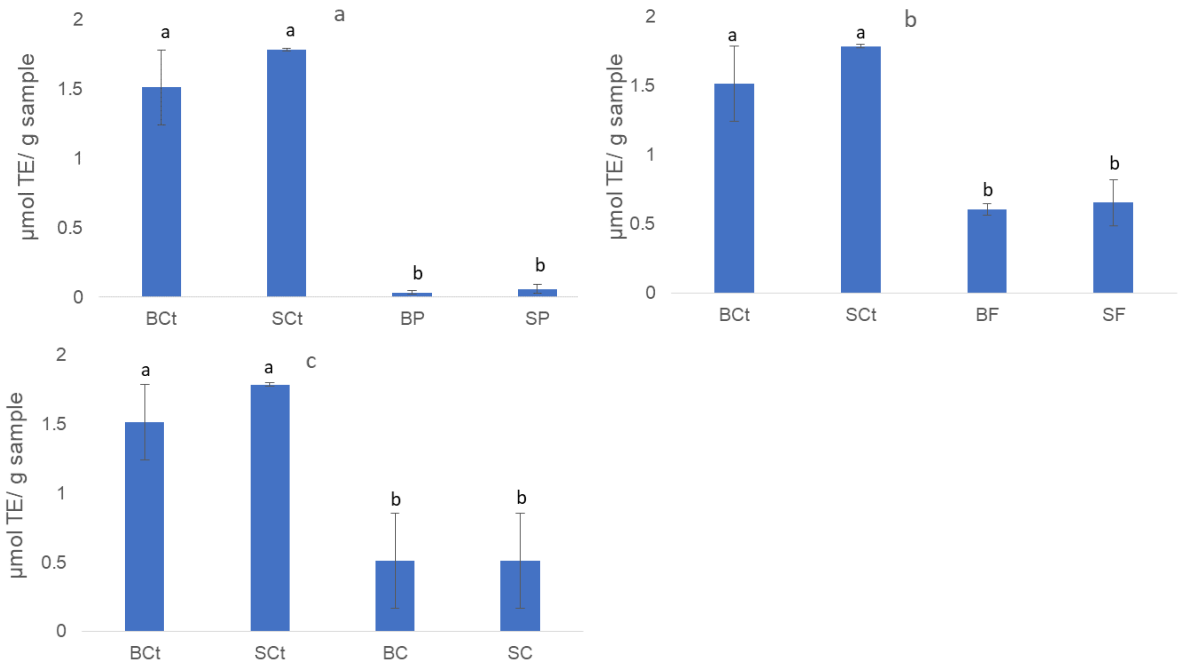

Figure 5.11. Antioxidant capacity content of extracts obtained before fermentation of sugarcane bagasse and straw (BCt and SCt) and bagasse and straw extracts after fermentation with (a) *Lactobacillus plantarum* (BP and SP); (b) *Lactobacillus fermentum* (BF and SF) and (c) *Saccharomyces cerevisiae* (BC and SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism,

These values of *L. plantarum* differ substantially from those found in the literature. According to the study of **Frediansyah, A. (2021)**, fruit juices fermented with the bacterium *L. plantarum* have an increase in antioxidant capacity and concentration of phenolic compounds and flavonoids **(Frediansyah *et al.*, 2021)**.

The results of BF and SF extracts show low antioxidant activity in comparison to the control extracts. These values differ substantially from those found in the literature. According to **Mishra, V., 2015**, the studies conducted for the microorganism *L. fermentum* has a dual functional properties: antimicrobial activity against pathogens and high total antioxidant activity and total antioxidant status of intact cells and lysates. It also revealed the antioxidant potential of the probiotic soft cheese spread containing different fatty acids **(Mishra *et al.*, 2015)**.

These values of experience of *S. cerevisiae* differ substantially from those found in the literature. According to **Jilani, H., *et al.*, (2016)** the value of the ORAC in Assam tea leaves increased from 17.64 to 121.55 µmol TE/ g DW after fermentation **(Jilani *et al.*, 2016)**. Similarly, **Moore *et al.*,** demonstrated

that the *S. cerevisiae* yeasts tested were able to significantly increase ORAC values compared to controls and this data suggest that the yeast may improve the freedom and bioavailability of antioxidants from wheat bran.

It can be concluded from the ORAC analysis we performed that the extracts of controls have higher antioxidant activity than the extracts fermented with the microorganisms. In the non-fermented and fermented extracts, the straw has a higher value than the bagasse and the fermented extract with better antioxidant activity is with the microorganism *L. fermentum* followed by the yeast *S. cerevisiae*. Hence, the fermentation with *L. plantarum* and *S. cerevisiae* does not bring any advantage in concerning the antioxidant capacity improvement, not only by ORAC but as also detected previously by ABTS and DPPH.

5.2.2.3. Antimicrobial

The antimicrobial property of extracts obtained after 48 h of fermentation of straw and bagasse with *L. plantarum* at a concentration of 5% against *S. aureus* (**Figure 5.12a** and **5.12b**). The results reveal that the straw extract does not suppress the growth of the food pathogenic bacteria, whereas the bagasse extract suppresses the growth of the same bacteria. The bagasse extract had a minimum inhibitory concentration (MIC) at a concentration of 5% and a minimum bactericidal concentration (MBC) at a concentration of 4%. This bacterium has a large production of organic acids such as acetic and lactic acid which increases the inhibitory effect.

By using this lactic culture, which presents antimicrobial activity, in the production of fermented foods, it makes the products safer for the consumer, as well as increasing the shelf life since it inhibits the growth of deteriorating and/or pathogenic microorganisms **(da Costa *et al.*, 2019)**.

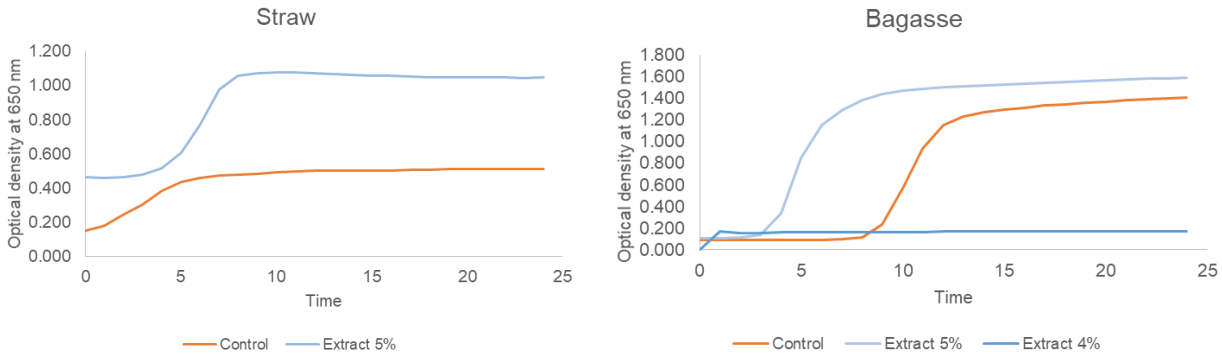

Figure 5.12. Optical densities curves of the fermentation extracts with straw and bagasse with *Lactobacillus plantarum* evaluated at 650 nm.

The antimicrobial property of extracts obtained after 48 h of fermentation of bagasse and straw with *L. fermentum* at a concentration of 5% against *S. aureus* (**Figure 5.13a** and **5.13b**). The results reveal that straw and bagasse extracts do not suppress the growth of food pathogenic bacteria. This is

due to the production of organic acids in combination with the production of a bacteriocin-like protein that acts in acidic states **(Lin *et al.*, 2007)**.

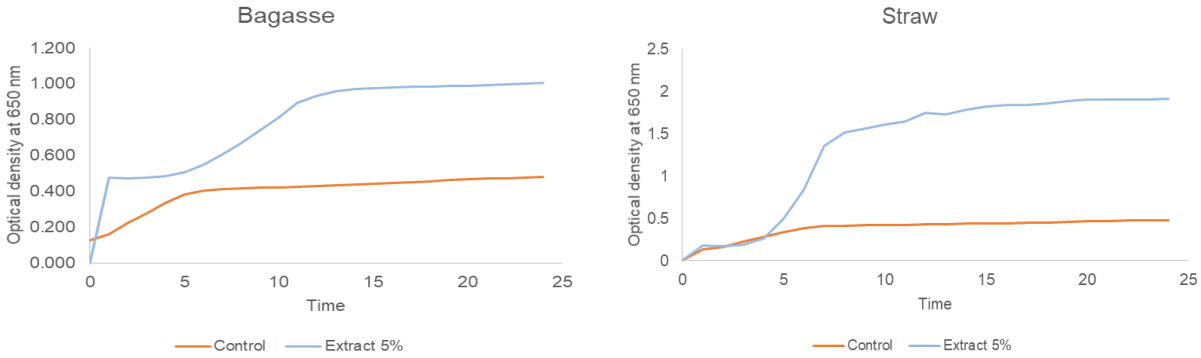

Figure 5.13. Optical densities curves of the fermentation extracts with straw and bagasse with *Lactobacillus fermentum* evaluated at 650 nm.

The antimicrobial property of extracts obtained after 72 h of fermentation of bagasse and straw with *S. cerevisiae* at a concentration of 5% against *S. aureus* (**Figure 5.14a** and **5.14b**). The results reveal that the straw and bagasse extracts do not suppress the growth of the food pathogenic bacteria.

This extract shows moderate antimicrobial activity against bacteria, having preference for gram-negative bacteria. Also, lysate cells have a better antimicrobial activity. The non-inhibition of the SA pathogenic bacteria, may be due to antagonism by production of inhibitory compounds or a low concentration of the inhibitory extracts **(Rajkowska 2012)**.

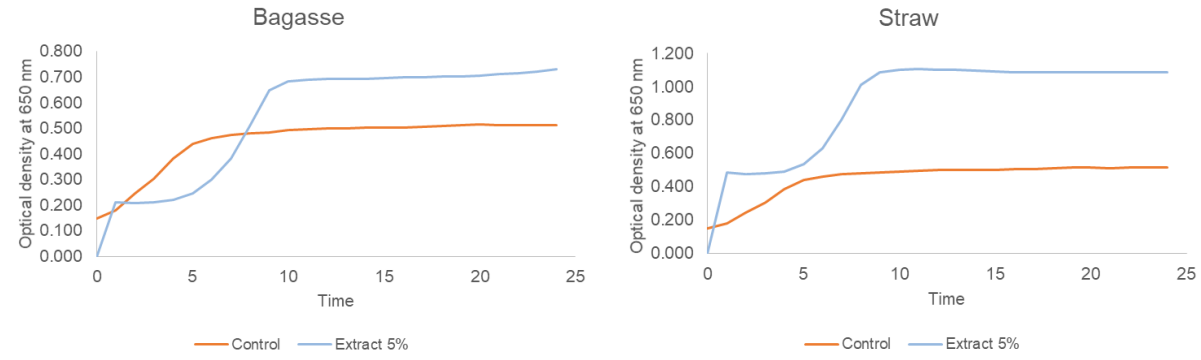

Figure 5.14. Optical densities curves of the fermentation extracts with straw and bagasse with *Saccharomyces cerevisiae* evaluated at 650 nm.

