Prebiotic effect of xylooligosaccharides produced from birchwood xylan by a novel fungal GH11 xylanase

Running title: Prebiotic xylooligosaccharides produced by a *T. amestolkiae* xylanase

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Abstract

A fungal endoxylanase belonging to the glycoside hydrolase gene family 11 (GH11) was obtained from the ascomycete *Talaromyces amestolkiae*. The enzyme was purified, characterized and used to produce a mixture of xylooligosaccharides (XOS) from birchwood xylan. A notable yield of neutral XOS was obtained (28.8%) upon enzyme treatment and the mixture contained a negligible amount of xylose, having xylobiose, xylotriose and xylotetraose as its main components. The prebiotic potential of this mixture was demonstrated upon analyzing the variations in microorganisms' composition and organic acids profile in breast-fed child feces fermentations**.** The strong production of acetic and lactic acid, the decrease of potentially pathogenic bacteria and the increase of bifidobacteria and possible beneficial commensals confirmed the prebiotic value of these xylooligosaccharides.

Keywords

Xylosidase, XOS, microbiome, breast-fed, SCFAs, *Staphylococcus hominis*.

1. Introduction

Hemicelluloses are a fundamental part of the renewable resources in the biosphere, since they constitute the second major carbon source in crop wastes, only preceded by cellulose. Unlike this one, hemicelluloses group a wide variety of polysaccharides, among which xylan is the most abundant and has attracted great interest as raw material both for bioethanol production and to obtain value-added compounds. However, the industrial exploitation of xylan still needs to overcome the challenges derived from its structural heterogeneity and complexity.

Xylans are heteropolysaccharides formed by a backbone of β-1,4 linked xylopyranoses, branched by other monosaccharides, especially arabinofuranose, and highly substituted with glucuronic or methyl-glucuronic acid and acetyl side-groups. Their branching degree and composition strongly depend on the xylan source. As a result of these properties, a coordinated action of several hydrolases is required for accomplishing the complete breakdown of the polymer, with endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) playing the main roles. The first type of hemicellulases cut the xylan backbone into soluble oligosaccharides (XOS), which can be depolymerized to xylose by the action of β-xylosidases (Polizeli et al., 2005). Interestingly, although the complete conversion of xylan into xylose is a main target for bioethanol production, there is a huge and growing interest in the XOS themselves. These oligosaccharides are composed of xylopyranose residues linked through β-1,4 bonds, with a degree of polymerization (DP) from 2 to 10 units. An expanding number of studies on their production and purification are being carried out, as their potential as emerging prebiotics is becoming evident (Aachary & Prapulla, 2009; Akpinar, Erdogan, Bakir, & Yilmaz, 2010; Chapla, Pandit, & Shah, 2012).

Gibson et al. (2004) defined prebiotics as "non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract." In this context, XOS have demonstrated their capacity to selectively stimulate the growth of probiotic microorganisms present in the lower gastrointestinal tract such as *Lactobacillus* and *Bifidobacterium* species (Chapla et al., 2012; Aachary & Prapulla, 2011). Besides this role as a classical prebiotic, XOS include other beneficial effects, among them possible antitumorigenic, anti-inflammatory, and antiallergic activities (Aachary & Prapulla, 2011). Together with these benefits, XOS have no negative effects on human health or objectionable organoleptic properties (Aachary &

Prapulla, 2009), which has made these oligosaccharides highly demanded as functional food ingredients.

In order to satisfy this demand, XOS are obtained from the xylan fraction of lignocellulosic materials by physicochemical or enzymatic methods. Physicochemical approaches mainly include autohydrolysis and acid hydrolysis. These processes are quite fast, but the substrate is partially converted into monosaccharides, reducing the production yield and releasing toxic by-products, making harder the subsequent purification phase. In contrast, the enzymatic approach is environmentally friendly, requires mild conditions, produces low monosaccharides' yields and non-toxic by-products (Linares-Pastén, Karlsson, & Aronsson, 2016; Chapla et al., 2012). The enzymatic hydrolysis of xylan is carried out by endoxylanases, a broad group of glycosidases that, according to the Carbohydrate Active Enzymes database (CAZy, http://www.cazy.org/), are distributed among the families GH8, GH10, GH11, GH30, GH43 and GH51. However, most of them are grouped in families 10 and 11, which include a rich representation of endoxylanases produced by filamentous fungi. These enzymes are extracellular and display high activity as compared to those from bacteria and yeasts (Polizeli et al., 2005). In this sense, the genera *Trichoderma* and *Aspergillus* have been extensively investigated in the search of cellulolytic and hemicellulolytic species, although *Penicillium* strains also seem to be good candidates as producers of these enzymes (Chavez, Bull, & Eyzaguirre, 2006).

In a previous study, a screening for lignocellulolytic fungi was carried out and the perfect state of a *Penicillium* species, identified as *Talaromyces amestolkiae*, was selected for encoding a large number of cellulases and hemicellulases (Nieto-Dominguez et al., 2015). The work presented here reports the production, isolation and biochemical characterization of a novel endoxylanase from this fungus. The capacity of

the enzyme for converting xylan into xylooligosaccharides was evaluated and the prebiotic potential of the resultant XOS mixtures demonstrated.

2. Material and methods

2.1. Materials

5 mL-HiTrap QFF cartridge, Superdex™ 75 10/300 GL column and Low Molecular Weight Gel Filtration Kit 17-0442-01 were purchased from GE Healthcare (Chicago, IL, USA).

Beechwood and birchwood xylan, Avicel, glucose, cellobiose, carboxymethylcellulose, nitrophenyl (NP) substrates and Coomassie Brilliant Blue R-250 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Xylooligosaccharides from DP1 to DP6 were purchased from Megazyme (Wicklow, Ireland).

