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Hydrolysates containing xylooligosaccharides produced by different strategies: Structural characterization, antioxidant and prebiotic activities

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ABSTRACT

This study explores the structural characterization, antioxidant and prebiotic activities of hydrolysates containing xylooligosaccharides (XOS) produced by different strategies: direct fermentation of beechwood xylan (FermBX) and enzymatic treatment of beechwood (EnzBX) and rice husk (EnzRH) xylans. EnzBX and EnzRH showed XOS with a backbone of (1 → 4)-linked-xylopyranosyl residues and branches of arabinose, galactose, and uronic acids. FermBX presented the highest content of total phenolic compounds (14 mg GAE/g) and flavonoids (0.6 mg QE/g), which may contribute to its antioxidant capacity –39.1 μmol TE/g (DPPH), 45.7 μmol TE/g (ABTS), and 79.9 μmol Fe II/g (FRAP). The fermentation of hydrolysates decreased the abundance of microorganisms associated with intestinal diseases from *Eubacteriales*, *Desulfovibrionales* and *Methanobacteriales* orders, while stimulating the growth of organisms belonging to *Bacteroides*, *Megamonas* and *Limosilactobacillus* genera. The production of short-chain fatty acids, ammonia, and CO_2 suggested the prebiotic potential. In conclusion, hydrolysates without previous purification and obtained from non-chemical approaches demonstrated promising biological activities for further food applications.

1. Introduction

Xylooligosaccharides (XOS) are sugar oligomers formed by xylose units linked through β-1,4 glycosidic bonds. XOS contain between 2 and 10 xylose units (de Freitas, Carmona, & Brienzo, 2019) and can be decorated with side groups linked to the main chain, namely acetyl, glucuronosyl, arabinosyl and galactosyl residues (Coelho, Rocha, Moreira, Domingues, & Coimbra, 2016). XOS are non-toxic, non-carcinogenic and generally recognized as safe (GRAS). Additionally, XOS present notable organoleptic properties, stability in a wide range of temperatures (up to 100 °C) and pH (2.5 to 8.0) conditions, and competitive prices comparing to other oligomers (Amorim, Silvério, Prather, & Rodrigues, 2019), hence making them attractive for several food applications.

Although XOS are naturally present in honey, fruits, vegetables, and other food products, they are found in small amounts (Mano et al., 2018), which justifies developing strategies to produce these oligomers at an industrial scale from other sources. Likewise, XOS have several biological properties of interest to the food and cosmetic industries. XOS are recognized as prebiotics due to their ability to stimulate the growth of beneficial gut microbiota and inhibit pathogenic and deteriorating microorganisms (Sajib et al., 2018). The gut microbiota utilizes XOS and produces gases (mainly H_2 and CO_2) and key metabolites to human intestinal health, e.g., lactate and short-chain fatty acids (SCFAs) (Álvarez et al., 2020; Moniz et al., 2016). The prebiotic potential of XOS has been successfully demonstrated through *in vitro* evaluation with human fecal inocula (Amorim et al., 2020a; Buruiana, Gómez, Vizireanu, & Garrote, 2017). Other studies indicate that XOS also have antioxidant properties (Jagtap, Deshmukh, Menon, & Das, 2017; Valls et al., 2018) and emerge as an alternative to traditional synthetic antioxidants. Together with these health benefits, XOS can act in diabetes and colon cancer prevention, improve lipid metabolism, and stimulate the immune response

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(Amorim et al., 2019).

XOS are produced by xylan hydrolysis, a heteropolysaccharide with a linear backbone of β-1,4 xylose residues (de Freitas et al., 2019). Among the available strategies to obtain XOS (e.g., autohydrolysis, chemical hydrolysis, enzymatic hydrolysis, or combined processes) (Gautério, da Silva, Hübner, & Ribeiro, 2021; Jagtap et al., 2017; Surek & Buyukkileci, 2017), the use of xylanases (and eventually auxiliary enzymes) stands out due to the specific deconstruction of the polysaccharide chain. Xylanases cleave β-1,4 glycosidic bonds in the xylan backbone and release XOS, while other auxiliary enzymes remove side groups (de Freitas et al., 2019). Enzymatic treatments operate under mild temperature and pressure conditions while contributing to the monosaccharides' low release and non-formation of toxic compounds (Amorim et al., 2019). Enzymatic hydrolysis generates XOS with a lower degree of polymerization (DP) (2–6) than other strategies, such as chemical hydrolysis and autohydrolysis (DP > 6) (Surek & Buyukkileci, 2017), desirable in food and pharmaceutical industries due to their prebiotic potential. Several studies involving the enzymatic production of XOS have used renewable materials as a xylan source (Álvarez et al., 2020; Jagtap et al., 2017). As a result, enzymatic processes have been established based on XOS character as the only oligomers produced from lignocellulosic biomass in the market (Moniz et al., 2016).

In general, XOS production by enzymatic hydrolysis requires a biomass pretreatment step to extract the xylan portion followed by the enzymatic reaction step. The enzyme must either be exogenous or produced *in situ*. Within this perspective, the use of lignocellulosic biomass by microorganisms has shown a remarkable potential to produce XOS, mainly due to the process's simplicity (Amorim et al., 2019). Some studies report both XOS and xylanolytic enzymes production in the culture medium (Reque, Pinilla, Gautério, Kalil, & Brandelli, 2019), while others focus on obtaining XOS themself (Amorim, Silvério, Gonçalves, et al., 2019). Promising results on XOS production involving the use of renewable biomass as a xylan source (Reque et al., 2019), as well as genetic engineering strategies for species improvement (Amorim, Silvério, Gonçalves, et al., 2019), have been reported. Similarly to enzymatic processes, the direct fermentation leads to mixtures containing high amounts of XOS and low xylose content due to the microorganisms' preference for readily available sugars (Amorim et al., 2019).

Different hydrolytic approaches can result in oligomers with distinct structural features, which have an impact on the biological properties of XOS. The choice of the most suitable hydrolytic process depends not only on the XOS amounts produced, but also on the XOS properties and their possible industrial applications. Thus, this study explores the characterization of hydrolysates containing XOS produced by different strategies – enzymatic treatment and direct fermentation – regarding XOS composition and structure, besides antioxidant and prebiotic activities. The relationship between the XOS structure in the unpurified mixtures and their antioxidant and prebiotic potential is also discussed. The non-chemical approaches used in this work to produce XOS are well-framed within the sustainable production concept, presenting several advantages over the conventional chemical methods. To the best of our knowledge, this is the first comparative study of the biological properties of XOS produced by enzymatic and fermentative processes.

2. Material and methods

2.1. Xylan sources and extraction

Rice husk was kindly supplied by rice processing industries (Rio Grande do Sul, Brazil). Commercial beechwood xylan was purchased from Apollo Scientific Ltd. (Stockport, Cheshire, UK) and Sigma-Aldrich Chemical Ltd. (St. Louis, Missouri, US). Beechwood xylan from different companies have similar compositions, being mainly composed of xylose (79% to 89%) with low quantities of methyl glucuronic acid (7.7% to 9.5%) (Díaz-Arenas et al. (2022); Gomes & Chimphango, 2015), which did not compromise the current study, as verified by our previous studies (Gautério, da Silva, Hübner, & Ribeiro, 2021; Gautério, Hübner, Ribeiro, et al. 2021).