5.2.3. Chemical and biological evaluation of best SSF extracts

After analysing all the fermentation times and concentrations of inoculum in the different microorganisms, it was decided not to use the bacteria *L. fermentum* because it does not produce extracts with good antioxidant and antimicrobial activity. Therefore, it was decided to use only the extracts obtained from *L. plantarum* and the yeast *S. cerevisiae* fermentation. The conditions used for the bacteria were 48 h of fermentation with an inoculum concentration of 10^4 UFC/ mL and for the yeast 72 h of fermentation with an inoculum concentration of 10^5 UFC/ mL. These extracts were further analysed with

four different tests: profile of organic acids and monosaccharides where the separation of the chemical compounds present in the solution occurs, total protein and phenolic content which serve to quantify the protein and nitrogen of the samples and determine the phenolic and polyphenolic antioxidants present, respectively and biologically the cytocompatibility was analysed to know the harmful value of the extracts in relation to the CaCo-2 cells. These tests were performed to evaluate the properties of the extracts with possible biological potential.

5.2.3.1. Profile of organic acids and monosaccharides

The number of organic acids (lactic acid, acetic acid, malic acid, formate and propionate), monosaccharides (arabinose) and ethanol were quantified in the end of each fermentation condition (**Table 5.1.**).

Table 5.1. Characterization of organic acids and monosaccharides of extracts obtained after fermentation of sugarcane and straw control (BCt and SCt); bagasse with *L. plantarum* and *S. cerevisiae* (BP and BC) and straw with *L. plantarum* and *S. cerevisiae* (SP and SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass and type and microorganism. ND – Not Detected

	Arabinose	Lactic	Acetic	Malic	Formate	Propionate	Ethanol
BCt	136.97 ± 2.26[a]	ND	65.92 ± 2.26[ab]	ND	ND	ND	ND
SCt	108.78 ± 0.35[a]	ND	80.69 ± 0.19[ab]	ND	ND	ND	ND
BP	95.55 ± 17.21[a]	88.72 ± 31.33[a]	59.75 ± 21.40[b]	ND	ND	29.13 ± 0.5[a]	ND
BC	103.18 ± 18.83[a]	ND	23.54 ± 16.86[c]	ND	7.73 ± 1.45[a]	7.72 ± 3.23[a]	7.64 ± 2.03[a]
SP	ND	89.11 ± 20.37[a]	91.96 ± 18.50[ab]	32.65 ± 12.62[a]	ND	2.46 ± 2.00[a]	ND
SC	70.40 ± 19.99[a]	2.37 ± 0.1[b]	132.51 ± 3.78[ab]	ND	6.07 ± 3.59[a]	ND	ND

In the experience, in samples before and after fermentation with bacteria and yeast, the only monosaccharide detected was arabinose and the values of this sugar in bagasse and straw extracts are similar ($p > 0.05$), with the values between 70.40 and 136.97 mg/ g of dry extract.

The acetic acid was the main acid present in all samples before and after fermentations. A decrease was observed in bagasse extracts after fermentation from 65.92 to 59.75 mg/ g of dry extract in samples with bacteria and 23.54 mg/ g of dry extract in samples with yeast.

Regarding the other organic acids, lactic acid was detected in bagasse and straw extracts obtained after bacteria and yeast fermentation (BP, SP and SC), the malic acid was only detected in straw extract obtained after yeast fermentation and reach amounts of 32.65 mg/ g of dry extract. The formate was detected in samples after yeast fermentation and reach amounts between 6.07 and 7.73 mg/ g of dry extract. Ethanol was only detected in bagasse extract obtained after yeast fermentation with amount

of 7.64 mg/ g of dry extract. Finally, propionate was present in bagasse and straw extract after bacteria and yeast fermentation (BP, BC and SP).

Bagasse and straw are composed of complex polysaccharides such as cellulose and hemicellulose. Cellulose is composed of glucose molecules linked together, whereas hemicellulose is formed by several sugars. The degradation of this polymers releases these sugars: β-D-xylose, α-arabinose, β-D-mannose, β-D-glucose and α-D-galactose.

Arabinose is present in extracts in more quantities than glucose and xylose because xylan is hydrolysates at a much higher rate than cellulose **(Du Toit *et al*., 1984)**. Arabinose is present in the hemicellulose of sugarcane in significant quantities. According to the study of **Chatterjee, S., *et al.,* (2021)**, the yeast *S. passalidarum* can ferment sugars released from lignocellulosic material previously saccharified as arabinose. In the study presented, after fermentation there is a reduction in the amount of arabinose present in the extracts.

Different microorganisms also produce during fermentation different organic acids. Bacteria mainly produces lactic acid and acetic acid. Yeast, on the other hand, normally produces acetic acid and ethanol. Regarding the organic acids present, in the bacteria and as expected, the ones found in larger quantities are the lactic acid and the acetic acid and in smaller quantities the propionate and malic acid, while in the yeast, the organic acid in larger quantities is the acetic acid, having in smaller quantities the propionate, formate, lactic acid and the alcohol **(Doores, 2005)**.

When fermentation of the biomass occurs with the bacteria of the LAB group, they transform the sugars into lactic acid, hence the large quantity of this organic acid present in the extracts. Acetic acid is present in the extract after fermentation in these bacteria due to e.g. the degradation of lactic acid that is produced, the result of citrate metabolism and/or originates from the heterofermentative pathway **(Zalán *et al.,* 2010)**. Propionate is one of the metabolites formed in the fermentation of bacteria due to the presence of carbohydrates and has great antimicrobial and nutritional benefits.

S. cerevisiae uses glucose to produce ethanol, and the higher the glucose, the faster the conversion of 2-ketobutyrate to propionate, which is found in yeast extract due to degradation of L-threonine from glucose **(Wentao *et al*., 2022)**. The lactic acid present in the yeast extract is derived from glucose and reduced pyruvate decarboxylase activity. The formate is produced by the degradation of 5-hydroxymethylfurfural and the furfural under acid conditions **(Zeng *et al*., 2022)**.

5.2.3.2. Total protein

For the two microorganisms with best antioxidant and antimicrobial activity, total protein at the end of fermentation was tested. Proteins are the main source of nitrogen, which is essential for the metabolism of bacteria and yeast. In both **Figure 5.15a** and **Figure 5.15b**, nitrogen is not a limiting factor for microbial growth.

The high percentage of protein in both bacteria and yeast extracts is due to the addition of supplements, such as yeast extract and peptone, at the beginning of saccharification.

The samples before and after fermentation with *L. plantarum* (**Figure 5.15a**), the total protein content of the bagasse and straw extracts was 33 and 40 %, respectively ($p > 0.05$). On the other hand,

bagasse and straw extracts obtained after *S. cerevisiae* fermentation (**Figure 5.15b**) were significantly different ($p < 0.05$) lower values of total protein content (30 %) for straw.

As it can be seen in **Figure 5.15a** and **5.15b**, fermentation did not impact the total protein content in bagasse and straw extracts since no differences were observed with controls.

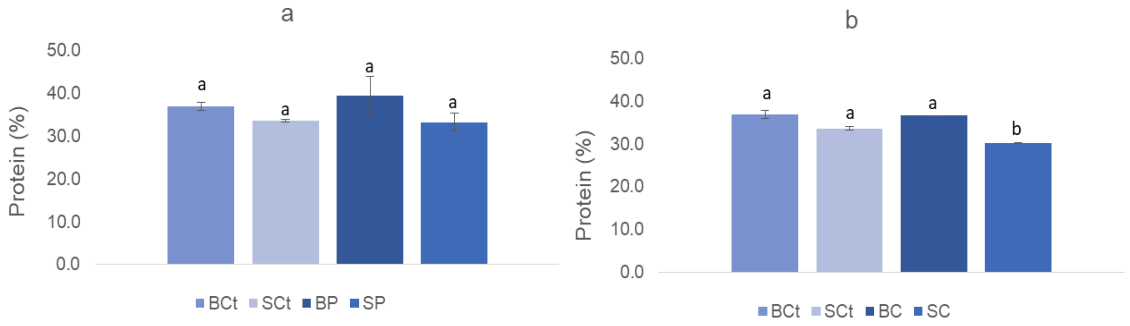

Figure 5.15. Total protein content of extracts obtained before fermentation of sugarcane bagasse and straw (BCt and SCt) and after fermentation with (a) *L. plantarum* (BP and SP) and (b) *S. cerevisiae* (BC and SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

5.2.3.3. Total Phenolic Content

The Folin-Ciocalteu assay, despite being characterized as a total phenolic determination assay, measures the reducing capacity of the samples based on an electron transfer mechanism.

Observing the values obtained, it is possible to see that the sugarcane straw extract obtained before fermentation (**Figure 5.16.**) had the highest phenolic values (14.04 ± 1.99 mg GA/ g). After fermentation, the extract with the highest phenolic content was obtained from straw with *S. cerevisiae* yeast (14.31 ± 0.05 mg GA/ g).

A possible explanation is the high phenolic content that straw has in their composition, besides sugars, proteins and aromatic amines that can interfere in this analysis. The phenolics are released due to the enzymatic treatment of the samples which leads to the structural rupture of the cell walls. Some authors demonstrated that fermentation has a positive influence on total phenolic content of extracts due to the metabolic activity of the microorganisms **(Dordevic *et al.*, 2010)**.

Straw fermentation with *S. cerevisiae* or *L. plantarum* did not change significantly the total phenolic content. However, bagasse biomass fermented with *S. cerevisiae* and *L. plantarum* presented differences with the yeast presenting a favourable increase in phenolic content of 2 % (**Figure 5.16.**).