2.2. Fungal strain and culture media. The *T. amestolkiae* CIB strain is deposited at the IJFM (Instituto "Jaime Ferrán" de Microbiología) culture collection with the reference A795.

Fungal cultures were carried out according to our previous work (Nieto-Dominguez et al., 2015). Mandels medium was supplemented with 2% (w/v) beechwood xylan as carbon source and inducer of xylanolytic enzymes. In order to compare the secretion of xylanase activity, 1% and 3% xylan, 1% D-glucose and 1% Avicel were used as alternative inducers. Samples were periodically withdrawn from three replicate flasks and centrifuged at 20,000 × *g* for 5 min to separate the culture liquids from the mycelium.

2.3. Enzyme and protein assays. Endo-β-1,4-xylanase activity was measured by the release of reducing sugars according to the Somogyi-Nelson method (Nelson, 1944). One unit of endoxylanase activity was defined as the corresponding to the release of 1 μmol of reducing sugar per minute. The standard reaction mixture consisted of 2.5% xylan, 50 mM sodium acetate (AcONa) buffer (pH 5) and the appropriate dilution of the purified enzyme or culture crude extract. Standard assays were incubated at 50 ºC and 1,200 rpm for 5 and 10 min, in order to check the linearity of the activity.

Enzymatic activity against glucose-containing substrates was measured following the release of glucose using the Glucose-TR kit (Spinreact, Vall d'en Bas, Spain) according to the manufacturer's instructions.

The BCA method was used to quantify proteins, using bovine serum albumin (BSA) as standard and Pierce reagents (Thermo scientific, Waltham, MA, USA), following the manufacturer's instructions.

2.4. Endoxylanase purification. Cultures were grown in 250 mL flasks with 50 mL of Mandels medium and 2% beechwood xylan and harvested after 3 days. Culture liquids were separated from mycelium by filtering through filter paper. The filtrate was collected and centrifuged at 10,000 × *g* and 4 ºC for 30 min. The supernatant was subsequently filtered through discs of 0.8, 0.45 and 0.22 μm pore size (Merck-Millipore, Darmstadt, Germany). The flow-through was concentrated and dialyzed against 10 mM phosphate sodium buffer (pH 6) by ultrafiltration using a 5-kDa cutoff membrane. An endoxylanase was isolated through two chromatographic steps using an ÄKTA Purifier chromatography system (GE Healthcare, Chicago, IL, USA). First, the dialyzed crude extract was loaded onto a 5 mL-HiTrap QFF cartridge equilibrated in 10 mM phosphate buffer (pH 6) and a flow rate of 1 mL/min. Proteins were eluted by

applying a linear gradient of 1 M NaCl from 0 to 50% for 25 min. Then, the mobile phase mix changed to 100% 1 M NaCl for 10 min and finally to 0% for 10 min in order to wash and re-equilibrate the column, respectively. Fractions displaying endoxylanase activity were collected, dialyzed in 10 mM phosphate buffer (pH 6) and concentrated using 5 kDa Amicon® Ultra-4 Centrifugal Filter Units (Merck-Millipore, Darmstadt, Germany). Next, the samples were analyzed in a Superdex™ 75 10/300 GL column equilibrated in 100 mM NaCl phosphate 10 mM (pH6) buffer, eluting at a flow rate of 0.5 mL/min for 60 min. The purified endoxylanase (XynM) was dialyzed and concentrated by ultrafiltration and stored at 4 ºC.

2.5. Physicochemical properties of XynM. The estimated molecular mass of the protein and its homogeneity were determined by SDS-PAGE in 12% acrylamide gels (Laemmli, 1970), staining with Coomassie Brilliant Blue R-250 and using Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA, USA). The accurate molecular mass of XynM was measured by MALDI-TOF in an Autoflex III (Bruker Daltonics). In order to determine its quaternary structure, XynM was also subjected to size exclusion chromatography in a Superdex™ 75 10/300 GL column as described above. Prior to analysis, the column was calibrated with molecular-weight standards (Low Molecular Weight Gel Filtration Kit 17-0442-01) analyzed in the same conditions as the samples.

The isoelectric point, thermal and pH stabilities and optimal pH and temperature of the purified enzyme, together with the effect of some common chemical compounds on the activity of XynM, were determined as previously described (Nieto-Dominguez et al., 2015). The compounds selected for the assay were KCl, $MgSO_4$, $CaCl_2$, $BaCl_2$,

$MnCl_2$, $FeSO_4$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $AgNO_3$, $ZnSO_4$, $HgCl_2$, $Pb(NO_3)_2$, ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol (2-ME).

2.6. Substrate specificity. XynM activity was tested against other substrates apart from xylan in order to study its specificity. *p*-NP-β-D-xylopyranoside and *p*-NP-β-D-glucopyranoside were assayed at a final concentration of 3.5 mM in 50 mM AcONa buffer (pH 5). The enzyme activity was also measured against 20 mM of the disaccharide cellobiose and the polysaccharides Avicel and carboxymethylcellulose at final concentrations of 10 and 20 g/L, respectively.

The kinetic parameters for hydrolysis of beechwood xylan by XynM were determined. K_m and V_{max} were obtained by measuring the enzymatic activity against a range of substrate concentrations from 1.5 to 70 g/L. Values were calculated by fitting the experimental data by least squares to the Lineweaver-Burk linear equation of the Michaelis-Menten model.

2.7. Peptide mass fingerprinting and N-terminal sequencing of XynM. Peptide mass fingerprinting of the purified endoxylanase was carried out using MALDI-TOF mass spectrometry and N-terminal amino acid sequence was analyzed by sequential Edman degradation. Both approaches were carried out as described elsewhere (Nieto-Dominguez et al., 2015).