The xylan was extracted from rice husk according to the method described by Hauli, Sarkar, Mukherjee, Chattopadhyay, & Mukhopadhyay (2013) with some modifications. Erlenmeyer flasks (500 mL) containing rice husk (particle size smaller than 0.5 mm) and 10% (w/v) NaOH at a ratio of 1:10 (w/v) were kept at 50 °C, under orbital shaking (100 rpm) for 16 h. After this period, the flasks were steamed at 100 °C for 3 h and then cooled in an ice batch. The mixture was centrifuged (4757 × g, 4 °C, 30 min) and the supernatant acidified with 12 mol/L HCl to pH 5.0. Xylan precipitation was carried out by adding 95% (v/v) EtOH (1.5 volumes) to the acidified fraction. The precipitate was kept in the ethanolic solution for 1 h and it was further recovered by centrifugation (4757 × *g*, 30 min). Finally, the precipitate was dried in a hot air oven (55 °C, 24 h) and powdered in a mixer.

2.2. Preparation of hydrolysates containing XOS

The hydrolysates containing XOS were obtained by microbial fermentation of beechwood xylan (FermBX) or by enzymatic hydrolysis of beechwood xylan (EnzBX) and rice husk xylan (EnzRH), as illustrated in Fig. 1. FermBX was obtained by xylan fermentation using a cloned *Bacillus subtilis* 3610 containing the xylanase gene xyn2 (Amorim, Silvério, Gonçalves, et al., 2019). At the point of maximal XOS production, the medium was centrifuged (5000 × *g*, 5 min) and the cell-free supernatant was lyophilized. FermBX presents a total XOS amount and XOS yield of 1.5 ± 0.1 mg/mL and 306.0 ± 4.0 mg XOS/g xylan, respectively, according to our previous study (Amorim, Silvério, Gonçalves, et al., 2019).

EnzBX and EnzRH were obtained by enzymatic hydrolysis of xylan using a crude xylanase extract from *Aureobasidium pullulans* CCT 1261 (Gautério, da Silva, Hübner, & Ribeiro, 2020). Enzymatic treatment occurred at 40 °C under orbital shaking (180 rpm) for 24 h in Erlenmeyer flasks (125 mL) containing the reaction mixture (40 mL) composed as follows: EnzRH – 3% (w/v) rice husk xylan in pH 5.3 and an enzyme load of 200 U/g; EnzBX – 6% (w/v) beechwood xylan in pH 6.0 and an enzyme loading of 260 U/g (Gautério, Hübner, Ribeiro, Ziotti, & Kallil, 2021). At the end of hydrolysis, the mixture was placed in a boiling bath for 5 min to enzyme denaturation. The mixture was centrifuged (4757 × *g*, 30 min) and the supernatant was lyophilized.

2.3. Carbohydrate analysis

2.3.1. Total sugar and uronic acid quantification

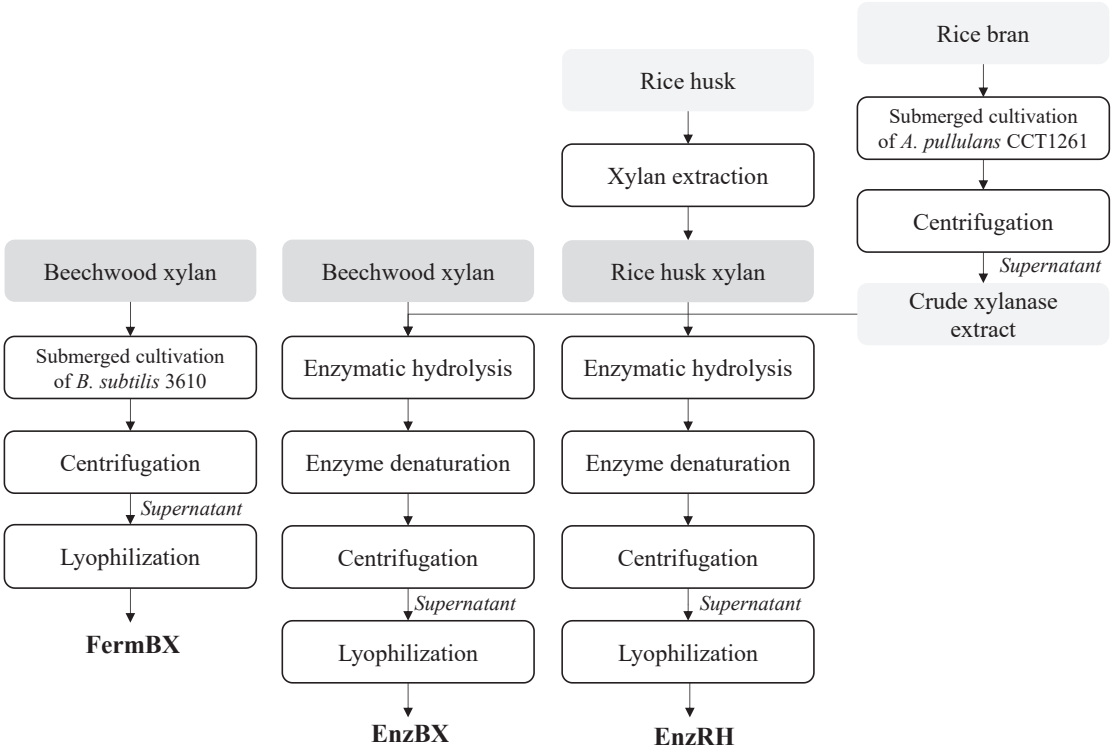
Neutral sugars were released from EnzRH and EnzBX by treatment with 72% (w/w) H_2SO_4 during 3 h at room temperature with occasional stirring followed by hydrolysis with 1 mol/L H_2SO_4 for 2.5 h at 100 °C. The sugars were then derivatized to their alditol acetates and analyzed by GC–FID Perkin Elmer-Clarus 400. Total sugar content was also determined according to a modification of the phenol–H_2SO_4 method by addition of 80 μL of sample dissolved in water, followed by 160 μL of 5 % (w/v) phenol and 1 mL of 96 % (w/w) H_2SO_4 (Coelho et al., 2016).

Uronic acids were determined colorimetrically according to a modification of the 3-phenylphenol colorimetric method (Coimbra, Delgadillo, & Waldron, 1996). Samples were prepared by pre-hydrolysis in 0.2 mL of 72% (w/w) H_2SO_4 for 3 h at room temperature followed by hydrolysis with 1 mol/L H_2SO_4 for 1 h in at 100 °C. A calibration curve was prepared with D-galacturonic acid (0.00–0.11 mg/mL).

2.3.2. XOS quantification and linkage analysis

XOS contents in the hydrolysates from enzymatic hydrolysis were quantified by HPLC-RID (Gautério et al., 2018) using a calibration curve with xylose (Sigma-Aldrich, San Luis, US) and XOS standards (Megazyme, Bray, IE).

The glycosidic linkages between the monomers that compose the



EnzBX – Hydrolysate from the enzymatic hydrolysis of beechwood xylan; EnzRH – Hydrolysate from the enzymatic hydrolysis of rice husk xylan; FermBX – Hydrolysate from the fermentation process of beechwood xylan.

Fig. 1. Hydrolysates containing XOS obtained by different strategies.

hydrolysate samples were identified by methylation analysis (Coelho et al., 2016). The permethylated carbohydrates were dissolved in $CHCl_3$: MeOH (1:1, v/v), and dialyzed with membranes with a cut-off of 1 kDa against 50% (v/v) EtOH. After evaporation to dryness, permethylated carbohydrates were hydrolyzed with 2 mol/L trifluoroacetic acid, followed by reduction ($NaBH_4$) and acetylation. The partially methylated alditol acetates were analyzed by GC–MS. The DP of XOS was obtained by the calculation of the relative amount of total xylose divided by the amount of terminally linked xylose. This estimation considers the non-occurrence of xylose (Xyl) as branching residue. The branching percentage was calculated as the ratio between the branching points in substituted Xyl residues (Xyl_{subst}) and the total amount of Xyl (Xyl_{total}), multiplied by 100. Xyl_{subst} was considered the sum of the amount of monosubstituted residues (2,4- Xyl + 3,4-Xyl) + twice the amount of disubstituted residues (2,3,4-Xyl).