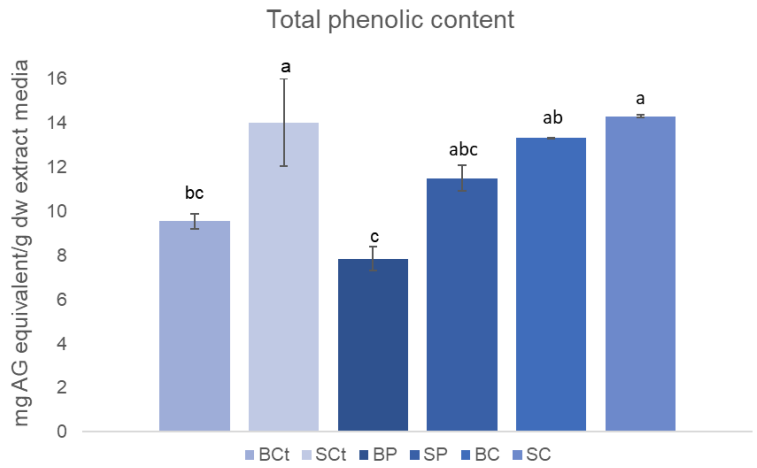

Figure 5.16. Total phenolic content of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation with *L. plantarum* (BP and SP) and *S. cerevisiae* (BC and SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

Sugarcane by-product is a source of phenolic compounds, and their colour has a positive correlation with the response given in the Folin-Ciocalteu assay **(Caderby *et al*, 2013)**. The phenol values observed are high for sugar products because these are extremely coloured materials and phenolic compounds are strongly involved in the formation of this colour. However, these values may have been overestimated due to the presence of sucrose, glucose and Maillard reaction compounds in these products, which may interfere with the test and therefore enhances the development of the blue colour of the test **(Payet *et al*, 2006)**. Other factors that may influence the phenolic composition of sugarcane by-products may be the maturity of the plant at harvest time, the amount of leaf crushing or environmental factors such as climatic conditions or pest infestations. A great variation in temperature and pressure during the process may modify the nature of the polyphenols **(Payet *et al*, 2006)**.

The bacterium *L. plantarum* has the ability to retain the oxygen present in the solution which is responsible for the auto-oxidation and depolymerisation of existing phenolic compounds **(Kachouri *et al.,* 2014)**.

The increase in total phenolic content in the straw substrate may be due to the enzymatic degradation of complex polyphenols to simpler and biologically active ones by bacteria during fermentation **(Jayabalan *et al.,* 2008)**.

S. cerevisiae possesses the ability to produce antioxidants such as ρ-coumaroyl-CoA **(Aung *et al*, 2022)**.

5.2.3.4. Cytocompatibility

The cytocompatibility assay was performed to detect which safe and sublethal concentration of the extracts could be used.

It was analysed cell viability for the extracts obtained from fermentation of different biomasses with the bacterium *L. plantarum*. As we can see (**Figure 5.17a**), exposure of cells to concentrations above 6.25 mg/mL of bagasse extract showed toxicity by exhibiting a metabolic inhibition above 30% on CaCo-2 cells. For straw extracts (**Figure 5.17b**) concentrations above 3.125 mg/mL showed toxicity.

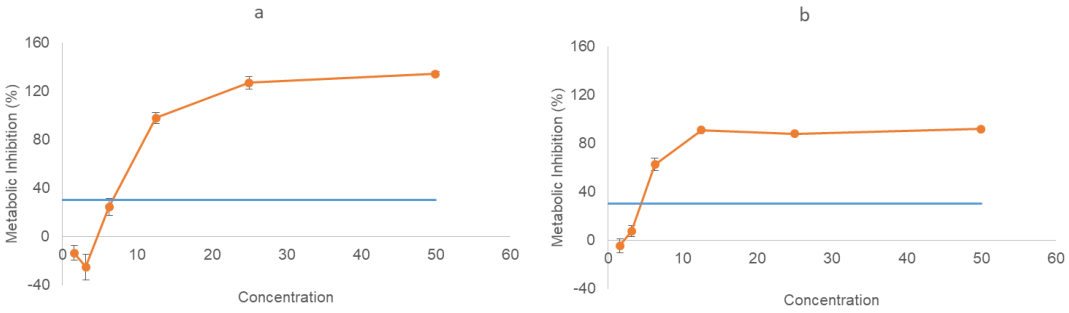

Figure 5.17. Cytocompatibility profile of the fermented sugarcane (a) bagasse and (b) straw extract fermented with *L. plantarum* dispersed in water when in contact with intestinal Caco-2 cell line, measured by the potential of the extract to inhibit the cell metabolic activity. The blue line represents the 30 % cytotoxicity limit.

Cell viability was analysed for the extracts obtained from fermentation of different biomasses with the yeast *S. cerevisiae*. As we can see in **Figure 5.18a** and **Figure 5.18b**, exposure of cells to concentrations above 6.25 mg/mL showed toxicity by exhibiting a metabolic inhibition above 30% on CaCo-2, for both bagasse and straw.

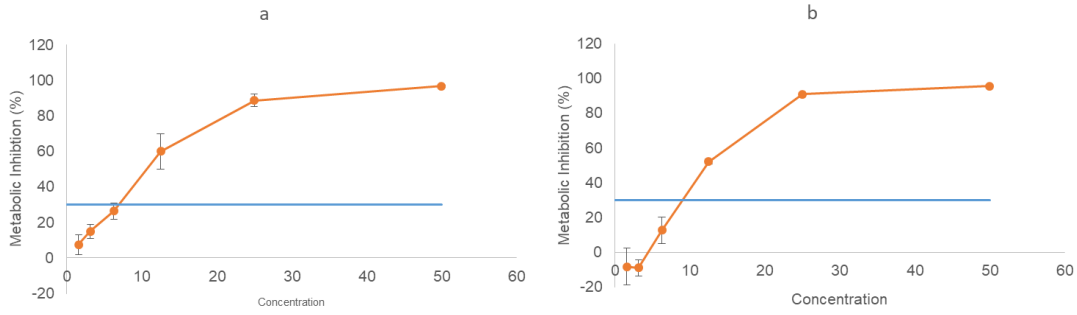

Figure 5.18. Cytocompatibility profile of the fermented sugarcane (a) bagasse and (b) straw extract with *S. cerevisiae* dispersed in water when in contact with intestinal Caco-2 cell line, measured by the potential of the extract to inhibit the cell metabolic activity. The blue line represents the 30 % cytotoxicity limit.

5.3. Sequential Saccharification and Fermentation

5.3.1. Biomass effect removal in sugar consumption

Initially, the extracts were subjected to simultaneous saccharification and fermentation (SSF) followed by ultra-sonication and centrifugation to remove all insoluble material from the samples. At the end of the whole process, the different extracts were analysed chemical and biologically by different methods described in the previous sections.

With these analyses, it can be concluded that the fermentation process should be modified and therefore we opted for a sequential saccharification and fermentation (SQSF), where initially a saccharification is performed for 24 h with the Celluclast enzyme, then all the biomass present in the samples is removed and finally fermentation occurs with the best conditions. These conditions were: fermentation with *S. cerevisiae* for 72 h with a cell concentration of 10^6 CFU/ mL and the bacteria *L. plantarum* for 48 h of fermentation with a cell concentration of 10^5 CFU/ mL. At the end of the fermentation, the extracts were subjected to ultra-sonication and differential centrifugation so that all the whole cells were removed and the fragments were kept. Finally, the extracts were analysed chemically (total antioxidant activity, total protein and phenolic content, characterization of monosaccharides, organic acids and the phenolic profile) and biologically (prebiotic potential, cytocompatibility and immunomodulatory effect).

In **Figure 5.19.** is represented the total sugars variation along 48 and 72 h fermentation of bagasse extract with *L. plantarum* and straw extract with *S. cerevisiae*, respectively. In bagasse with *L. plantarum* was visible *a* reduction ($p < 0.05$) in sugars after 48 h, however no differences were observed after 24 h. Over the fermentation time, the total sugar concentration decreases from 1.30 to 0.36 mg/ mL. On the other hand, the straw with *S. cerevisiae* was visible a significant decrease ($p < 0.05$) of total sugars immediately after 24 h fermentation that continues to decrease significantly ($p < 0.05$) after 48 h and 72 h besides not being significantly representative ($p > 0.05$). Based on those results it was possible to conclude that removal of biomass had an influence on the consumption of sugars by microorganisms. These values are lower than the ones presented before without the removal of the biomass at the end of saccharification (**Figure 5.5.** and **Figure 5.7)**. These changes may be due to fact that microorganisms consumption was limited by the amount of sugars released from the biomass trough the enzyme. *Lactobacillus plantarum* after 48 h present a higher sugar consumption than *Saccharomyces cerevisiae* due to the fact that yeast is not capable to consume the xylose derived from the hemicellulose **(Cong *et al.,* 1981)**.

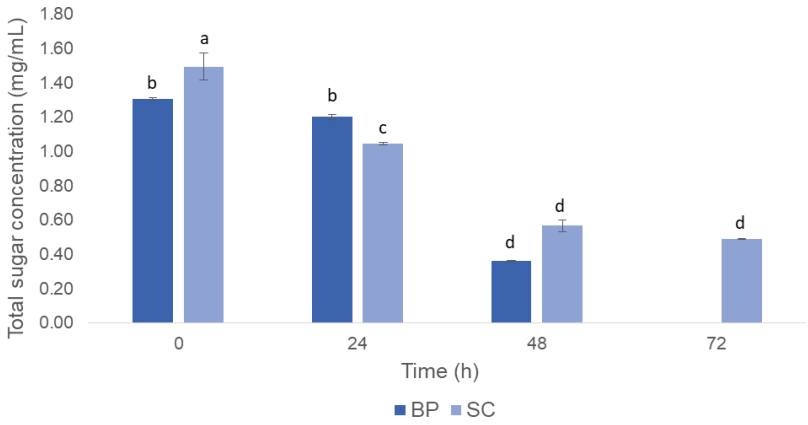

Figure 5.19. Total sugar concentration (mg/ mL) of extracts obtained during fermentation of sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

5.4. Characterisation of final extracts under optimal fermentation conditions (SQSF)

To verify the antioxidant capacity of the extracts three different methods (ABTS, DPPH and ORAC) were tested for each condition (BCt, SCt, BP and SC).

5.4.1. Antioxidant Capacity by ABTS and DPPH

In this experience (**Figure 5.20.**) we can see that the extract of straw after fermentation with yeast have the higher ($p < 0.05$) antioxidant activity measured by the two methods ABTS and DDPH. In ABTS method before fermentation no differences were observed between bagasse and straw (BCt and SCt) and after fermentation a significant decrease in antioxidant capacity was observed for bagasse and straw (BP and SC) since EC increase ($p < 0.05$). In DPPH method, no differences were observed ($p > 0.05$) between bagasse and straw before fermentation (BCt and SCt) and bagasse after fermentation (BP). In the same method, no differences were observed ($p > 0.05$) between straw before and after fermentation (SCt and SC). The results of this experience show that the extracts with best antioxidant capacity by ABTS was straw fermented with the yeast (SC) presenting an EC50 of 2.62 ± 0.98 mg/ mL. In DPPH method, the extract with best antioxidant capacity was straw extract with and without yeast fermentation (SC and SCt) with an EC50 of 6.85 ± 0.24 and 13.08 ± 0.34 mg/mL, respectively.