2.8. Sequencing and classification of XynM. A BLASTP search against the NCBInr database was carried out using the sequences of the N-terminal region and internal peptides. The gene sequences of the best hits were used as queries to run a local BLASTN against the assembled genome of *T. amestolkiae* CIB (GenBank accession

number MIKG00000000). A predicted gene was identified and its sequence submitted to the SignalP 4.1 server for locating the signal peptide. An alignment between the gene and the best hits of the former BLAST search was performed for introns' identification. The putative coding reading region of XynM, without introns and signal peptide, was translated to protein and submitted to dbCAN server (Yin et al., 2012) to assign XynM to a glycosyl hydrolase family. Local BLAST and alignments were performed on BioEdit sequence alignment editor (version 7.2.5).

2.9. Production of XOS by enzymatic hydrolysis of birchwood xylan. The hydrolysis reactions contained 20 g/L birchwood xylan in 10 mM AcONa buffer pH 4.6 with 1 U/mL XynM, and the control sample had the same composition without enzyme. All samples (5 mL) were incubated at 50 ºC and 600 rpm, for 96 h, and 100 µL-aliquots were mixed, at different time points, with pure ethanol (final ethanol concentration 70% v/v) to inactivate the enzyme and precipitate the remaining xylan. The inactivated samples were centrifuged at 3,000 rpm in order to analyze the supernatant by High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD).

The analysis was carried out in an ICS3000 Dionex (Sunnyvale, CA, USA) system consisting on a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. An anion-exchange 3 × 250 mm Carbo-Pack PA-200 column (Dionex, Sunnyvale, CA, USA) was used at 30 °C. Eluent preparation was performed with Milli-Q H_2O. The initial mobile phase was 15 mM NaOH at 0.5 mL/min for 12 min. An 8 min-gradient from 15 mM to 75 mM NaOH and from 0 mM to 80 mM AcONA was applied. Then, the mobile phase composition varied from 75 mM to 100 mM NaOH and

220 from 80 mM to 320 mM AcONa for 10 min. A final 15 min-gradient was programmed
221 to return to the initial conditions (15 mM NaOH and 0 mM AcONa). The peaks were
222 analyzed using the Chromeleon software. The flow rate was constant at 0.5 mL/min and
223 25 µL of each sample were injected. The identification and quantification of xylobiose,
224 xylotriose, xylotetraose, xylopentaose and xylohexaose was done using commercial
225 standards. All samples were previously diluted 1:10 with H_2O and filtered through 0.45
226 µm nylon filters.
227
228 **2.10. Characterization of products.** The molecular weight of the hydrolysis products
229 from birchwood xylan was assessed using a mass spectrometer with hybrid QTOF
230 analyzer (model QSTAR, Pulsar I) (AB Sciex, Framingham, MA, USA). Samples were
231 analyzed by direct infusion and ionized by electrospray with methanol as ionizing phase
232 both in positive and negative reflector modes.
233
234 **2.11. Prebiotic effect of XOS from birchwood xylan**.
235 **2.11.1. Fecal fermentations.** Assays were carried out in 10 mL microfermentors (24
236 multi-well plates; µ-24 Bioreactor) (Pall Corporation, Port Washington, NY, USA). The
237 fresh fecal sample came from a female breast-fed baby who had not received antibiotic
238 treatment for at least 3 months prior to experimentation and had no history of bowel
239 disorders. Stools were vacuum stored and refrigerated until fermentation took place.
240 Fresh feces (800 mg) were weighted and dissolved in the culture medium described by
241 Macfarlane and coworkers (1998) in a proportion 1:5 (feces:medium) to hydrate them.
242 Each microfermentor was inoculated with 500 µL of the homogenized mixture (fecal
243 slurry). Before starting fermentation, the headspace was displaced with nitrogen, and the
244 whole assay was performed in anaerobiosis, maintaining temperature (37 °C) and pH

(pH 5.5). A sample was taken from the initial fecal slurry as the time 0 control, and fermentations were harvested after 24 h and immediately frozen and stored at -20 °C.

2.11.2. Production of short-chain fatty acids (SCFAs) and other organic acids. The content of acetic, propionic, butyric, lactic and succinic acid in the slurries from fermentations was evaluated by high-performance liquid chromatography (HPLC). For organic acids quantification, 0.2 mL of the fermented samples were centrifuged at 14,000 rpm for 60 min, filtered by 0.45 µm pore size filters and diluted by 1:2 in MilliQ quality water. An aliquot of 20 µL of processed samples was analyzed using a HPLC Acquity equipped with a 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and isocratically analyzed with 5 mM H_2SO_4 as eluent (0.6 mL/min flow rate). Peaks were detected with a refractive index detector.

2.11.3. Microbiome analysis. DNA from fecal fermentation samples was isolated according to Yuan and coworkers (2012) with minor modifications, with the aid of the MagnaPure Compact System (Roche Life Science, Indianapolis, IN, USA), to avoid bias in DNA purification toward misrepresentation of Gram positive bacteria. For massive sequencing, the hypervariable region V3-V4 of bacterial 16S rRNA gene was amplified using key-tagged eubacterial primers (Klindworth et al., 2013) and sequenced with a MiSeq Illumina Platform, following the Illumina recommendations for Library preparation and sequencing for metagenomics studies.

The resulting sequences were split taken into account the barcode introduced during the PCR reaction. Quality control of the sequences was performed in different steps: i) quality filtering (minimum threshold of Q20) was performed using FASTX-Toolkit version 0.013, ii) primer (16S rRNA primers) trimming and length selection

(reads over 300 nt) was done with cutadapt version 1.2. These FASTQ files were converted to FASTA files and UCHIME program version 7.0.1001 was used in order to remove chimeras that could arise during the amplification and sequencing step. Those clean FASTA files were subjected to analysis with QIIME version 1.8 with the parameters by default and SILVA 16s rRNA database version 123 in order to annotate each sequence at different phylogenetic levels (phylum, family and genus). Putative species level was afterward annotated comparing the taxonomical association found in QIIME database with NCBI database species annotation. Alpha diversity and beta diversity was conducted with QIIME.