2.4. Phenolic compounds and antioxidant activity

For the analysis of total phenolic compounds and antioxidant activity, 50 mg per mL solutions of lyophilized hydrolysates (EnzBX, EnzRH and FermBX) were prepared in ultrapure water, filtered through Whatman 0.22 μm nylon membranes (Milipore, Burlington, US), and then reserved for analyzing the antioxidant potential of the samples. Commercial prebiotics (lactulose, raffinose, and inulin) and synthetic antioxidants (Butylated hydroxytoluene – BHT, and Butylated hydroxyanisole – BHA) were used as standards.

The total content of phenolic compounds was determined using the Folin-Ciocalteu colorimetric method according to Ballesteros, Teixeira, & Mussatto (2014) and was expressed in mg gallic acid equivalent per g of hydrolysate (mg GAE/g). On the other hand, the total flavonoids were quantified through the aluminum chloride colorimetric assay (Ballesteros, Teixeira, & Mussatto, 2014) and were expressed in mg quercetin equivalent per g of hydrolysate (mg QE/g). The antioxidant activity was determined by using three different assays, namely the radical cation decolorization (ABTS), free radical scavenging activity (DPPH), and ferric reducing antioxidant power (FRAP), as described by Ballesteros, Cerqueira, Teixeira, & Mussatto (2015). ABTS and DPPH data were plotted as a function of antioxidant concentration to obtain the ABTS and DPPH inhibition concentration at 50% (IC_{50}). The antioxidant activities were expressed as μmol of Trolox equivalent per g of hydrolysate (μmol TE/g), while FRAP values were expressed as μmol of ferrous equivalent per g of hydrolysate (μmol Fe (II)/g).

2.5. Prebiotic activity

2.5.1. Fecal inoculum

One healthy human volunteer aged 26, non-smoker and with no associated diseases to the metabolic and gastrointestinal systems, provided the fecal sample. The volunteer did not consume any antibiotics, pre- or probiotic supplements for at least 3 months before the fecal donation. A voluntary agreement was obtained from the fecal donor prior to this study. The fecal sample was collected on site and diluted with anaerobic (100% nitrogen) phosphate-buffered saline solution (PBS, 0.1 mol/L, pH 7.0) at the proportion of 1:10 (w/w), respectively. Afterwards, the diluted sample was stored at 4 °C overnight until the inoculation.

2.5.2. In vitro fermentation of hydrolysates containing XOS

The growth medium was prepared according to Amorim et al. (2020a) with the addition of FermBX, EnzBX and EnzRH at a final concentration of 10 g/L. *In vitro* fermentations were carried out in a static batch culture model and under anaerobic conditions (37 °C, 48 h). Fermentation experiments initialized by adding the fecal inoculum at 11% (v/v). Liquid samples were taken at different time intervals (0, 12,

24, 36 and 48 h), centrifuged (4000 × *g*, 10 min), and the supernatant was collected and stored at −20 °C for further analysis. Gas samples from the headspace were collected to access gas production. The final pH of the medium was measured at 48 h, while the biomass samples were centrifuged, washed, resuspended in PBS (0.1 mol/L, pH 7.0), and stored at −20 °C for DNA extraction and sequencing analysis. All fermentations were performed in duplicate using a blank with no hydrolysate addition as a negative control.

2.5.3. Microbiota analysis

Liquid fraction from fecal inoculum and fermentation medium (48 h) were used for DNA extraction using the Fast DNA SPIN kit for soil (MP Biomedicals, Solon, US) according to the manufacturer's instructions. The extracted DNA samples were analyzed using High-throughput sequencing (16S rRNA gene) by Illumina MiSeq Technology (Illumina, San Diego, US), as described early (Salvador et al., 2019). All the sequencing analysis were performed in duplicate and at the RTL Genomics (Lubbock, US). The sequences were submitted as FASTQ files at the European Nucleotide Archive (ENA) under the BioProject accession number PRJEB46334 (https://www.ebi.ac.uk/ena/browser/view/PRJEB46334).

2.6. Statistical analysis

The software Statistica 5.0 was used to perform the statistical analysis by *t*-test or ANOVA followed by Tukey's post hoc test at a significance level of 0.05 ($p < 0.05$). The precepts of independence, normality, and homoscedasticity of the residuals were checked before all tests.

3. Results and discussion

3.1. Sugar composition and structure of hydrolysates from enzymatic treatments

EnzBX and EnzRH were characterized in terms of the content and composition of XOS, as well as the XOS yield, by HPLC-RID. Both XOS content and yield were higher in EnzBX (10.3 ± 0.2 mg/mL and 170.8 ± 4.0 mg XOS/g xylan) than in EnzRH (2.0 ± <0.1 mg/mL and 65.5 ± 2.4 mg XOS/g xylan). The hydrolysates from enzymatic treatment were mainly composed of xylobiose (5.6 ± 0.1 mg/mL for EnzBX and 0.9 ±<0.1 mg/mL for EnzRH), xylotriose (3.3 ± 0.1 mg/mL for EnzBX and 0.5±<0.01 mg/mL for EnzRH), and XOS with DP 4–5 (1.3 ± 0.2 mg/mL for EnzBX and 0.6±<0.1 mg/mL for EnzRH).

EnzBX was composed mainly of xylose residues (86.6 mol%) and uronic acids (13.4 mol%), accounting for 67% of the sample. In contrast, EnzRH presented xylose.

(37.6 mol%) and glucose (49.0 mol%) in higher quantities than arabinose (7 mol%) and uronic acids (6.3 mol%), comprising 32% of the sample. These differences in mol percentages can be attributed to the nature (e.g., grass or wood), purity, and extraction method of xylan used to obtain the hydrolysates (Pinales-Márquez et al., 2021). Another explanation may be related to the differences in the reaction conditions, particularly in the xylan concentration and enzyme loading, thus impacting XOS composition. The phenol–sulfuric acid method revealed a higher total sugar content for EnzBX (972.9 ± 16.2 mg/g) than the sulfuric acid method (671.5 ± 31.7 mg/g). Xylose is more acid-labile than glucose and can be degraded during the hydrolysis analysis, which can justify the different values of total sugar contents obtained for EnzBX. Total sugar content for EnzRH was similar in both methodologies (i.e., 322.9 ± 17.7 mg/g after the carbohydrate hydrolysis with sulfuric acid and 310.8 ± 4.8 mg/g by phenol–sulfuric acid method).

As observed by the glycosidic-linkage analysis (Table 1), EnzBX was mainly composed of xylose oligosaccharides. XOS had a backbone composed of (1 → 4)-linked-xylopyranosyl residues (86.6%) with an average DP of 16 and 6% of branching. A higher average DP was found with the GC–MS analysis which is a more sensitive technique than the HPLC-RID that only quantifies XOS with DP < 6 under the experimental conditions applied. In addition, the high DP value can be linked to the non-degraded xylan in the EnzBX. Most branches occur in the *O*-2 (5.1 %) and *O*-3 in small amounts (0.4 %), and the side chains are composed of arabinose (0.5 %), galactose (0.3 %) and uronic acids. Glucuronic acid is the uronic acid component of xylans, as reported for arabinoxylans from brewers' spent grain (Coelho et al., 2016) and xylans from lignified tissues, and should comprise the remaining percentage of substituted xylose (4.7%).