The yeast *S. cerevisiae* can influence the antioxidant capacity observed for sugarcane straw due to its capacity to transform sugars into metabolites such as phenolic compounds **(Brandolini *et al.*, 2007)**.

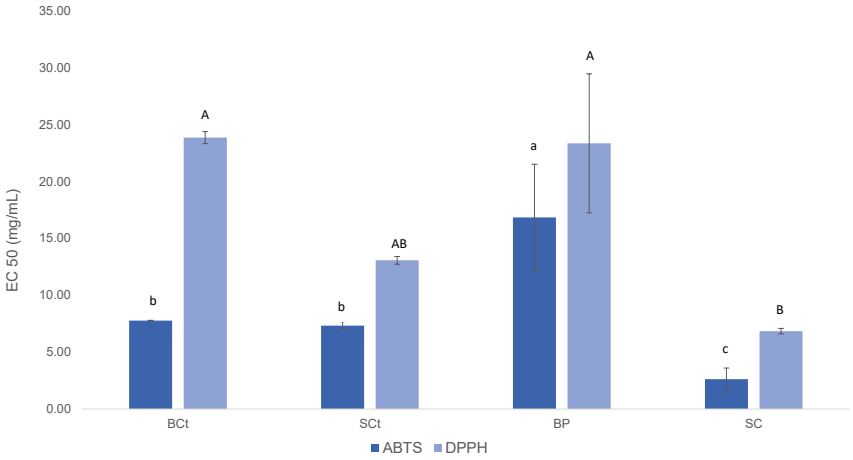

Figure 5.20. Total antioxidant capacity of extracts obtained before fermentation of sugarcane bagasse and straw (BCt and SCt) and after fermentation of sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism. a Different lowercase (a) and uppercase (A) letters represent significant differences between samples measured for ABTS and DPPH methods respectively.

5.4.2. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC method gives the total antioxidative capacity of the analysed sample rather than the capacity of the individual components of the system. Several studies demonstrated that cell-free extracts

from lactic acid cultures show some degree of antioxidant activity, by showing capacity to release antioxidants in the intestine after exposure to bile salts when they are consumed **(Saide *et al.*, 2005)**. The concentration and accumulation of many biologically actives substances in sugarcane like phenolic compounds are responsible for the peroxyl radical scavenging capability antioxidant properties in the extracts and this is agreement with previous reports **(Sun *et al.*, 2002; Thaipong *et al.*, 2006)**. Thus, plants with rich phenolic contents are a valuable source of antioxidants **(Zheng *et al.*, 2017)**.

In ORAC method (**Figure 5.21.**), we can see that there are no significant differences ($p > 0.05$) between bagasse control (BCt) and straw control (SCt). The extract obtained from bagasse fermented with *L. plantarum* (BP) had no significant differences ($p > 0.05$) with straw fermented with *S. cerevisiae* (SC), but both extracts presented a decrease in their radical scavenging capacity comparing with bagasse and straw control (BCt and SCt). The higher antioxidant activity was observed for straw and bagasse before fermentation (SCt and BCt) presenting values of 1.58 ± 0.32 and 1.29 µmol TE/ g sample, respectively. The values of bagasse and straw decrease after fermentation (BP and SC) with a value of 0.89 ± 0.28 and 0.91 ± 0.03 µmol TE/ g sample.

This value for ABTS differ substantially from those found in lentil extracts fermented with *L. plantarum* (12.5 µmol Trolox/ g DW) that show higher antioxidant activity according to **Fernandez-Orozco et al., 2007**. **Đorđević, säiler-Marinković**, and **Dimitrijević-Branković (2010)** evaluated the influence of fermentation by *L. rhamnosus* and *S. cerevisiae* on the antioxidant activities of 4 cereals (wheat, buckwheat, wheat germ, barley and rye) and compared them with their unfermented counterparts and concluded that antioxidant activity increase through fermentation by lactic acid and yeast **(Masisi *et al.*, 2016)**.

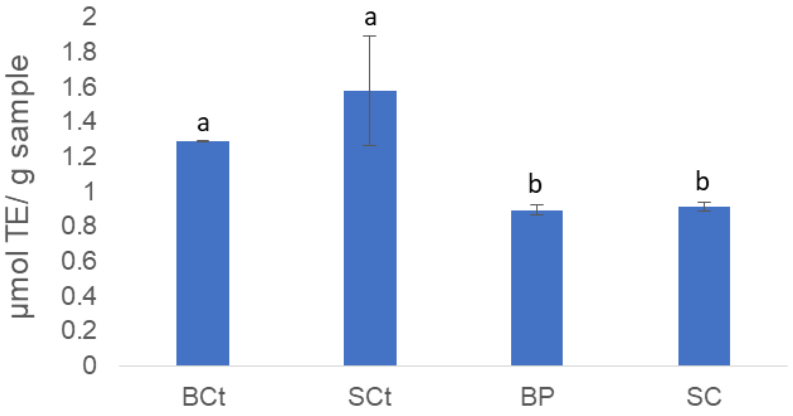

Figure 5.21. Antioxidant capacity content of extracts obtained before fermentation of sugarcane bagasse and straw (BCt and SCt) and after fermentation of sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

5.4.3. Profile of organic acids and monosaccharides

The number of organic acids (lactic acid, acetic acid and propionate) and monosaccharides (cellobiose, glucose and sorbitol) were quantified in the end of each fermentation condition trough high performance liquid chromatography (**Figure 5.22.**).

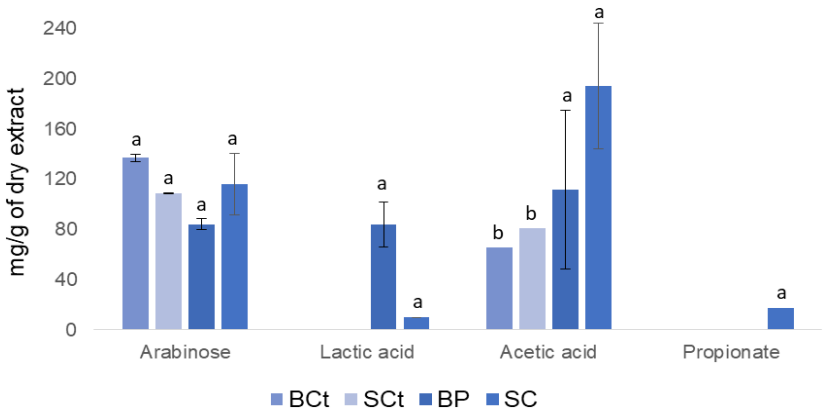

Figure 5.22. Characterization of monosaccharides and organic acids of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism for each compound.

The monosaccharides, glucose and cellobiose were only detected in the bagasse and straw samples before fermentation. Glucose reached amounts of 38.89 ± 1.13 for bagasse and 41.51 ± 2.63 mg/ g of dry extract for straw. Cellobiose reached amounts of 4.80 ± 2.52 for bagasse and 12.59 ± 0.96 mg/ g of dry extract for straw.

Monosaccharides are the main source of carbon and energy in plants **(Büttner *et al.,* 2000)**. For efficient hydrolysis of cellulosic substrates, the enzyme celluclast acts at the end of the cellulose and lignin chains and causes the release of cellobiose. Then a second hydrolysis of the soluble materials to glucose occurs **(Gabiatti Junior *et al.,* 2020)**. According to the study of **Gabiatti Junior *et al*., (2020)**, after 25 h of hydrolysis of washed residual cellulose casings with the Celluclast enzyme, there is a greater increase in glucose than in cellobiose with values of 15 and 8 mg / mL, respectively. These data are in agreement with those presented in **Figure 5.22.**, where glucose is significantly higher than cellobiose.

In organic acid, the acetic acid was the main acid present in all samples before and after fermentation. An increase after bacteria and yeast fermentation were observed from 53.73 ± 2.31 to 111.82 ± 44.74 mg/ g of dry extract to bagasse and from 83.88 ± 0.59 to 194.16 ± 35.50 mg/ g of dry extract to straw ($p < 0.05$). Lactic acid was only detected in extracts obtained after yeast and bacteria fermentation and propionate was detected in extract after yeast fermentation. The lactic acid reached amounts of 84.02 ± 12.54 for bagasse and 9.83 ± 0.55 mg/ g of dry extract for straw. Propionate reached amounts of 17.05 ± 0.24 mg/ g of dry extract.

Organic acids are known as products or metabolites of microbial fermentations. Under anaerobic conditions, heterofermentative LAB produce lactic and acetic acids as end products. These organic acids have antimicrobial and antioxidant characteristics **(Kuley *et al*, 2020)**. The main activity of the lactic acid bacteria is to produce the end product lactic acid by metabolizing the sugar. The presence of acetic acid in the media after fermentation by the *L. plantarum* can be the result of the different biochemical pathway, e.g. the degradation product of produced lactic acid, the result of the citrate metabolism and/or it originates from the heterofermentative pathway **(Zalán *et al.,* 2009)**. The yeast *S. cerevisiae* has a low lactic acid value which is derived from glucose and reduced pyruvate decarboxylase activity.

Sugarcane by-products such as bagasse and straw are mainly composed of nitrogen, carbon and potassium. The high carbon content causes these by-products to produce chemicals such as organic acids **(Soltanian *et al.,* 2019)**. With the hydrolysis of the lignocellulosic structure, the acetyl and uronic groups present in the hemicellulose chains are removed and acetic acid is formed **(Lopes Silva *et al.*, 2017)**.

The hydrolysate of lignocellulosic materials mainly consists of sugars such as xylose, glucose and arabinose. Sugarcane consists mainly of two carbohydrates (cellulose and hemicellulose) and when efficient hydrolysis is used, the hemicellulose is completely hydrolysed into D-xylose and L-arabinose **(A. Pessoa *et al.,* 1997)**.

5.4.4. Total protein

In **Figure 5.23.**, nitrogen is not a limiting factor for microbial growth. The high percentage of protein in both bacteria and yeast extracts is due to the addition of supplements, such as yeast extract and peptone, it the beginning of saccharification.

In this experience, in samples before and after fermentation with *S. cerevisiae* the total protein content of straw is similar ($p > 0.05$) with a value between 32 – 33 %. The bagasse extract before and after fermentation with *L. plantarum* do not show significant differences ($p > 0.05$) as well as with the straw after fermentation with *S. cerevisiae.* The values of total protein in bagasse extract range from 35 – 38 %.