Microbiomes were grouped by treatment (control 0 h, control 24 h, 200 g/L XOS and 400 g/L XOS) and means were compared in order to determine if XOS displayed a demonstrable prebiotic effect in the tested conditions.

3. RESULTS AND DISCUSSION

3.1. XynM production. At laboratory scale it is usual to select a commercial xylan of high purity and homogeneity in order to induce xylanase activity, maximizing reproducibility and making results comparable with others from different sources (Chavez et al., 2006). For this reason, endoxylanase activity and total secreted proteins were measured in *T. amestolkiae* cultures with different commercial carbon sources. The endoxylanase-inducer effect of 1% glucose, 1% Avicel or 1%, 2% or 3% beechwood xylan in 6 day-old cultures was assayed (Fig S1, Supplementary). As expected, no endoxylanase activity was detected when glucose was the carbon source, since this monosaccharide has been extensively reported as a strong repressor of the hemicellulolytic metabolism in the genus *Penicillium* (Chavez et al., 2006). However, cultures with Avicel, a cellulosic substrate, produced a small endoxylanase induction

(15% of the maximal activity). Induction of xylanolytic enzymes in fungi by this kind of substrates has already been reported (Polizeli et al., 2005) suggesting an overlap between the expression pathways of cellulases and xylanases (Chavez et al., 2006). Cultures induced with 2% and 3% xylan showed the highest extracellular endoxylanase activity, although maximal activity was achieved two days before with 2% xylan, which implies a more cost-effective enzyme production. Based on these results, 2% beechwood xylan was chosen as the best inducer for endoxylanase, which was produced, purified and characterized from these crude extracts.

3.2. Purification of XynM. Cultures were harvested at day 3 post-inoculation, (maximal activity), using crudes for enzyme isolation. Purification resulted in a final yield of 5.3% recovered activity. The pure enzyme (Fig. 1A) was stored at 4 ºC, maintaining its activity for at least six months. After the process, the specific activity increased from 61.4 to 118.3 U/mg, which represents a degree of purification of 1.9. These apparently low values may be understood taking into account that the initial endoxylanase activity was measured using the concentrated crudes against xylan, a complex polymer which needs the coordinate action of different enzymes to be completely hydrolyzed. Along the purification process, the progress in XynM isolation proceeded in parallel with a decrease in the auxiliary enzymes that participate in xylan hydrolysis, causing an unavoidable loss of activity that may explain the low final yield and degree of purification.

3.3. Characterization of XynM. The enzyme was determined to have an accurate molar mass of 19,861 Da (Fig. 1B), an isoelectric point around 5.5, and a monomeric active form. XynM displayed maximal activity at pH 3-4 and 50 °C and was stable

320 across the wide range of pH assayed, although its thermostability was low at 50 °C and
321 higher temperatures. These are typical properties previously reported for endoxylanases
322 from *Penicillium* species (Chavez et al., 2006).

323 XynM fitted into Michaelis-Menten kinetics for beechwood xylan, while it was
324 inactive against the other substrates tested. This high specificity has been reported as a
325 characteristic of GH11 xylanases (Polizeli et al., 2005). Its kinetic characterization
326 revealed K_m and V_{max} values of 5.5±1.4 mg/mL and 129.0±12.6 U/mg, respectively,
327 which are similar to those reported for several endoxylanases from *Penicillium* and
328 *Talaromyces* species. It was the addition of some common chemical compounds to the
329 reaction mix that revealed the most distinguishing characteristic of XynM: its high
330 tolerance to Cu^{2+} and Hg^{+}. The case of Cu^{2+} is especially remarkable because it is
331 commonly detected upon ash analysis of lignocellulosic biomass (Bin & Hongzhang,
332 2010). Both cations have been reported as strong inhibitors of endoxylanases (Ryan et
333 al., 2003; Belancic et al., 1995).

334 Additional data on enzyme characterization are presented in the Supplementary
335 information (Fig. S2, and Table S1).

336

337 **3.4. Sequencing and classification of XynM.** The preliminary identification of XynM
338 relied on its peptide mass fingerprint and N-terminal sequence. MALDI–TOF and
339 TOF/TOF data from the fingerprint and N-terminal sequences were used to interrogate
340 the NCBI non-redundant protein database. The search returned as the best matches a
341 GH11 protein from *Thielavia terrestris* NRRL 8126 (gi:367042760) and Xylanase B
342 from *Talaromyces purpurogenum* (gi:1004289) (Belancic et al., 1995), respectively.

343 The sequences from the mRNA encoding for *T. purpurogenum* (GenBank
344 accession number Z50050.1) and *T. terrestris* (NCBI Reference Sequence

XM_003651712.1) xylanases were used as queries to run local BLASTN against the assembled genome of *T. amestolkiae*. Both queries matched the same genome region with E values of $2 \cdot 10^{-18}$ and $4 \cdot 10^{-23}$ respectively. The located region was validated by identifying the reported internal peptide and N-terminal sequence. Then, alignment of this region with the gene sequences of XynB and the GH11 xylanase from *T. terrestris* allowed predicting the start and stop codons and the presence of a single intron.

The putative sequence of XynM, analyzed using the SignalP 4.1 server, indicated a signal peptide that fitted perfectly with the beginning of the N-terminal sequence determined experimentally. The mature gene sequence without the signal peptide and the intron was analyzed by the ExPASY Bioinformatics Resource Portal and resulted in a theoretical molecular mass of 19,785 Da, very close to the value obtained by MALDI-TOF for the purified protein. This good agreement and the matches of the N-terminal sequence and the internal peptide suggest that it was correctly identified. The nucleotide sequence was then submitted to the GenBank database under the accession number KX641268.