Table 1
Glycosidic linkage composition (% mol) determined for EnzBX and EnzRH.

Glycosyl linkage	EnzBX		EnzRH	
	$\bar{X}$	RSD (%)	$\bar{X}$	RSD (%)
t-Ara*f*	0.5	14	15.7	3
2-Ara*f*			3.8	14
3-Ara*f*			2.6	7
5-Ara*f*			1.0	14
Total	**0.5**		**23.1**	
t-Xyl	6.3	8	8.6	10
3-Xyl			0.8	24
4-Xyl	86.6	1	32.1	6
2,4-Xyl	5.1	6	4.9	7
3,4-Xyl	0.4	14	17.6	8
Total	**98.4**		**64.0**	
t -Glc	tr		2.5	18
4-Glc	0.4	30	5.2	12
6-Glc			1.4	17
3,6-Glc	0.2	20		
4,6-Glc			1.1	13
Total	**0.5**		**10.3**	
t -Gal	0.3	12	2.6	15
6-Gal	0.2	16		
Total	**0.5**		**2.6**	
XOS DP	16		7	
XOS % branching	6		35	

EnzBX – Hydrolysate from the enzymatic hydrolysis of beechwood xylan; EnzRH – Hydrolysate from the enzymatic hydrolysis of rice husk xylan; XOS DP – degree of polymerization (DP) calculated as $Xyl_{total}/t\text{-}Xyl$; XOS % branching - calculated as $[(2,4\text{- Xyl} + 3,4\text{-Xyl} + 2 \times (2,3,4\text{-Xyl}))/Xyl_{total}] \times 100$.

As shown in Table 1, EnzRH was also composed of XOS with a backbone of (1 → 4)-linked-xylopyranosyl residues (32.1 %). However, XOS present a lower average DP (7) and a higher branching percentage (35%) than EnzBX. EnzRH XOS's side chains were composed mainly of arabinose (15.7%) and contain galactose (2.6%) and uronic acids linked in *O-3* (17.6%) and *O*-2 (4.9%). The high amount of 3,4-Xyl residue further indicates the presence of oligosaccharides with DP higher than 4. As previously reported, XOS resulting from the enzymatic hydrolysis of corncob arabinoxylans showed a DP up to 19 (Ribeiro et al., 2018). The glucose oligosaccharides (10.3%) may result from the presence of lignocellulolytic enzymes in the crude enzymatic extract (Gautério, da Silva, Hübner, et al., 2021). The average DP of the glucooligosaccharides, obtained by the ratio between total glucose and *t*-Glc, was found to be 4.

3.2. Antioxidant phenolic compounds

From the ABTS assay, it was found that the inhibition percentages increased according to the concentration of hydrolysates, reaching 76.5%, 65.7%, and 62.6% at concentrations of 20 mg/mL, 30 mg/mL, and 8 mg/mL for the EnzRH, EnzBX, and FermBX, respectively (Fig. 1S, Supplementary Material). The same behavior was observed for EnzBX and EnzRH using the DPPH method. The inhibition percentages reached 65.5% at 50 mg/mL and 63.6% at 20 mg/mL (Fig S2, Supplementary Material), while FermBX, achieved a similar inhibition (61.8%) with only 8 mg/mL, thus showing a higher antioxidant potential.

XOS from the enzymatic hydrolysis of wheat straw (Jagtap et al., 2017) and sugarcane straw (Ávila, Martins, & Costa, 2020) showed 58%

and 78% DPPH inhibition, respectively, at 4 mg/mL. Similarly, commercial XOS (purity over 90%) from the enzymatic hydrolysis of corncob showed 65.3% and 59.4% of DPPH and ABTS inhibition, respectively, at 4 mg/mL (Yu et al., 2015). At the same concentration, FermBX showed an inhibition of 31% for both DPPH and ABTS assays and reached 62% at 8 mg/mL. In contrast, XOS from the enzymatic hydrolysis of prehydrolyzate Moso bamboo achieved an DPPH inhibition above 85% at 3 mg/mL (Huang et al., 2019). Considering that FermBX was obtained from the cultivation medium without any previous concentration or purification stages, its antioxidant activity is promising in comparison to the studies mentioned.

The IC_{50} of FermBX in DPPH and ABTS assays (6.6 mg/mL and 6.1 mg/mL, Table 1S, Supplementary Material) was lower than the ones of EnzBX (34.6 mg/mL and 20.9 mg/mL) and EnzRH (14.5 mg/mL and 10.8 mg/mL), which indicates that a low concentration of FermBX is enough to achieve 50% of radical inhibition. The IC_{50} values for EnzBX and EnzRH in both DPPH and ABTS assays were much higher than for other XOS obtained by enzymatic hydrolysis (i.e., IC_{50} between 1 and 3 mg/mL) (Zhang et al., 2019; Huang et al., 2019). However, most of the studies on the antioxidant activity of XOS analyze the purified or concentrated fractions of these oligomers (Valls et al., 2018; Veenashri & Muralikrishna, 2011), while EnzBX and EnzRH are mixtures of XOS and non-hydrolyzed xylan.

For the three hydrolysates evaluated, the ABTS method showed higher values of Trolox equivalent (TE) than those obtained with the DPPH method (Table 2), a behavior also reported by Valls et al. (2018) and Zhang et al. (2019). Results in the FRAP method showed the same trend observed using the ABTS and DPPH methods, i.e., FermBX showed greater antioxidant capacity in comparison to EnzBX and EnzRH (Table 2).

At low concentrations (1 mg/mL), the synthetic antioxidants showed strong antioxidant activity in comparison to the hydrolysates (Table 2). Nevertheless, the BHT and BHA are pure and highly reactive compounds, which makes it difficult to compare with the biological samples. No antioxidant activity was detected for commercial prebiotics (raffinose, lactulose, and inulin) even at high concentrations (40 mg/mL). Based on the results obtained for the commercial prebiotics, the hydrolysates containing XOS can be considered promising mixtures due to their biological properties, namely the proven antioxidant activity and the potential prebiotic activity.

Table 2
Total phenolic compounds and flavonoids and antioxidant activities of hydrolysates containing XOS, synthetic antioxidants or commercial prebiotics.

Hydrolysate	TFC (mg GAE/g)	TFV (mg QE/g)	DPPH (μmol TE/g)	ABTS (μmol TE/g)	FRAP (μmol Fe II/g)
EnzBX	8.0 ± 0.1^{c}	0.2 ± < 0.1^{b}	7.5 ± 0.3^{c}	13.4 ± 0.2^{c}	28.8 ± 0.9^{c}
EnzRH	9.9 ± 0.1^{b}	0.2 ± < 0.1^{b}	14.5 ± 1.1^{b}	26.5 ± 2.4^{b}	44.1 ± 1.1^{b}
FermBX	14.0 ± 0.2^{a}	0.6 ± 0.1^{a}	39.1 ± 2.2^{a}	45.7 ± 1.4^{a}	79.9 ± 2.8^{a}
BHA*			445.9 ± 1.7	473.3 ± 4.5	13080.0 ± 48.1
BHT*			447.9 ± 0.5	442.1 ± 2.7	4338.2 ± 241.5
Prebiotics*			nd	nd	nd

Mean ± standard deviation (n = 5 or 6). Equal lowercase letters in the same column and between the hydrolysate samples indicate that there is no significant difference among the values by Tukey test ($p > 0.05$). EnzBX – Hydrolysate from the enzymatic hydrolysis of beechwood xylan; EnzRH – Hydrolysate from the enzymatic hydrolysis of rice husk xylan; FermBX – Hydrolysate from the fermentation process of beechwood xylan; TFC – Total phenolic compounds; TFV – Total flavonoids; BHT – Butylated hydroxytoluene; BHA – Butylated hydroxyanisole; Prebiotics – Commercial prebiotics (lactulose, raffinose and inulin) tested individually; nd – not detected. *TFC and TFV were not quantified for BHA, BHT and commercial prebiotics.