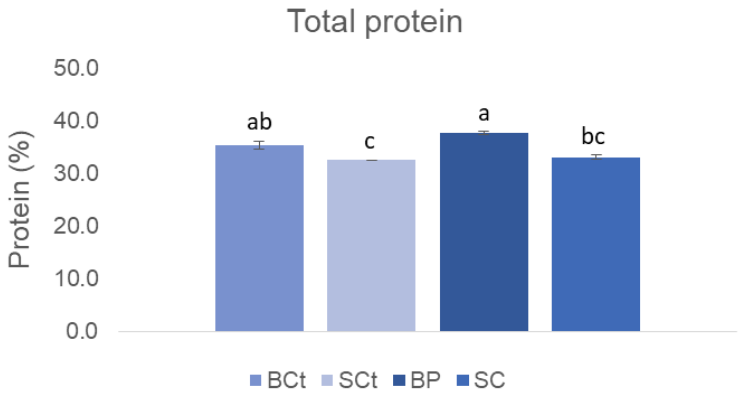

Figure 5.23. Total protein content of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation of sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

5.4.5. Total Phenolic Content

The total phenolic content of bagasse and straw extracts was evaluated before and after fermentation and it was possible to observe that straw extract had more total phenolic ($p < 0.05$) than bagasse and the fermentation of straw with *S. cerevisiae* presented the higher value (16.15 ± 0.29 mg GA/ g dw). On the other hand bagasse extracts did not shown significant differences with *L. plantarum* fermentation process (**Figure 5.24.**). This higher content of polyphenols maybe related with the fact that straw normally has more phenolic compounds than bagasse and in addition, the yeast during the fermentative process can also causes a greater release of phenolics.

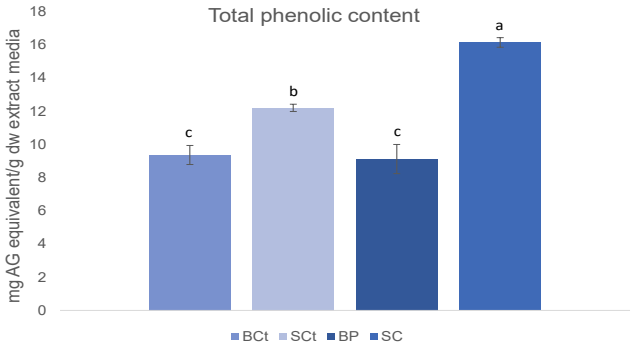

Figure 5.24. Total phenolic content of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

5.4.6. Individual Phenolic Compounds

The phenolic profile and organic acids of the different obtained extracts (BCt, SCt, BP and SC) was performed trough LC-ESI-UHR-QqTOF-MS.

In this experience, three organic acids (**Figure 5.25.**) were detected in extracts. Citric acid and azelaic acid were detected in samples before and after fermentation with bacteria and yeast. In citric acid a decrease was observed after straw extract fermentation ($p < 0.05$) from 7.79 ± 1.83 to 2.90 ± 0.24 mg/ g of dry extract while a slight decrease was observed in bagasse extract ($p > 0.05$) from 7.13 ± 1.55 to 6.98 ± 0.59 mg/ g of dry extract. Azelaic acid had a similar value before and after bagasse fermentation with *L. plantarum* ($p > 0.05$) and a slight increase after straw fermentation with *S. cerevisiae* from 0.064 ± 0.01 to 0.088 mg/ g of dry extract ($p < 0.05$). Malic acid was only detected in samples after fermentation without significant differences between samples.

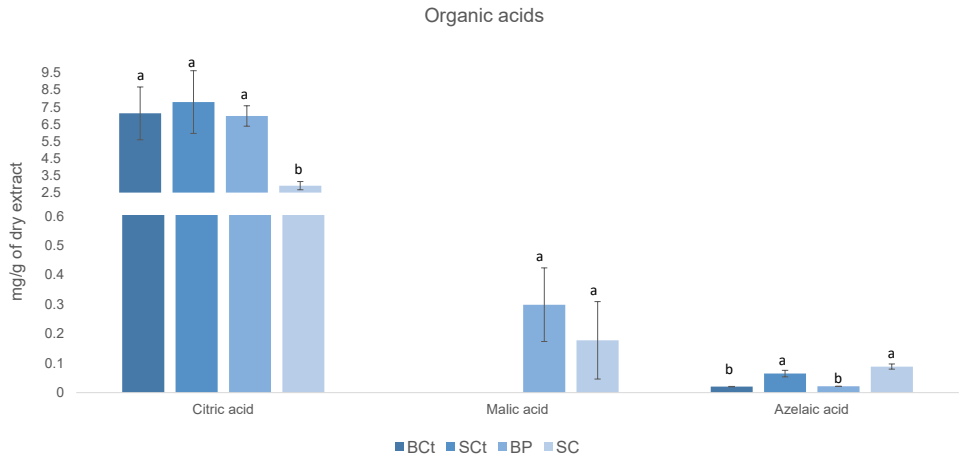

Figure 5.25. Characterization of organic acids of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism for each compound.

Organic acids are the natural products or natural intermediates of the main metabolic pathways of microorganisms and can be synthesized from sugars such as glucose, xylose and sucrose or by the hydrolysis of lignocellulosic biomass. Citric acid and malic acid are two types of antioxidants that are also safe additives, widely used in the food, medical, daily chemical and healthcare product industries. In addition, citric and malic acid can be used as carbohydrate source to provide energy for microbial activities which accelerates the growth of LAB. It can reduce pH and inhibit the growth of yeast and moulds **(Li *et al.*, 2020)**. Malic acid is an intermediate of the fundamental cycle as it enters it through its aspartate-argininosuccinato derivative **(Gupta *et al.*, 2018)**. Yeast cannot effectively degrade malic acid because its malic enzyme has a low affinity for the substrate used **(Redzepovic *et al.*,2003)**. Its low value cannot be attributed to malolactic fermentation because the amount of lactic acid produced is small. According to Colorretti the low value of malic acid present may be due to D-malic acid molecules entering *S. cerevisiae* cells by passive diffusion **(Chen *et al.*, 2014)**. Citric acid is a natural component and common metabolite of plants and animals. This is the second most profitable product of fermentation. **Hamissa *et al.*, 1981** report that high yields of citric acid were obtained when glucose was used as substrate with the yeast *S. cerevisiae*. The use of yeast extract and peptone as a supplement used in the extract with the yeast provides an increase in citric acid **(Afolabi *et al.*, 2018)**. *L. plantarum* bacteria are able to metabolise citric acid as an accessible carbon source present in biomass **(Mousavi *et al.*, 2013)**.

The azelaic acid is a C7 saturated dicarboxylic with antimicrobial and antioxidant properties. Upon hydrolysis by the selected enzyme, azelaic acid was found in small amounts presumably resulting from the degradation of lignin building blacks containing **(Jiang *et al.*, 2016)**. Fermentation with *L. plantarum* increases lipid components and other bioavailable molecules such as azelaic acid. This or-

ganic acid was reported to be produced through central carbon metabolism and is found in the supernatant **(Siedler *et al.*, 2019)**. During fermentation, the yeast *S. cerevisiae* can promote the production of acids, as it was described for azelaic acid **(Shi *et al.*, 2022)**.

After analysing the organic acids present before and after the fermentation with bacteria and yeast, the polyphenolic profile was also analysed and it was possible to quantify 6 compounds in the bagasse and straw extracts (**Figure 5.26.**).

Phenolic compounds are important constituents of food products of plant origin. These compound are the secondary metabolites of plants which are not only responsible for the colouring shades in fruits and vegetables but also protect plants from pathogens, parasites and predators. In addition, the presence of phenolic compounds on the diet is beneficial to health. The health effects of phenolic compounds depend on the amount consumed and on their bioavailability **(Rodríguez *et al.*, 2009)**.

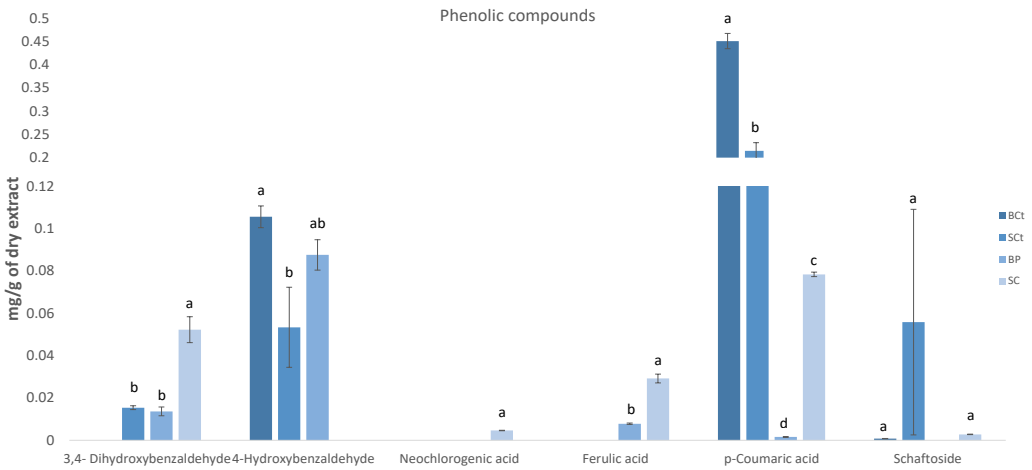

Figure 5.26. Characterization of phenolic compounds of extracts obtained before fermentation of sugarcane bagasse and straw control (BCt and SCt) and after fermentation sugarcane bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism for each compound.

Three compounds belonging to the hydroxycinnamic acid class were identified in bagasse and straw extracts. The *p*-coumaric acid presented a large decrease in the bagasse and straw extract after fermentation ($p < 0.05$). Ferulic acid was only detected in extracts after fermentation of bacteria and yeast with different values ($p < 0.05$). Neochlorogenic was only detected in straw extract of yeast after fermentation.

Within hydroxybenzoic acids, two different compounds were identified and these are 3,4- dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. The 3,4-dihydrozybenzaldehyde was detected in straw extract before fermentation and in extracts after fermentation. On the other hand, the 4 hydroxybenzaldehyde was detected in extracts before fermentation and in bagasse extract after bacteria fermentation.

Within flavonoids class the schaftoside was the only compound identified and it was detected in extracts before fermentation and in straw extract with yeast fermentation with similar values ($p > 0.05$).

Fermentation of bagasse with *L. plantarum* (BP) showed an increase in antioxidant activity, and this may be attributed to polyphenolic compounds. The changes in phenolic compounds might be due to the glycosidase produced by *L. plantarum*, which led to phenolic metabolism and release. Studies report that *ρ*-coumaric acid is present in sugarcane products and this acid is very common in the diet and has antioxidant activity. The phenolic compounds 4-hydroxybenzaldehyde and ferulic acid are present not only in the extracts with microorganisms but also in the extracts of sugarcane molasses, cane juice, cane syrup and other sugar products. The phenolic compound schaftoside is a flavone-C-glycoside and is present not only in extract of straw but also in extracted cane juice with scavenging activity against DPPH radicals **(Asikin *et al*., 2013)**. In the straw extract with the yeast, a compound derived from hydroxycinnamic acid, neochlorogenic acid, was found. This compound was also found in sweet cherries, blackberries, plums and cherry wine. This phenolic compound is stable and not susceptible to the action of enzymes **(Cryzowska *et al*., 2002)**.