The predicted gene was analyzed by the dbCAN server in order to annotate the enzyme into a glycosyl hydrolase family, indicating that XynM belongs to the GH11 family (E-value $4.7 \cdot 10^{-58}$). This was not unexpected attending to its low molecular mass and high specificity reported above (Polizeli et al., 2005).

3.5. Production of XOS by hydrolysis of birchwood xylan catalyzed by XynM. XOS production reached the maximum yield after 10 h reaction, and the profile of DPs barely changed for the following 14 h (Fig. 2A, Table 1). The thermal and end-product inhibition of XynM as well as the decrease of accessible hydrolytic sites in the polysaccharide may explain the stagnation of the global yield (Akpinar et al., 2010).

The maximum output of neutral XOS (28.8%) is expressed as percentage of the initial amount of birchwood xylan, and was calculated from the sum of the concentrations of uncharged XOS from Xyl_2 to Xyl_6 measured from HPAEC-PAD (Table 1). The high content of Xyl_2 and Xyl_3 as products of the reaction catalyzed by XynM agrees with the fact that GH11 endoxylanases do not display significant activity towards these small products (Pollet, Delcour, & Courtin, 2010). It is noteworthy the negligible formation of free xylose. Other reaction products could not be identified, and probably comprise substituted xylooligosaccharides. Indeed, the high retention times observed for the unidentified XOS suggested that these substituents may be negatively charged, which would be in good agreement with the presence of glucuronic or methyl-glucuronic acids, some of the most common side-chains in xylans.

Mass spectrometry analysis confirmed the presence of 4-*O*-methyl-D-glucuronic acid derivatives in the reaction. The peaks were found as $[M + Na]^+$ and $[M + K]^+$ adducts in the positive mode (Fig. 2B) and as $[M - H]^-$ and $[M + Cl]^-$ adducts in the negative mode (Fig. 2C). The presence of xylose (173.04 m/z), glucose (203.05 m/z), xylobiose (305.08 m/z; 321.05 m/z), xylotriose (437.20 m/z; 453.09 m/z) and xylotetraose (569.16 m/z) was confirmed, in agreement with the previous results obtained by HPAEC-PAD (Fig. 2A). In the negative mode spectra, 4-*O*-methyl-α-D-glucuronopyranosyl derivatives of xylooligosaccharides Xyl_3-Xyl_5 (603.18 m/z; 735.22 m/z; 867.26 m/z) were also found. Larger neutral XOS may not be detected by this technique because of their low proportion in the reaction mixture and the reduced ionizability of this type of oligosaccharides (Ahn & Yoo, 2001).

The use of endoxylanases for obtaining XOS from xylan as an alternative to the physicochemical approaches has attracted great interest and several reports have come out during the last decade (Table S2, Supplementary). It should be emphasized that

comparison of the published information on different xylanases is a difficult task, since parameters as enzyme dosage and purity (especially regarding the presence or absence of xylanolytic auxiliary activities), xylan source, and type of pretreatment vary depending on the report. However, the analysis of data from the literature revealed that XynM produced XOS in a comparable yield to those from most of the reported xylanases (Bian et al., 2013; Akpinar et al., 2010), releasing a virtually negligible amount of monomeric xylose, which is indeed the main advantage of the enzymatic production of XOS as compared with physicochemical approaches.

3.6. Prebiotic effect of XOS from birchwood xylan.

3.6.1. Production of SCFAs and other organic acids. SCFAs, lactate and succinate, some of the main end-products of fermentation by colonic bacteria (Fooks, Fuller, & Gibson, 1999; Rodriguez-Colinas et al., 2013), were determined in fecal fermentations supplemented with the whole mixture of birchwood XOS (containing both the neutral XOS and the 4-*O*-methyl-D-glucuronic acid derivatives) produced by catalysis with XynM. The global production of organic acids was remarkably higher in the presence of these XOS, especially for acetic and, to a lesser extent, for lactic acid, and the differences observed seemed to be dose-dependent (Fig. 3). In general, this profile is in good agreement with other reports for fecal fermentations of XOS (Kabel, Kortenoeven, Schols, & Voragen, 2002). However, the amount of butyric acid, an end-product usually found upon fermentation of other mixtures of XOS by probiotic bacteria (Lecerf et al., 2012), changed from 14 mg/L (0 h) to be negligible regardless of the presence or not of the XOS mixture.

Even though the presence of these organic acids, which are well-known biomarkers for probiotic species (Aachary & Prapulla, 2011; Lecerf et al., 2012; Li,

Summanen, Komoriya, & Finegold, 2015), suggested the prebiotic effect of birchwood XOS, their role was further confirmed analyzing the composition of the microbial communities in these samples.

3.6.2. Microbiome determination. Microorganisms were identified up to the genera and putative species level and quantified in terms of relative abundance (Fig. 4). According to the microbiome data, *Bifidobacterium* was one of the predominant genera in the initial control (25%). The abundance of these probiotic bacteria in the feces of breast-fed children has been previously reported and related to the presence of prebiotic oligosaccharides in breast-milk (Barile & Rastall, 2013). However, the proportion of *Bifidobacterium* species dropped off remarkably (18%) after 24 h in the control without XOS, while a clear beneficial effect of the XOS mixture on bifidobacteria was observed. The presence of 200 g/L XOS allowed maintaining its initial population (23%) in the same time period, and with 400 g/L it raised up to 32%.

In the case of *Lactobacillus,* the other fully established probiotic genus, the presence of this XOS combination had no apparent impact on its relative abundance, as has been observed for other XOS mixtures (Li et al., 2015).