Due to the diversity of microbial enzymes and their synergistic action to degrade the substrate in the medium, the release of XOS and other compounds with antioxidant activity can occur simultaneously. Besides, depending on the nature of xylan, the hydrolysate resulting from the enzymatic treatment may contain other compounds (Cheng et al., 2012) that contribute to its antioxidant activity, in addition to XOS. Enzymes from different sources and microorganisms can also have an impact in the products resulting from hydrolysis. Some studies have demonstrated that phenolic compounds in the xylan structure can exert antioxidant activity (Zheng et al., 2021). Thus, the contents of total phenolic compounds (TFC) and flavonoids (TFV) were determined in all the hydrolysates, as shown in Table 2. As a result, FermBX showed higher contents of TFC and TFV than the other hydrolysates, which may also contribute to its higher antioxidant capacity.

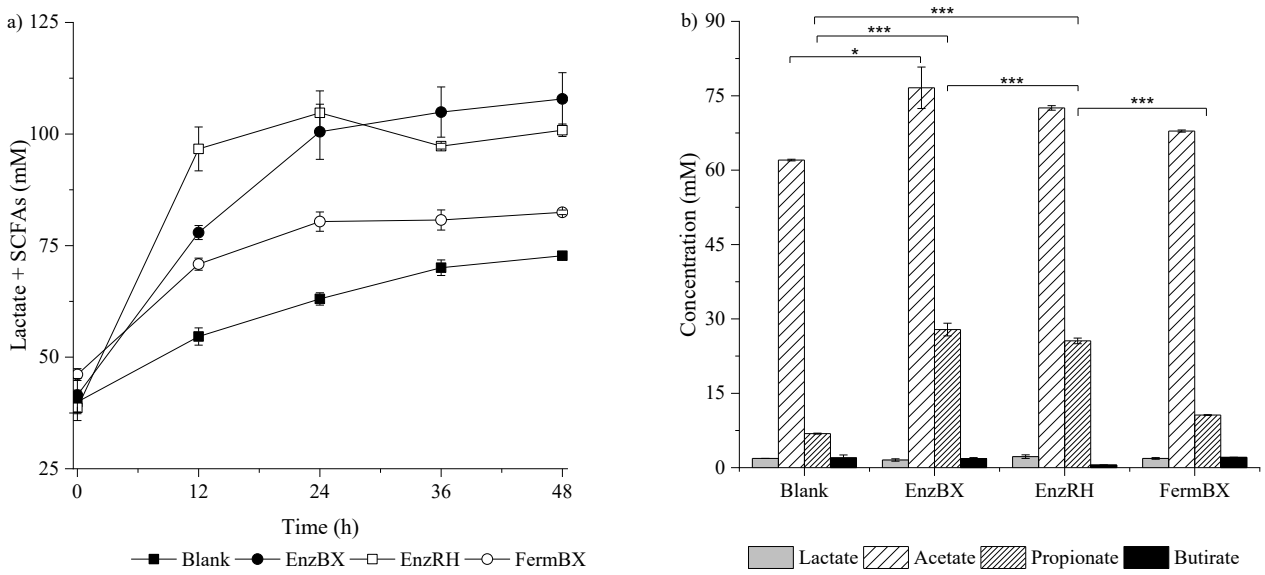
The antioxidant activity of FermBX and EnzBX can be attributed in part to the presence of methyl glucuronic acid residues from the beechwood xylan structure, as reported by Valls et al. (2018). Soluble and insoluble phenolic compounds present in rice husk, namely ferulic acid, ρ-coumaric acid, ρ-hydroxybenzoic acid, gallic acid, and vanillic acid, are related to the antioxidant activity of this biomass (Butsat, Weerapreeyakul, & Siriamornpun, 2009). Other studies have reported that the antioxidant activity of XOS from enzymatic hydrolysis of rice husk is due to the presence of ρ-coumaric acid, ferulic acid and syringic acid (Veenashri & Muralikrishna, 2011). According to Table 1, both EnzBX and EnzRH contain a 5-Ara*f*, diagnostic of a phenolic compound linked to the primary hydroxyl group of the arabinose residue, as previous reported by Amorim et al. (2019) for the FermBX structure.

Overall, the three hydrolysates containing XOS obtained by different production strategies showed antioxidant activity using the three methodologies employed, highlighting the antioxidant potential of these non-purified hydrolysates in future applications. The presented results suggest that the antioxidant activity of hydrolysates is not only due to the presence of XOS, but also to other compounds (Zheng et al., 2021) that can be linked to their structure and/or present in the obtained mixtures.

3.3. Organic acids and ammonia production, pH changes and gas generation

As shown in Fig. 2a, the addition of hydrolysates at 10 g/L in the medium increased the total amount of lactate and short-chain fatty acids (SCFAs) until 1.5-fold in comparison to the blank and after 48 h of fermentation. This result agrees with previous studies on the prebiotic effect of XOS using human fecal inocula (Amorim et al., 2020a; Buruiana et al., 2017; Sajib et al., 2018). The maximum production of lactate and SCFAs were observed at 24 h for both FermBX (80.4 ± 2.2 mM) and EnzRH (104.7 ± 4.9 mM), and at 48 h for EnzBX (107.9 ± 5.9 mM) and the blank (70.0 ± 1.7 mM). Particularly for EnzRH, the production slightly dropped after the maximum point, while for the FermBX and EnzBX the production remained constant after 24 h or gradually increased during the fermentation, respectively (Fig. 2a). These differences may be associated with the size of the XOS chain (Table 1), being the lower DP XOS probably fermented faster than those with higher DP, a behavior also observed by Buruiana et al. (2017). The amount of lactate and SCFAs produced using EnzRH or EnzBX is in the same magnitude as that obtained using lactulose (113.6 ± 0.7 mM), a commercial prebiotic (Amorim et al., 2020b), and two purified XOS fractions from barley's straw (102.7 mM and 117.20 mM) (Álvarez et al., 2020). Furthermore, the amount of organic acids produced using FermBX is in agreement with that obtained using purified XOS from corn straw (75–80 mM) (Moniz et al., 2016). All these comparisons consider the same initial substrate content in the assays (i.e., 10 g/L).

The total amount of lactate and SCFAs (Fig. 2a) is generally considered as a good indicator of the prebiotic character. Fig. 2b shows the production profiles of lactate, acetate, propionate, and butyrate, obtained at 48 h of fermentation. Acetate was the main SCFAs produced



EnzBX – Hydrolysate from the enzymatic hydrolysis of beechwood xylan; EnzRH – Hydrolysate from the enzymatic hydrolysis of rice husk xylan; FermBX – Hydrolysate from the fermentation process of beechwood xylan. Asterisks indicate a statistically significant difference among the values determined by Tukey test (*p<0.05, **p<0.01, ***p<0.001).