Gallic acid is decarboxylated to pyrogallol by the bacterium *L. plantarum* of 4-hydroxybenzaldehyde acid occurs. The 3,4 - dihydroxybenzaldehyde content have increased with *S. cerevisie* fermentation and also it has been reported to have antioxidant properties by reactive oxygen species (ROS), chelating metal ions and indirectly inducing antioxidant **(Ofosu *et al.*, 2022)**. The straw control extract has an amount of lignin of about 25 % that can be extracted through saccharification, and this extract is rich in phenolic compounds that are antioxidant and antimicrobial.

Fitzgerald observed that when the cultured *S. cerevisiae* with vanillin the likely breakdown products would be the oxidation of vanillin to vanillic acid, the demethoxylation of vanillin to protocatechuic aldehyde (3,4 – dihydroxybenzaldehyde) or the demethoxylation of vanillic acid to 3,4 – dihydroxybenzoic acid **(Fitzgerald *et al*., 2003)**.

5.4.7. Antidiabetic (α-Glucosidase inhibition)

For the antidiabetic activity, an enzyme, α-glucosidase, which breaks down starch and disaccharides into glucose was used. For this assay, Acarbose was used for its validation. Acarbose is an antidiabetic drug, which inhibits the enzyme in the intestines. This inhibition reduces the rate of glucose absorption through delayed carbohydrate digestion and prolonged digestion time **(Martin *et al.,* 1996)**.

In this assay, two different extracts were used, first the bagasse fermented with *L. plantarum* at a concentration of 1.5625 mg/mL and a second the straw with *S. cerevisisae* at a concentration of 3.125 mg/mL. The selected concentrations were based on the non-cytotoxic concentrations evaluated in **Section 5.4.9.** . The two extracts, with these initial concentrations had no capacity to inhibit the enzyme and therefore there was no activity of any kind (data not shown).

5.4.8. Screening of prebiotic potential

To assess the potential prebiotic effect of extracts obtained after straw and bagasse saccharification with Cellulase followed by fermentation with *S. cerevisiae* and *L. plantarum*, a simulation of gut microbiota fermentation was performed through an *in vitro* fermentation model using faeces from five donors. Sampling was performed at times 0, 24 and 48 h, in order to quantify the pH, the total and specific bacteria, such as *Enterobacteriaceae*, LAB and *Bifidobacterium*, present in the sample and also

to quantify the short chain fatty acids (SCFA). This data was compared with two controls, one positive designated as Fruto oligosaccharides (FOS) and one negative only with faecal inoculum control (IC).

Normally, the pH of the human colon varies between 5 and 7 and in this experience the negative control (IC) and bagasse extract no significant differences ($p > 0.05$) were observed after 48 h. On the other hand, FOS presented a high decrease from 7.14 ± 0.01 to 3.64 ± 0.05 and in straw extract pH decreased from 7.24 ± 0.02 to 6.72 ± 0.05 (**Figure 5.27.**). The pH decrease, although slight, in BP and SC extracts in the intestinal tract may promote the increase of some beneficial bacteria and inhibit harmful microorganisms. FOS, on the other hand, is not digestible and therefore passes intact through the small intestine to the colon to support the growth of healthy bacteria in the digestive tract.

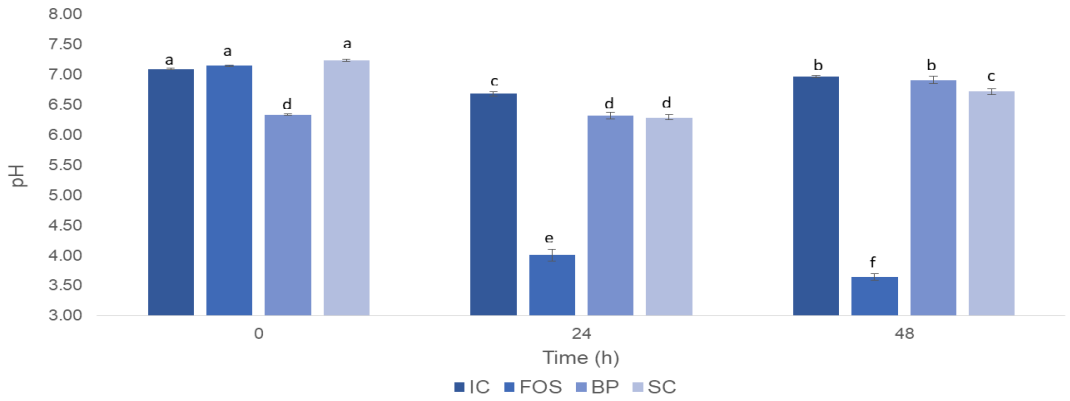

Figure 5.27. pH Value of the inoculum control (IC), Fructooligosaccharides (FOS), bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC). [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

For each condition and over time, viable cell counts were taken for total / facultative anaerobic bacteria (a) acid lactic bacteria (LAB) (b), *bifidobacteria* (c) and Gram negative bacteria and *enterobacteria* (d), which are normally found in the healthy gut microbiota and **Figure 5.28.** shows the changes in bacterial population during the 48 h of this treatment.

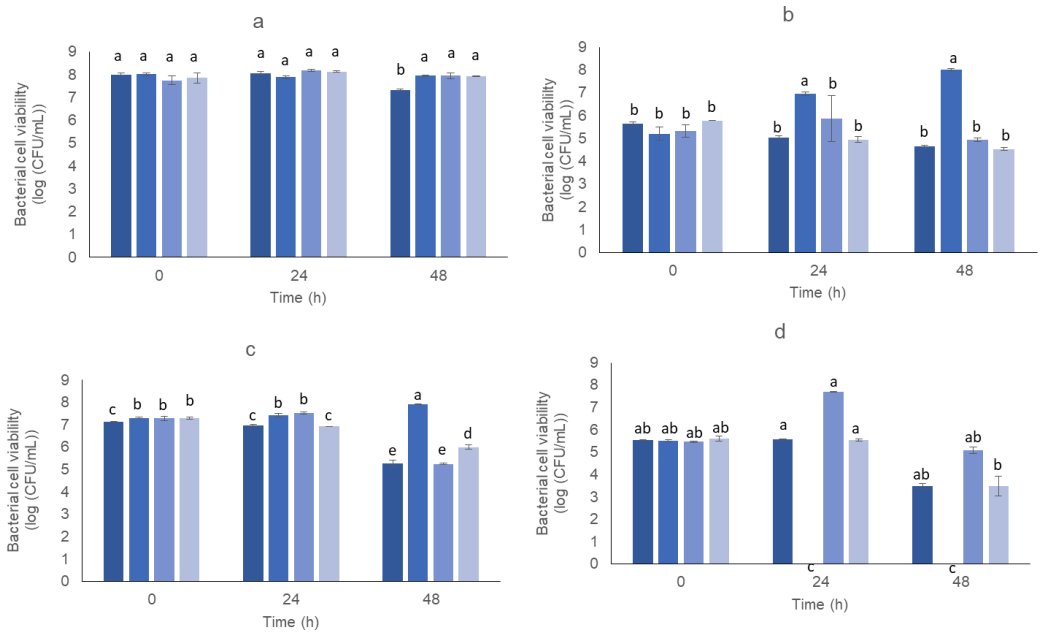

Figure 5.28. Bacterial viable cell counts (log CFU/ mL) in inoculum control (■ IC), Fructooligosaccharides (■ FOS), bagasse with *L. plantarum* (■ BP) and straw with *S. cerevisiae* (■ SC) obtaining using different culture media incubated during 48 h at 37 ºC in anaerobic. (a) Total bacteria, (b) Lactic acid bacteria, (c) Bifidobacterium, (d) Gram negative and Enterobacteriaceae. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

In total bacteria no significant differences (p< 0.0) were detected between extracts by presenting value of 8 log (CFU/ mL) over 48 h. The BP and SC extracts have some oscillations over the 48 h of fermentation on all culture media.

In (LAB) no significant differences (p > 0.05) were seen along fermentation time in the inoculums with bagasse extract and straw extract neither in negative control. On the other side, the positive control (FOS) increased from 5.20 to 8 log (CFU/mL) after 48 h. The BP extract has a good ability to increase the production of LAB in the first 24 h which does not happen in the following.

In *bifidobacterium* in negative control a decrease by the end of 48 h was observed, while in positive control the presence of FOS stimulated this group of bacteria's to increase from 7.3 to 7.9 log (CFU/mL), in the presence of bagasse extract decreased ($p < 0.05$) from 7.2 to 5.2 and in the presence of straw extract decreased ($p < 0.05$) from 7.3 to 6.0 log (CFU/mL). The BP and SC extracts did not promote and increase in their growth.

The group of *enterobacteriaceae* cells were not detected in positive control after 24 and 48 h fermentation, while in negative control and in the presence of straw extract decreased significantly only after 48 h fermentation from 5.5 to 3.5 log (CFU/mL) and in the presence of bagasse extract a slowly decrease after 48 h fermentation from 5.4 to 5.0 log (CFU/ mL). The extracts analysed promoted the growth of *enterobacteria*. Although BP and SC extracts present interesting characteristics for being prebiotics, they need to be improved due to the growth of gram-negative and *enterobacteria* that may harm the host's health.

The pH modulates bacterial populations, so differences in bacterial population composition between inocula are expected. FOS has a large pH variation over time and therefore a large variation in bacterial population in different media. In the different culture media that were evaluated, the inoculum with FOS was the only one that promoted the growth of LAB and *bifidobacteria* and the only one where it did not inhibit the growth of *enterobacteria* and this is due to it being a prebiotic. In IC it would be expected that there would be no major changes in the bacterial populations as no nutrient source was added.

At the end of each time point in the fermentations performed, SCFA and lactate production was analysed (**Figure 5.29.**). Each set included four conditions in triplicate: (1) IC (fecal inoculum with basal medium), (2) FOS with faecal inoculum, (3) BP *(*bagasse with *L. plantarum* and faecal inoculum) and (4) SC (straw with *S. cerevisiae* and faecal inoculum). The IC provided information about the production of the intestinal microbiota without interference from a nutritional source, while FOS is a prebiotic fibre that can reach the intestine unaltered and therefore has the capacity to reinforce the immune system and minimise invasion and colonisation by pathogenic microorganisms. These two controls allow comparison of the function of the extracts.