Apart from the effect on conventional probiotic bacteria, the most remarkable outcome was the dramatic increase of the proportion of *Staphylococcus* sp., and specifically *S. hominis*, in the presence of XOS respect to the control samples at 0 h and 24 h. Given the extent of the increase, the identity of this species was further confirmed by PCR-based analysis (Table S3, Supplementary). *S. hominis* is a coagulase-negative staphylococcus naturally found in the microbiome of breast-fed children (Martin et al., 2007). Strains of this species were recently suggested as potential probiotics for its ability to produce bacteriocins, a sort of substances that act as growth inhibitors of

445 relevant pathogens as *Staphylococcus aureus* or *Helicobacter pylori* (Sung, Kim, Kim,
446 Joo, & Kim, I, 2010; Lopez-Brea, Alarcon, Domingo, & Diaz-Reganon, 2008). The
447 enrichment in *S. hominis* may be related to the observed profile of organic acids. The
448 high quantities of acetic and lactic acids determined in samples containing XOS may
449 seem surprising when compared to the modest increase in the abundance of
450 bifidobacteria. As observed in Figure 3, the content of acetic acid increased 3.6- and
451 5.4-fold in fermentations with 200 g/L and 400 g/L XOS samples, respectively, as
452 compared with the 24 h control. Indeed, the production of these metabolites is related to
453 the two main bacterial populations positively affected by XOS: *Bifidobacterium* (~1.8-
454 fold increment) and *S. hominis* (12.0-fold increment with 200 g/L XOS and 14.7-fold
455 with 400 g/L). Considering the evident stimulation of the growth of the last
456 microorganism by XOS, and taking into account that it was found to produce both
457 acetic and lactic acid (Julak, Stránská, Procházková-Francisci, & Rosová, 2000; Kloos
458 & Schleifer, 1975), the organic acids profile determined in these experiments could be
459 mostly attributed to *S. hominis* metabolism. However, it is worth mentioning that,
460 besides the role of *S. hominis*, there are other potential explanations for the acids'
461 profile, as this pattern can differ for the same bacterial population depending on the
462 available substrates.

463 To a minor extent, results regarding *Streptococcus salivarius* are also interesting.
464 This species was also reported as a potential probiotic (Burton, Chilcott, Moore,
465 Speiser, & Tagg, 2006) and its content increased respect to the 24 h control from 11%
466 to 19% in samples with 400 g/L XOS.

467 The presence of two clostridia in control samples is also notable (16% at 0 h and
468 12% after 24 h). One of them, *Clostridium perfringens*, is a normal member of the gut
469 microbiota that has been related to several diseases when its population increases to

pathogenic levels (Smedley, Fisher, Sayeed, Chakrabarti, & McClane, 2005). The data in Figure 4 show that the presence of birchwood XOS led to a decrease in the abundance of clostridia below 0.5% for both concentrations tested. XOS also prevented the development of other pathogens, as *Actinomyces radingae* (7% of the microbiome of the 24 h control sample) that can produce actinomycosis, a human chronic disease (Smego & Foglia, 1998). Enterobacteriaceae was the most represented family in the control microbiomes, constituting 32% and 37% at 0 h and 24 h respectively. Among the members of this family, *Citrobacter freundii* was a clearly dominant species in controls, while its representation in the microbial community decreased notably. Just the opposite effect was observed for *Kluyvera cryocrescens,* which displayed a remarkable growth with 400 g/L XOS and was the main enterobacteria with 200 g/L. The biological relevance of this change in the profile of the microbiota is unclear and more information on both species is required. In global terms, 200 g/L XOS avoided the increase of enterobacteria while 400 g/L dropped off its presence to 26%. This decrease is considered a characteristic feature of prebiotics, which prevent the development of higher concentrations of potentially pathogenic species from this family (Macfarlane, Macfarlane, & Cummings, 2006).

Xylooligosaccharides are considered novel candidate prebiotics (Rastall & Gibson, 2015). Together with this work, rising evidences support their role in selectively affecting gut microbiota, in particular stimulating the growth of several species from *Bifidobacterium* and *Lactobacillus* (Aachary & Prapulla, 2011). However, most of these studies were carried out *in vitro* using single-species cultures (Li et al., 2015; Chapla et al., 2012), while the number of *in vivo* reports is much more limited and restricted to the effect of commercial XOS mixtures on the adult population (Lecerf et al., 2012; Aachary & Prapulla, 2011). Fermentation of fecal slurries constitute an *in*

vitro alternative to pure cultures that provides a useful representation of the diversity of the gut microbiota without requiring large quantities of the prebiotic tested (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). However, the investigations based on the effect of XOS in fecal fermentations are scarce (Kabel et al., 2002).

Most studies conducted on infants focus on formulations of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) that mimic the composition of human milk oligosaccharides (HMOS) and the prebiotic effect of human milk, without paying attention to other potential prebiotics (Barile & Rastall, 2013; Thomas & Greer, 2010). The main goal for those works was to restore the predominance of gut bifidobacteria, present in children fed with human breast milk, but lost in formula-fed infants (Thomas & Greer, 2010). Using this model, the XOS mixture obtained in this work has demonstrated not only bifidogenic effect, but also a certain antimicrobial action, which is typical from HMOS. Indeed, the growth inhibition of species from *Clostridium* and *Citrobacter* has been previously demonstrated for human milk (Marcobal et al., 2010; Newburg & Walker, 2007). This evidence suggests the potential interest of including XOS as components of infants' formula and opens the field for determining the effect of other prebiotics beyond GOS and FOS.