Fig. 2. (a) Total production of lactate and short chain fatty acids (SCFAs) and (b) production of lactate and SCFAs (acetate, propionate, and butyrate) during 48 h of fecal inocula growth in the absence of prebiotics (blank) or in a medium enriched with hydrolysates containing XOS at 10 g/L. Results are the average of duplicate analysis of each sample ± standard error (n = 2).

at 48 h, followed by propionate, for both blank and hydrolysates containing XOS (Fig. 2b). Among the hydrolysates, the EnzBX and EnzRH showed the highest production of acetate (76.6 ± 4.2 mM and 73 ± 0.45 mM) and propionate (27.8 ± 1.3 mM and 26.6 ± 0.6 mM), both differing statistically to the blank ($p < 0.05$). Previous studies also reported acetate as being the most abundant SCFA when XOS are available for *in vitro* fermentation with human fecal microbiota (Amorim et al., 2020a; Buruiana et al., 2017). The highest production of SCFAs in the culture medium containing EnzBX or EnzRH may be related to the presence of xylobiose and xylotriose in these hydrolysates, which are more easily fermentable than XOS with higher DP. Contrarily, FermBX is rich in xilotetraose, xilopentose, and xilohexose, as previously characterized by Amorim et al. (2020a).

Lactate production was induced in the first 12 h of fermentation and declined until the end of fermentation (data not shown), thus low amounts of this organic acid were detected at 48 h (Fig. 2b). Lactate is an intermediary metabolite produced by bifidogenic and lactic bacteria, which is converted into other organic acids by gut microbiota. Therefore, low amounts of lactate are expected at the end of fermentation, mainly when a high production of SFCAs occurs. This trend is in good agreement with the studies of Álvarez et al. (2020).

Due to the consumption of prebiotic oligosaccharides by gut bacteria and the consequent production of SCFAs, a decrease in pH throughout the fermentation is expected. Hence, the pH and ammonia concentration were determined after 48 h of fermentation (Table 2S, Supplementary Material). The addition of hydrolysates to the culture medium led to a pH and ammonia decrease when comparing to the blank (Table 2S, Supplementary Material), being this drop significant for EnzRH. In the culture media containing FermBX, EnzBX, and EnzRH the pH drop was 0.24, 1.71, and 1.68, respectively, considering the initial pH value. Similar behaviors regarding the pH drop were reported by other studies (Álvarez et al., 2020; Amorim et al., 2020a) of *in vitro* fermentation of XOS using human fecal inocula. In the current study, the pH decrease agrees with the amount of lactate and SCFAs produced during 48 h.

Fermentation of hydrolysates by human fecal inoculum also promoted an effect on gas production, as shown in Fig. 3. Higher amounts of CO_2 and H_2 were produced when the hydrolysates were added to the culture medium, contrarily to the blank (Fig. 3a), a trend also observed by Sajib et al. (2018) and Buruiana et al. (2017) in similar *in vitro* experiments with XOS from brewer's spent grain and corn stover, respectively. The maximum production of CO_2 obtained with EnzBX, EnzRH and FermBX were.

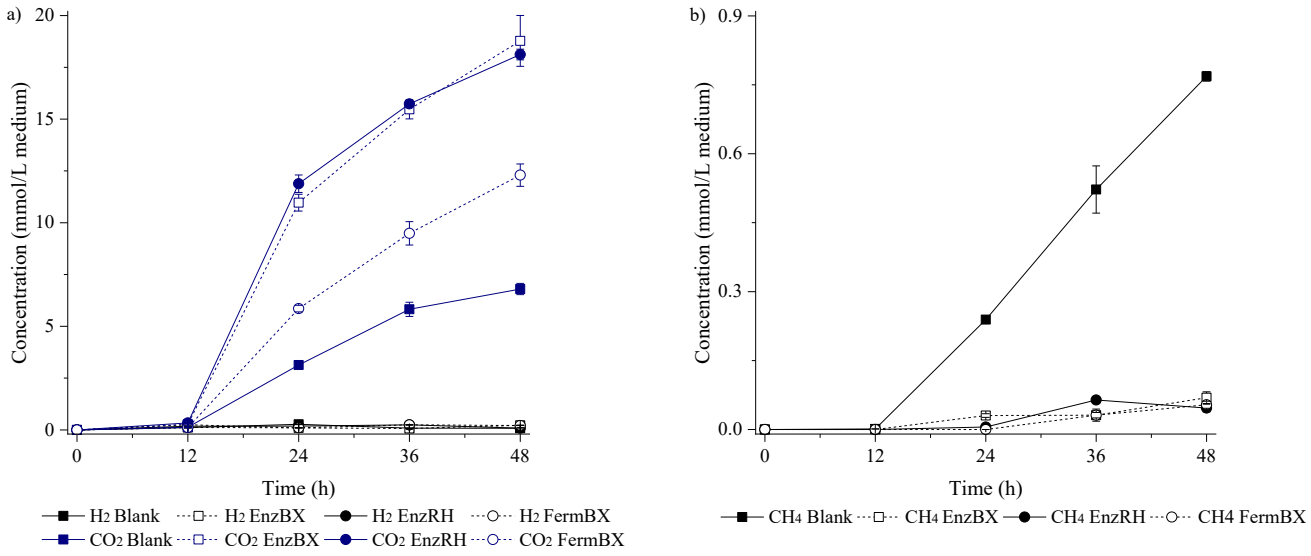
18.8 ± 1.2 mmol/L_{medium}, 18.2 ± 0.3 mmol/L_{medium} and 12.3 ± 0.5 mmol/L_{medium}, respectively. Gas production leads to the occurrence of flatulence, which can limit the inclusion of prebiotics in food formulations. Therefore, future *in vivo* models can be evaluated to establish the appropriate dose for the consumption of these specific mixtures of prebiotics.

As shown in Fig. 3b, methane production was reduced by 11 to 16-fold compared to the blank. Low levels of CH_4 may be associated with a possible reduction in the growth of methanogenic archaea, probably due to the presence of XOS in the culture medium.

3.4. The effect of hydrolysate containing XOS on intestinal microbiota

As shown in Fig. 4 and Table 3S (Supplementary Material), the hydrolysate containing XOS led to a distinct modulation of the gut microbiota after 48 h of fermentation when comparing to the blank. The supplementation of the culture medium with EnzBX and EnzRH resulted in a significant increase ($p < 0.05$) above 2-fold in *Firmicutes* considering the total bacterial community (Fig. 4b and 4c). Besides that, a significant decrease ($p < 0.05$) in *Bacteroidetes* was achieved by the fermentation of EnzBX (7.8-fold, Fig. 4b) and EnzRH (23.5-fold, Fig. 4c). The fermentation of EnzBX also significantly ($p < 0.05$) reduced the percentage of *Proteobacteria* (Fig. 4b) in 1.68-fold, but this reduction was not significant ($p > 0.05$) when using EnzRH (Fig. 4c). After adding FermBX to the culture medium, no significant ($p > 0.05$) modifications in the percentage of the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were verified (Fig. 4d) as compared to the blank sample.

Both EnzBX and EnzRH showed an outstanding ability to stimulate



EnzBX – Hydrolysate from the enzymatic hydrolysis of beechwood xylan; EnzRH – Hydrolysate from the enzymatic hydrolysis of rice husk xylan; EnzBX – Hydrolysate from the fermentation process of beechwood xylan.

Fig. 3. Production of (a) H_2 and CO_2 and (b) CH_4 by fecal inocula in the absence of prebiotics (blank) or enriched with medium enriched with hydrolysates containing XOS at 10 g/L. Results are the average of duplicate analysis of each sample ± standard error (n = 2).