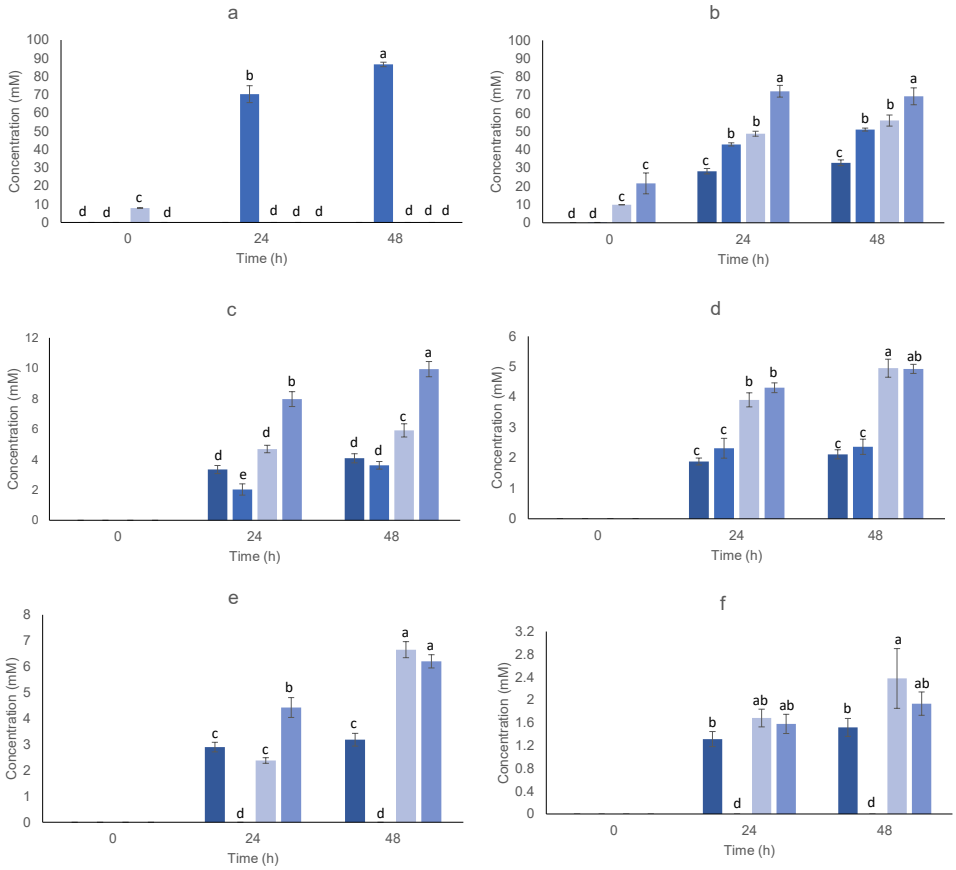

Figure 5.29. Concentration (mM) of the SCFA and lactate produced during 48 h of colonic fermentation using bagasse with *L. plantarum* (BP) and straw with *S. cerevisiae* (SC), inoculum control (IC) and Fruto oligosaccharides (FOS). (a) Lactate; (b) acetate; (c) butyrate; (d) propionate; (e) valerate and (f) isovalerate. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

The organic acid lactate appears normally in the first hours of fermentation and tends to disappear after 24 h because it is consumed by the bacteria in the intestine. This organic acid is produced by lactic acid bacteria and bifidobacteria which is then converted into butyrate and propionate **(Duncan *et al.,* 2004)**. This process did not occur in the study performed, because although we have concentrations of butyrate and propionate in all conditions, we do not have lactate in the initial time of any compound analysed, with the exception of BP (7.93 ± 0.16 mM). After 48 h, lactate is present in the positive control (FOS) with concentrations between 70 ± 4.64-90 ± 1.22 mM and an increase in the concentrations of acetate, propionate and butyrate is observed.

As expected, at the beginning of fermentation of all conditions there is no production of SCFA, except acetate which is found in minimal concentrations in BP and SC extracts, because they are produced by the microorganisms during the initial production of the extracts. The concentration of BP over 48 h increase from 10 ± 0.06 to 56 ± 3.06 mM and the concentration of SC increase from 22 ± 5.68 to 70 ± 4.63 mM. It is only after the first 24 h that acetate is present in the IC and FOS inoculums and an increasing ($p > 0.05$) slightly occurs in the following 24 h, ending with a value of 56 ± 1.58 and 51 ± 0.81 mM, respectively.

At the beginning of the fermentation and in the controls (IC, FOS, BP and SC) the organic acid butyrate was not detected. In the IC at the end of 48 h of fermentation a slight increase from 3.3 ± 0.27 to 4 ± 0.30 mM but insignificant ($p > 0.05$) occurred. In the positive control (FOS) and the fermented bagasse and straw extracts a significant increase ($p < 0.05$) was recorded. Propionate was only detected in the extracts after the first 24 h of fermentation. In controls (IC and FOS) a slight increase occurred after 48 h ($p > 0.05$). In the bagasse extract fermented with *L. plantarum* an increase ($p < 0.05$) from 3.90 ± 0.23 to 5 ± 0.30 mM was recorded at the end of 48 h. In the straw extract fermented with *S. cerevisiae* an increase from 4.3 ± 0.16 to 5 ± 0.15 mM ($p < 0.05$) occurred at the last 24 h.

Valerate and isovalerate are found in three of the four conditions tested (IC, BP and SC) at 24 and 48 h. The most significant increase in valerate and isovalerate concentrations is in the BP inoculum (2.4 ± 0.11- 6.7 ± 0.31 and 1.7 ± 0.16 - 2.4 ± 0.52 mM, respectively), followed by SC (4.4 ± 0.38 - 6.2 ± 0.26 and 1.6 ± 0.17 - 2.0 ± 0.21 mM, respectively) and lastly the IC inoculum (2.9 ± 0.19 - 3.2 ± 0.25 and 1.3 ± 0.13 - 1.5 ± 0.16 mM, respectively). The IC did not show significant differences ($p > 0.05$) over the 48 h in valerate and isovalerate organic acids. The bagasse extract fermented with *L. plantarum* shows significant differences ($p < 0.05$) in valerate and isovalerate over 48 h. Finally, the straw extract fermented with *S. cerevisiae* presents significant differences ($p < 0.05$) in valerate but no significant difference ($p > 0.05$) in isovalerate throughout the 48 h of fermentation.

For the BP extract and at 48 h, the concentration for acetate, propionate, butyrate, valerate and isovalerate were 56.04 ± 3.06, 4.95 ± 0.30, 5.92 ± 0.44, 6.66 ± 0.31 and 2.38 ± 0.52 mM, respectively. For the same conditions, the SC extract had a higher concentration for acetate and butyrate (69.32 ± 4.63 and 9.94 ± 0.50 mM, respectively), lower for valerate and isovalerate (6.21 ± 0.26 and 1.93 ± 0.21 mM) and equal to propionate (4.93 ± 0.15 mM). The SCFA produced by the two controls (IC and FOS) show lower concentrations than the extracts (BP and SC) propionate (4.93 ± 0.15 mM).

The condition with the lowest SCFA production is the IC since there was no added substrate to feed the intestinal bacteria. After FOS administration an increase in SCFA production was observed in

different areas of the colon. Thus, the administration of prebiotics induces the synthesis of SCFA which exerted positive effects against inflammatory of the intestinal barrier. These results suggest that prebiotics are not only used for the growth and development of specific colonic bacteria, but also promote the production of bacterial metabolites with potential health benefits **(Peredo-Lovillo et al., 2020)**.

Lactate normally appears in the first hours of fermentation and disappears after 24 hours due to its consumption by intestinal bacteria. It is produced by LAB and bifidobacteria and is then converted into acetate, propionate and butyrate. This happens with the BP extract which only shows values at time 0 and after 24 h presents values for acetate, propionate and butyrate. In all inocula tested, a gradual production of acetate occurred, and this is due to the fact that acetate production pathways are distributed among the bacterial groups in the gut and that it plays a direct and important role in appetite regulation. Furthermore, acetate is subject to bacterial transformation into other metabolites such as butyrate through the process of cross-feeding. Propionate has potential health-promoting effects due to its antilipogenic, cholesterol-lowering, anti-inflammatory and anti-carcinogenic actions. Butyrate is an important energy source for intestinal epithelial cells and is believed to counteract colorectal cancer and inflammatory **(Fehlbaum *et al.*, 2018)**.

5.4.9. Cytocompatibility

We analysed cell viability for the extracts obtained from fermentation of bagasse with the bacterium *L. plantarum* and straw with *S. cerevisiae* (**Figure 5.30a** and **Figure 5.30b**). As we can see (**Figure 5.30a**), exposure cells to concentrations above 1.5625 mg/mL show toxicity for bagasse extract fermented with *L.* plantarum since it was observed an metabolic inhibition above 30% on CaCo-2 intestinal cells. For straw (**Figure 5.30b**) concentrations above 3.125 mg/mL showed toxicity. These values are lower than in previous experiments. This may be due to the centrifugation procedures that were changed, at the beginning the whole cells were removed but it the current experiments some cells fragments were left in the medium.

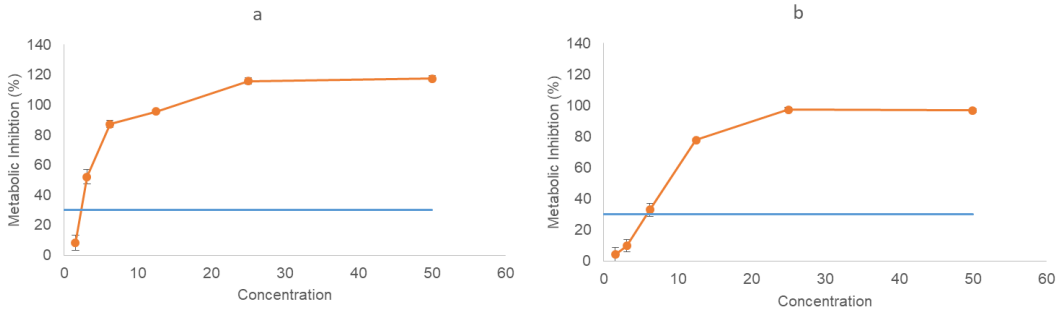

Figure 5.30. Cytocompatibility profile of the fermented sugarcane extract of (a) bagasse with *L. plantarum* and (b) straw with *S. cerevisiae* dispersed in water when in contact with intestinal Caco-2 cell line, measured by the potential of the extract to inhibit the cell metabolic activity. The blue line represents the 30 % cytotoxicity limit.

5.4.10. Immunomodulatory effect on CaCo-2

To measure the immunomodulatory effects of the extracts of bagasse and straw with microorganism (BP and SC), three major types of macrophage activation signals, including TNF-α (**Figure 5.31.**), IL-6 (**Figure 5.32.**) and IL-8 (**Figure 5.33.**), were evaluated in our study. The control group without the extracts was used to determine the basal levels of TNF-α, IL-6 and IL-8.