Conclusions

The GH11 endoxylanase XynM from *T. amestolkiae* was isolated, characterized, genetically sequenced and used to obtain a prebiotic mixture of XOS from birchwood xylan. The enzymatic hydrolysis resulted in a neutral XOS yield of 28.8%, mainly Xyl_2 to Xyl_4. To the best of our knowledge, this is the first report on the effect of a non-commercial XOS mixture on the fecal microbiota of breast-fed children. The prebiotic effect is well supported and the beneficial effect of birchwood XOS consisted not only

in stimulating the growth of beneficial bacteria, but also in preventing the development of potential pathogenic species.

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Author declaration

The authors declare that they have no conflict of interest.

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Table 1. Composition of the reaction mixture in the hydrolysis of 2% (w/v) birchwood xylan with XynM. Glc: glucose; Xyl: xylose; Xyl_2: xylobiose; Xyl_3: xylotriose; Xyl_4: xylotetraose; Xyl_5: xylopentaose; Xyl_6: xylohexaose; GA: glucuronic acid; XOS: xylooligosaccharides.

Reaction time (h)	**[Products] (g/L)[a]**								**XOS yield (%)[c]**
	Glc	**Xyl**	**Xyl_2**	**Xyl_3**	**Xyl_4**	**Xyl_5**	**Xyl_6**	**Total[b]**	
0.25	0.05	0.01	0.50	0.93	0.76	0.23	0.43	2.90	14.2
1	0.11	0.02	1.03	1.55	1.11	0.27	0.40	4.50	21.80
3	0.12	0.03	1.18	1.79	1.26	0.31	0.47	5.16	25.0
5	0.11	0.04	1.21	1.70	1.19	0.29	0.45	4.98	24.1
7	0.12	0.05	1.33	1.85	1.29	0.31	0.49	5.45	26.4
8	0.13	0.06	1.39	1.90	1.32	0.32	0.50	5.61	27.1
10	0.13	0.06	1.49	2.01	1.39	0.33	0.53	5.95	28.8
24	0.14	0.08	1.53	1.78	1.24	0.31	0.57	5.65	27.2

[a] Determined by HPAEC-PAD.
[b] Total identified products.
[c] Percentage of Xyl_2-Xyl_6 referred to the initial amount of birchwood xylan.

676 **FIGURE LEGENDS**

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679 **Figure 1.** A) SDS-PAGE analysis of pure XynM. B) Determination of the molecular

680 mass of XynM by MALDI-TOF.

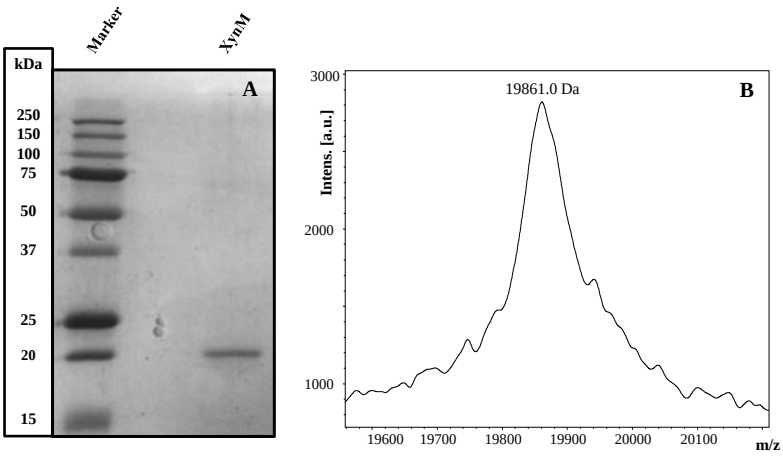

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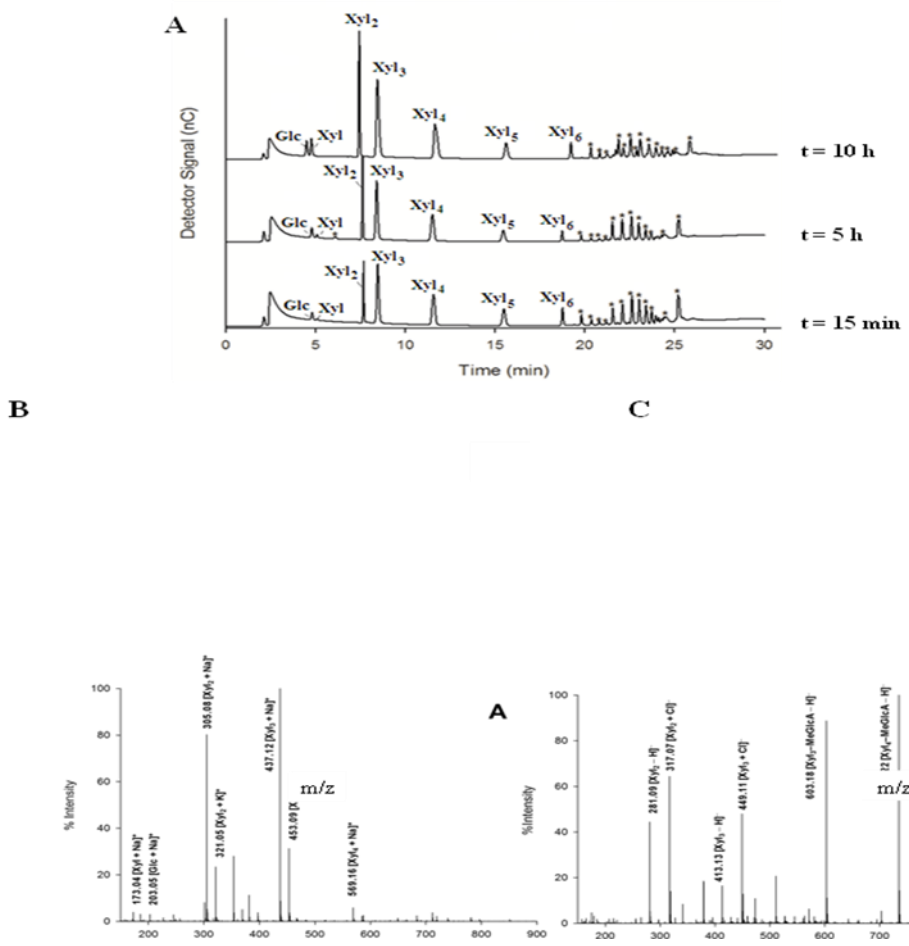
Figure 2. Hydrolysis of 2% (w/v) birchwood xylan with XynM. A) HPAEC-PAD chromatograms from 15 min, 5 h and 72 h-reaction mixtures. B) ESI/MS spectra recorded in the positive ion mode; C) ESI/MS spectra recorded in the negative ion mode. Glc: glucose; Xyl: xylose; Xyl_2: xylobiose Xyl_3: xylotriose; Xyl_4: xylotetraose; Xyl_5: xylopentaose; Xyl_6: xylohexaose; MeGlcA: 4-*O*-methyl-D-glucuronic acid; *: unidentified.