Megamonas funiformis (from *Selenomonadaceae* family) (Fig. 4 b-c), which represented 70.5 ± 0.5% and 58.5 ± 0.5%, respectively, of the total bacterial population. This stimulation is in good agreement with the high production of acetate and propionate (Fig. 2b) since *Megamonas* species are recognized as producers of these SCFAs (Zhao, Dong, Zhang, & Li, 2019). The significant increase of *M. funiformis* with the EnzRH addition may also possibly explain the slight drop on the lactate and SCFAs production at 12–48 h (Fig. 2a), namely through the production of propionate and butyrate from lactate (Duysburgh et al., 2020). In fact, the production of lactate dropped from 5.0 ± 0.1 mM (12 h) to 2.2 ± 0.4 mM (48 h) for EnzRH, while the production of propionate and butyrate slightly increased from 24.7 ± 1.3 and 0 ± 0 mM (12 h) to 25.6 ± 0.6 and 0.53 ± 0.01 mM (48 h), respectively.

Moreover, the results above agree with studies reporting the ability of *M. funiformis* to utilize other prebiotics, such as lactulose and galactooligosaccharides, for its growth (Mao et al., 2014). An expressive stimulation of the *M. funiformis* growth was also observed by the supplementation of the culture medium with a extract rich in arabinoxylooligosaccharides (AXOS) with average DP of 6 (Van Den Abbeele et al., 2018). Some studies have reported the reduction of *M. funiformis* in the gut microbial community of patients with multiple system atrophy (Wan et al., 2019) and Crohn's disease (Chen et al., 2014), being this species commonly present in healthy individuals and associated with a benefic microbiota.

Although the fermentation of EnzBX and EnzRH significantly decrease ($p < 0.05$) the relative abundance of organisms from *Bacteroides* genus (Fig. 4b-c), the FermBX slightly stimulated the growth of this group of bacteria (1.6-fold, Fig. 4d). This stimulation can be associated with the production of butyrate (Fig. 2b) since bacteria belonging to the *Bacteroides* genus produce this metabolite as an energy source to gut epithelial cells (Hwang et al., 2017).

The low abundance of lactic acid bacteria, either in the presence or absence of XOS, may be explained by the drop in lactate production (Fig. 2b), probably used for the growth of other bacteria and production of acetate and propionate. Nevertheless, the growth of Limosilactobacillus reuteri was observed after the addition of EnzRH in the medium, representing 89 ± 2% of the total Lactobacillaceae family. The *L. reuteri* is a probiotic bacterium with a strong modulatory effect on the host microbiota and immune response, promoting several benefits for gut health (Mu, Tavella, & Luo, 2018). Moreover, members of *Lactobacillaceae family* (e.g., *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii* or *Lacticaseibacillus rhamnosus*) have been investigated to improve the microbial pattern in vaginal dysbiosis (de Vrese, Laue, Papazova, Petricevic, & Schrezenmeir, 2019) and to treat or prevent gingivitis and periodontitis (Gruner, Paris, & Schwendicke, 2016).

All the hydrolysates reduced the relative abundance *of Proteobacteria* species, which are commonly associated with intestinal diseases (Rizzatti, Lopetuso, Gibiino, Binda, & Gasbarrini, 2017). In detail, the hydrolysates studied in this work reduced the relative amount of bacteria belonging to *Desulfovibrionales* order (25.4-fold for EnzRH, 18.3-fold for EnzBX, and 5.5-fold for FermBX), which were also found in submucosal tissue of patients with Crohn's disease (Chiodini et al., 2015). In addition, the fermentation of hydrolysates reduced the relative abundance of methanogenic archaea belonging to *Methanobacteriacea* family, being this reduction more expressive for EnzRH (35.0-fold) and EnzBX (23.3-fold) than FermBX (4.1-fold). This reduction is expected since the production of CH_4 decreased when the hydrolysates were included in the fermentation medium (Fig. 3b). The CH_4 production by methanogenic archaea can be associated with a delay in the gut transit (Ghoshal, Shukla, Srivastava, & Ghoshal, 2016).

The EnzBX and EnzRH hydrolysates reduced the relative abundance of ammonia-producing bacteria, such as *Clostridium* species, in 2500-fold and 1000-fold, respectively, reaching percentages values near to zero. The FermBX also collaborate to reduce the percentage of organisms from *Clostridium* genus in 3-fold. These results agree with the ammonia production during the fermentation (Table 2S, Supplementary Material), especially with the lowest NH_3 quantities obtained after the medium supplementation with EnzRH.

As showed above, supplementation of the culture medium with hydrolysates containing XOS resulted in differences in the stimulation or suppression of certain microorganisms. These differences may be related not only to the XOS structure (size and presence of branches) but also to the cross-feeding mechanism that occurs during substrate metabolization by the gut microbiota (Nordberg Karlsson, Schmitz, Linares-Pastén, & Adlercreutz, 2018). For instance, bacteria that do not have the specific enzymes for removing xylan substituents (e.g., α-arabinose and