In **Figure 5.31.**, the results show that basal extracts have no significant differences ($p > 0.05$) for all the interleukins. The extracts from bagasse and straw after fermentation presented significant differences ($p < 0.05$) among themselves and with basal extracts. The extract with a higher value was bagasse after fermentation (BP +) with a value of 89.87 pg/ mL of sample for interleukin TNF-α. TNF-α plays dual functions in cancer cells, where in some cases it induces apoptosis and necrosis, and in others it promotes tumor growth. However, there is strong evidence that TNF-α is pro-tumorigenic, promoting progression and metastasis of cancer cells. The TNF-α production in BP extract stimulated CaCo-2 cells as biomarkers of inflammatory response. On the other hand, the extract SC inhibited production of TNF-α.

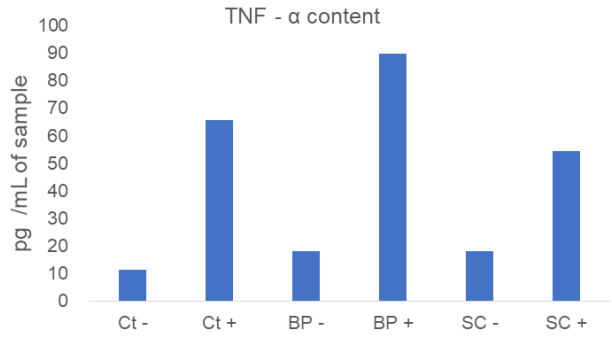

Figure 5.31. Tumor necrosis factor (TNF-α) concentrations produced by Caco-2 cells when treated with sugarcane bagasse extract (3.125 mg/ mL) with (BP+) and without *L. plantarum* (BP-) fermentation and treated with sugarcane straw extract (6.25 mg/ mL) with (SC+) and without *S. cerevisiae* (SC-) fermentation. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

In **Figure 5.32.**, the results show that control extracts have no significant differences but there was an increase in straw control extract ($p < 0.05$). The extracts obtained after fermentation for both bagasse and straw stimulated an increase ($p < 0.05$) in IL-6 content when compared with positive control and with both control extracts. The extract that stimulated higher content of IL-6 was straw after fermentation (SC +) with a value of 142.63 pg/ mL of samples. Overexpression of IL-6 has been documented in several neoplastic disorders, including but not limited to colorectal, ovarian and breast cancer and several haematological malignancies. IL-6 has been shown to play an important role in tumour cell growth and survival, angiogenesis, immunomodulation of the tumour microenvironment, stromal cell activation, and ultimate disease progression **(Taher *et al.*, 2020)**. Given this background, non fermented extracts BP- and SC- decrease IL-6 secretion by human colorectal cancer cell line CaCo-2 but fermented extracts (BP+ and SC+) stimulated an increase of IL-6.

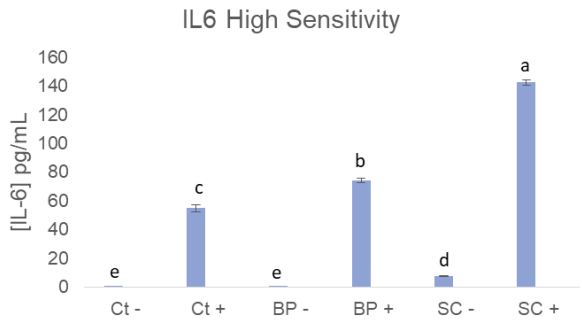

Figure 5.32. Interleukin-8 (IL-8) concentrations produced by Caco-2 cells when treated with sugarcane bagasse extract (3.125 mg/ mL) with (BP+) and without *L. plantarum* (BP-) fermentation and treated with sugarcane straw extract (6.25 mg/ mL) with (SC+) and without *S. cerevisiae* (SC-) fermentation. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

In **Figure 5.33.**, the results of IL-8 show that control extracts have no significant differences ($p > 0.05$) for all interleukins. The extracts from bagasse and straw after fermentation presented significant differences ($p < 0.05$) among themselves and with control. The samples with higher content of IL-8 were observed in cells treated with bagasse fermented extract (BP +) with a value of 1197.56 ± 1.72 pg/ mL of samples. However, when cells were treated in straw extract after fermentation a decrease in IL-8 was observed of 875.98 pg/ mL to 806.57 pg/ mL.

The IL-8 is a pro-inflammatory cytokine and involved in chemotaxis. Expression of IL-8 by cancer cells aids angiogenesis, increases proliferation and survival of cancer cells and promotes tumour escape from immune cells. In addition, expression of IL-8 by cancer cells is associated with poor prognosis in cancer patients **(Prakash *et al.*, 2021)**. BP decrease the secretion of IL-8 by human colorectal cancer cell line Caco-2, suggesting that BP exerts anti-cancer effects via down regulation of IL-8. On the other hand, SC extract increase the secretion of IL-8 and don't exerts anti-cancer effects.

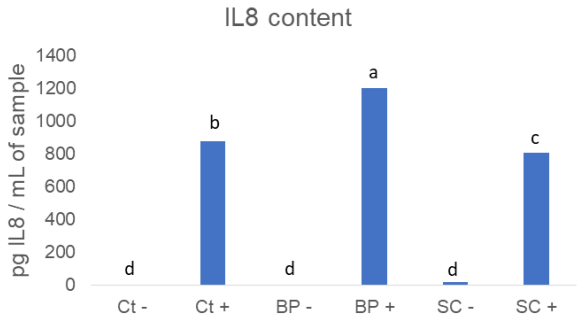

Figure 5.33. Interleukin-6 (IL-6) concentrations produced by Caco-2 cells when treated with sugarcane bagasse extract (3.125 mg/ mL) with (BP+) and without *L. plantarum* (BP-) fermentation and treated with sugarcane straw extract (6.25 mg/ mL) with (SC+) and without *S. cerevisiae* (SC-) fermentation. [a] Different letters mean significant differences ($p < 0.05$) between biomass type and microorganism.

6. Conclusions

Postbiotic refers to a preparation of inanimate microorganisms and/or their components that confers a health benefit to the host by enhancing the bioactivities of probiotics. Eating a diet rich in probiotic and prebiotic foods can ensure that the human gut has an adequate level of these essential compounds, as postbiotics are produced when probiotics feed on prebiotics. Postbiotics comprise metabolites such as short-chain fatty acids, extracellular polysaccharides and theionic acid. Their bioactivities include anti-inflammatory, anti-proliferative, antioxidant and immunomodulatory effects.

This work highlights the potential use of two by-products derived from the sugarcane industry, sugarcane straw and bagasse, to produce postbiotics, trough simultaneous and sequential saccharification with cellulase. For the fermentation process different microorganisms were tested, one yeast such as *S. cerevisiae* and a bacteria like *L. plantarum*, with potential application as a food ingredient.

Firstly, to obtain the maximum amount of total sugar released before went to the fermentation a saccharification process was optimized for both sugarcane biomasses (straw and bagasse) and it was possible to conclude that the best conditions were unmilled biomass with only celluclast enzyme for a period of hydrolysis of 48 h.

Secondly, after saccharification the fermentation conditions demonstrate that microorganisms of the genus *Lactobacillus* and the yeast *Saccharomyces cerevisiae* presented higher fermentation performance by decreasing the total sugar concentration obtained after a saccharification process. With the best extracts (*Lactobacillus plantarum* and *Saccharomyces cerevisiae* fermented with bagasse and straw) the monosaccharides and organic acids were characterised, where the following results were observed: arabinose was observed in all extracts, lactic and malic acid was only found in the extracts fermented with *Lactobacillus*, formate was observed in the extracts fermented with *Saccharomyces* extracts and propionate was present in the extracts with the microorganisms. The total protein level increased with fermentation, the total phenolic content was similar in fermentations and controls. The cytotoxicity with the yeast and the bacteria with bagasse has its maximum cell exposure at the concentration of 6.25 mg/ mL and the bacteria with straw has its maximum cell exposure at the concentration of 3.125 mg/ mL.

Thirdly, a sequential fermentation of saccharification with biomass removal was analysed and the results show that the microorganisms consume the sugars by presenting a decrease over time. Regarding the antioxidant capacity, in the ABTS and DPPH analyses, the extracts obtained after fermentation with bacteria had lower capacity than non-fermented extracts, while the extracts obtained through yeast fermentation had higher capacity than the respective non-fermented extracts. On the other hand, trough ORAC method, the fermentation led to a reduced antioxidant capacity for both biomasses. It was analysed the monosaccharides and organic acids present in the extracts where the results showed that in all the extracts it is present arabinose and acetic acid, in the extract with yeast it is observed propionate and lactic acid is present in the extracts with microorganisms. The protein content of the extracts increases slightly with fermentation as does the total phenolic content. Individual phenolic compounds present in the extracts were also identified, where the results were as follows: citric acid, azelaic *ρ*-coumaric, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde and schaftoside were present in all extracts. On the other hand, malic acid and ferulic were only detected in the extracts after

fermentation. Regarding the prebiotic effect was shown, through the increase in short-chain fatty acid, namely acetate, propionate and butyrate. The non-toxic levels of extracts for intestine Caco-2 cells were considered for concentrations lower of 3.125 mg/ml in the bagasse extract and 1.5625 mg/ml in the straw extract. Finally, the extracts obtained after bacteria fermentation stimulated the cells and an inflammatory response occurs trough TNF-α increase and modulates the pro-inflammatory mediators like IL-6 and IL-8. Extracts obtained through yeast fermentation induced inhibition of TNF-α production, and IL-6 secretion while stimulated an increase in IL-8 secretion. It was thus concluded that for the inflammatory system the intake of the bagasse extract is harmful, on the other hand, the straw extract with some modifications would be beneficial.

In conclusion, to achieve health benefits, the qualities of novel postbiotics must be thoroughly evaluated and understood. To ensure that the food sector is profitable and sustainable, the long-term health benefits of using these supplements must also be considered. Thus, postbiotics are constantly evolving and future research directions should emphasise establishing the relationship between health and the specific mechanisms of postbiotics.

7. Future work

The present work studied the production of postbiotic extracts for food application through sustainable fermentation at laboratory scale. To ascertain further health benefits to the consumer one should study the cholesterol and antihypertensive effect of the extracts. We should study the interaction in the fermentation of lactic acid bacteria with yeast because together we would have better results of antioxidant and antimicrobial capacity. We should also carry out a study of the interaction of the extracts in the various already existing foods in order to see the interaction between them. In addition, it would be interesting to ingest the product already incorporated into a food to understand how continuous ingestion could modulate and affect the human intestinal microbiota. An economic evaluation should be carried out to find out the cost of production and a market study of similar products already in existence to compare with the one developed in this work.

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