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691 **Figure 3.** Profile of SCFAs, lactic and succinic acid in the fermentations with XOS and

692 controls.

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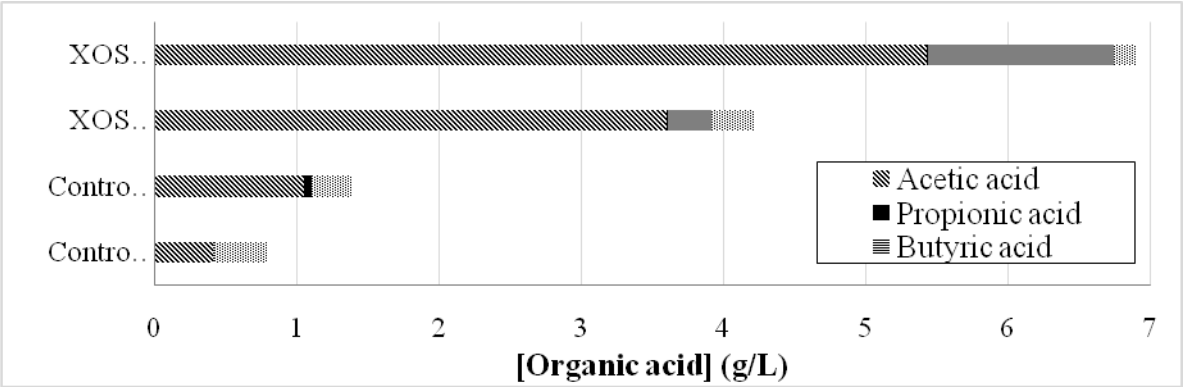

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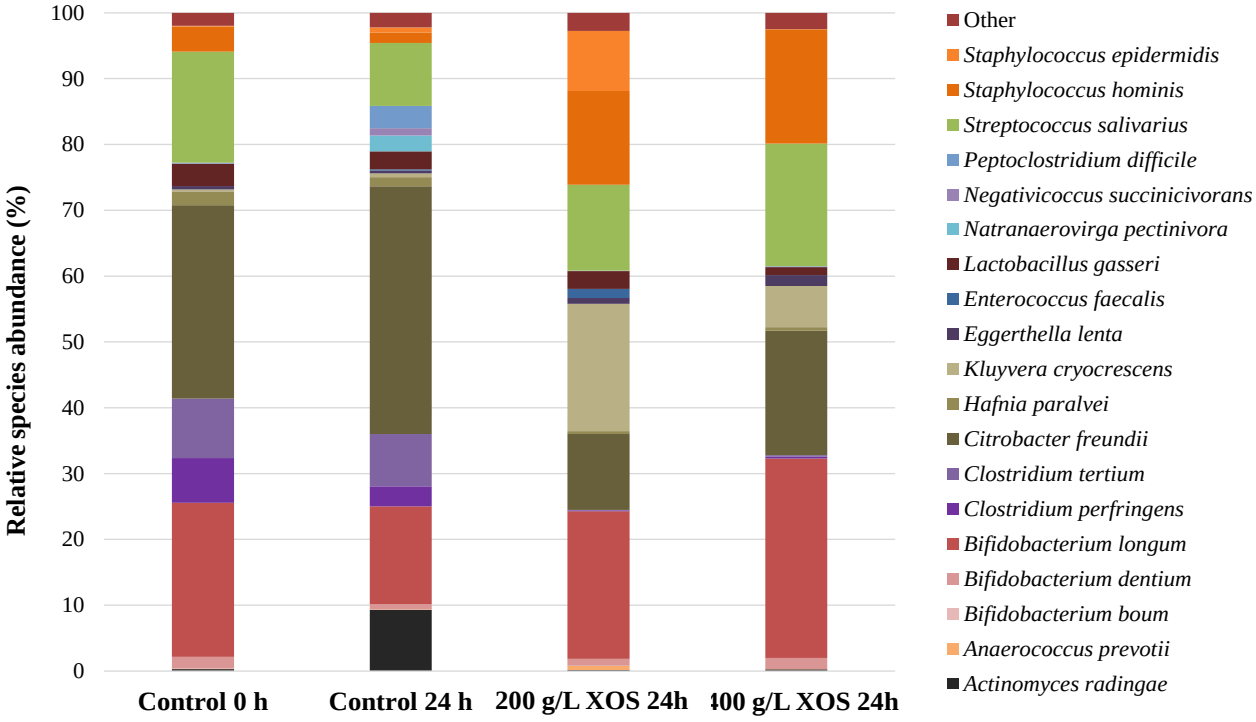
Figure 4. Relative species abundance of bacterial microbiomes from fermentations of control fecal samples (0 and 24 h) and in 24 h-samples containing a mixture of XOS.



700 Fig 1