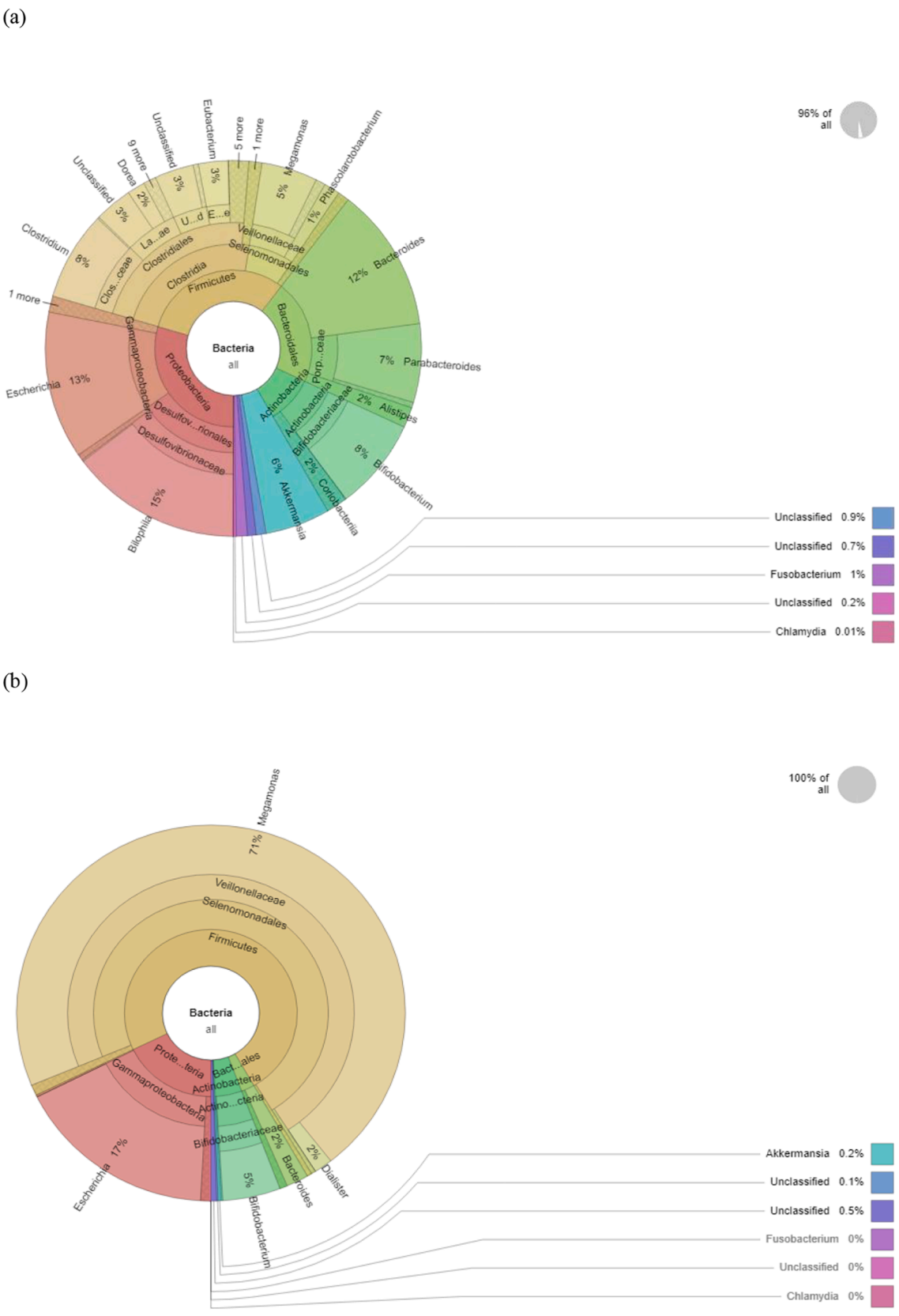


Fig. 4. Relative abundance of different bacteria after 48 h of *in vitro* fermentation by fecal inocula in the absence of prebiotics (blank) (a) or enriched with (b) EnzBX, (c) EnzRH or (d) FermBX at 10 g/L.

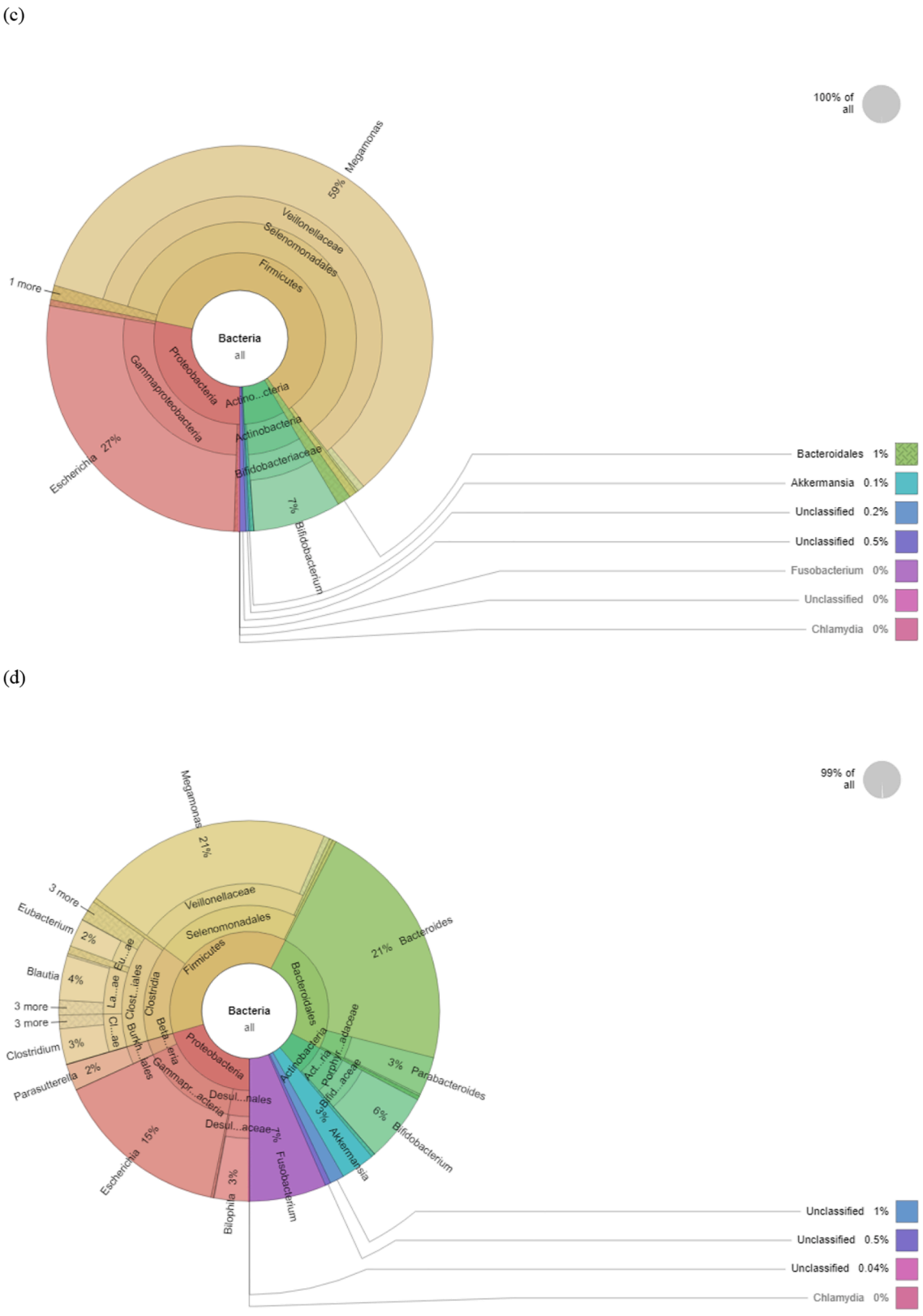


Fig. 4. (*continued*).

methyl glucuronic acid), such as *M. uniforms* (De Paepe, Kerckhof, Verspreet, Courtin, & Van de Wiele, 2017), may have been strongly stimulated due to the presence of other bacteria that produce these enzymes, thus making it possible to consume both more linear and branched XOS.

Based on the results herein presented, it is possible to assume that the biomass modulation was more process-dependent than substrate-dependent. Both EnzBX and FermBX used the same xylan source to produce XOS, which were obtained by different hydrolysis processes. In turn, different production strategies resulted in hydrolysates with

distinct XOS amounts, non-hydrolyzed xylan, DP, glycosidic linkages, and presence of phenolic compounds. Although the effect of these differences is still not well understood, their contribution to the biomass modulation needs to be considered, as the relative abundance of certain organism differed between the hydrolysates. Furthermore, the hydrolysates demonstrated potential to be used as prebiotics by reducing the undesirable bacteria and stimulating the benefic microbiota.

4. Conclusion

Hydrolysates produced by different strategies and sources resulted in XOS with different concentrations, sizes, linkages, and branches. The hydrolysates containing XOS showed distinct antioxidant potential, a fact also attributed to the presence of phenolic compounds. Regarding microbiota modulation, the production lactate and SCFAs, as well as ammonia and gas generation, suggest the prebiotic potential of the hydrolysates. Moreover, all the hydrolysates could reduce the undesirable bacteria and stimulate the beneficial microbiota, thus demonstrating their potential for gut health applications. Finally, hydrolysates without any previous purification steps showed promising antioxidant and prebiotic activities for several food applications.

CRediT authorship contribution statement

Gabrielle Victoria Gautério: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Cláudia Amorim:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing, Funding acquisition. **Sara C. Silvério:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing, Funding acquisition. **Beatriz B. Cardoso:** Investigation, Formal analysis, Data curation. **Lina F. Ballesteros:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Joana I. Alves:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Maria Alcina Pereira:** Methodology, Investigation, Writing – review & editing. **Soraia P. Silva:** Investigation, Formal analysis, Data curation. **Elisabete Coelho:** Conceptualization, Resources, Methodology, Investigation, Writing – review & editing. **Manuel A. Coimbra:** Conceptualization, Resources, Methodology, Investigation, Writing – review & editing. **Susana Juliano Kalil:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Lígia R. Rodrigues:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133231.

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