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

1. Introduction

Hemicelluloses are a fundamental component of the renewable resources in the biosphere, since they constitute the second major carbon source in crop wastes, only preceded by cellulose. Unlike cellulose, hemicelluloses comprise 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 as a source of 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, the coordinated action of several hydrolases is required to accomplish 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 large 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. A growing 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, Probert, Van Loo, Rastall, and Roberfroid (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 (Aachary & Prapulla, 2011; Chapla et al., 2012). Besides this role as a classical prebiotic, XOS

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include other beneficial effects, among them possible antitumorigenic, anti-inflammatory, and antiallergic properties (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 the subsequent purification phase more difficult. In contrast, the enzymatic approach is environmentally friendly, requiring mild conditions, and producing low monosaccharide yields and non-toxic by-products (Chapla et al., 2012; Linares-Pastén, Karlsson, & Aronsson, 2016). 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 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 prepared according to our previous work (Nieto-Dominguez et al., 2015). Mandels medium was supplemented with 2% (w/v) beechwood xylan as a 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 °C and centrifuged at 1200 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 °C 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% over 25 min. Then, the mobile phase mix was 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 (pH 6) 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 °C.

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 the 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 to locate 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 °C and centrifuged at 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 3000 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 CarboPac PA200 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 from 80 mM to 320 mM AcONa for 10 min. A final 15 min-gradient was programmed to return to the initial conditions (15 mM NaOH and 0 mM AcONa). The peaks were analyzed using the Chromeleon software. The flow rate was constant at 0.5 ml/min and 25 μl of each sample were injected. The identification and quantification of xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose were performed using commercial standards. All samples were previously diluted 1:10 with H_2O and filtered through 0.45 μm nylon filters.

2.10. Characterization of products

The molecular weight of the hydrolysis products from birchwood xylan was assessed using a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar I) (AB Sciex, Framingham, MA, USA). Samples were analyzed by direct infusion and ionized by electrospray with methanol as ionizing phase both in positive and negative reflector modes.

2.11. Prebiotic effect of XOS from birchwood xylan

2.11.1. Faecal fermentations

Assays were carried out in 10 ml microfermentors (24 multi-well plates; μ-24 Bioreactor) (Pall Corporation, Port Washington, NY, USA). The fresh faecal sample came from a female breast-fed baby who had not received antibiotic treatment for at least 3 months prior to experimentation and had no history of bowel disorders. Stools were vacuum stored and refrigerated until fermentation took place. Fresh faeces (800 mg) were weighted and dissolved in the culture medium described by Macfarlane and coworkers (1998) in a proportion 1:5 (faeces:medium) to hydrate them. Each microfermentor was inoculated with 500 μl of the homogenized mixture (faecal slurry). Before starting fermentation, the headspace was displaced with nitrogen, and the whole assay was performed in anaerobiosis, maintaining temperature (37 °C) and pH (pH 5.5). A sample was taken from the initial faecal 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 the 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 faecal 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, taking 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 performed 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. These clean FASTA files were subjected to analysis with QIIME version 1.8 with the parameters by default, and SILVA 16 s 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 substrate 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 °C, 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 by 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 across the wide range of pH assayed, although its thermostability was low at 50 °C and higher temperatures. These are typical properties previously reported for endoxylanases from *Penicillium* species (Chavez et al., 2006).

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

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

3.4. Sequencing and classification of XynM

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

The sequences from the mRNA encoding for *T. purpurogenum* (GenBank 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 the prediction of 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 considering its low molecular mass and high specificity reported above (Polizeli et al., 2005).

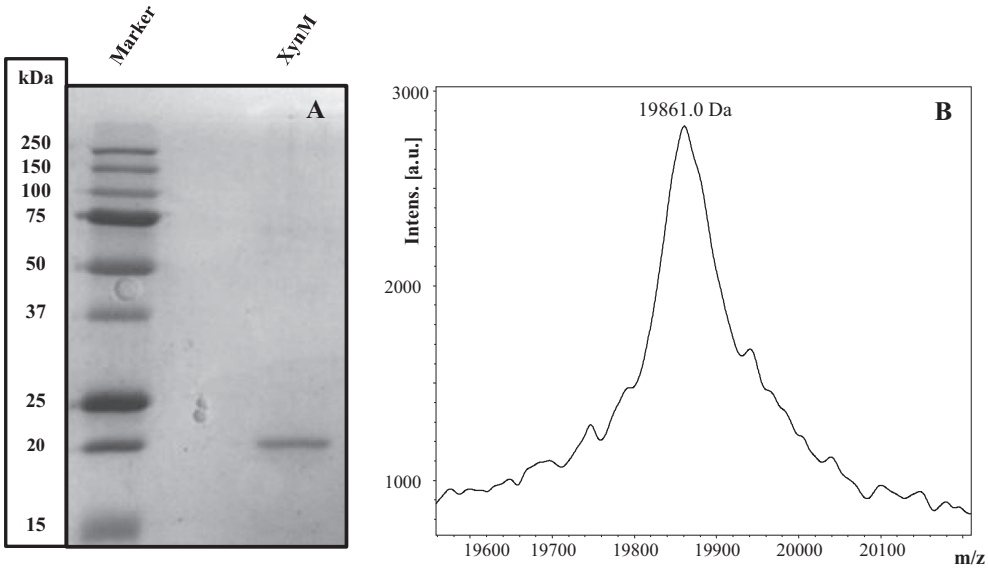

Fig. 1. A) SDS-PAGE analysis of pure XynM. B) Determination of the molecular mass of XynM by MALDI-TOF.

3.5. Production of XOS by hydrolysis of birchwood xylan catalyzed by XynM

XOS production reached the maximum yield after 10 h reaction time, 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 a 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). Also noteworthy is the negligible formation of free xylose. Other reaction products could not be identified, and probably contain substituted xylooligosaccharides. Indeed, the high retention times observed for the unidentified XOS suggests 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 been published 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 such 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 (Akpinar et al., 2010; Bian et al., 2013), 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 identified in faecal 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 faecal 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 by 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 faeces of breast-fed

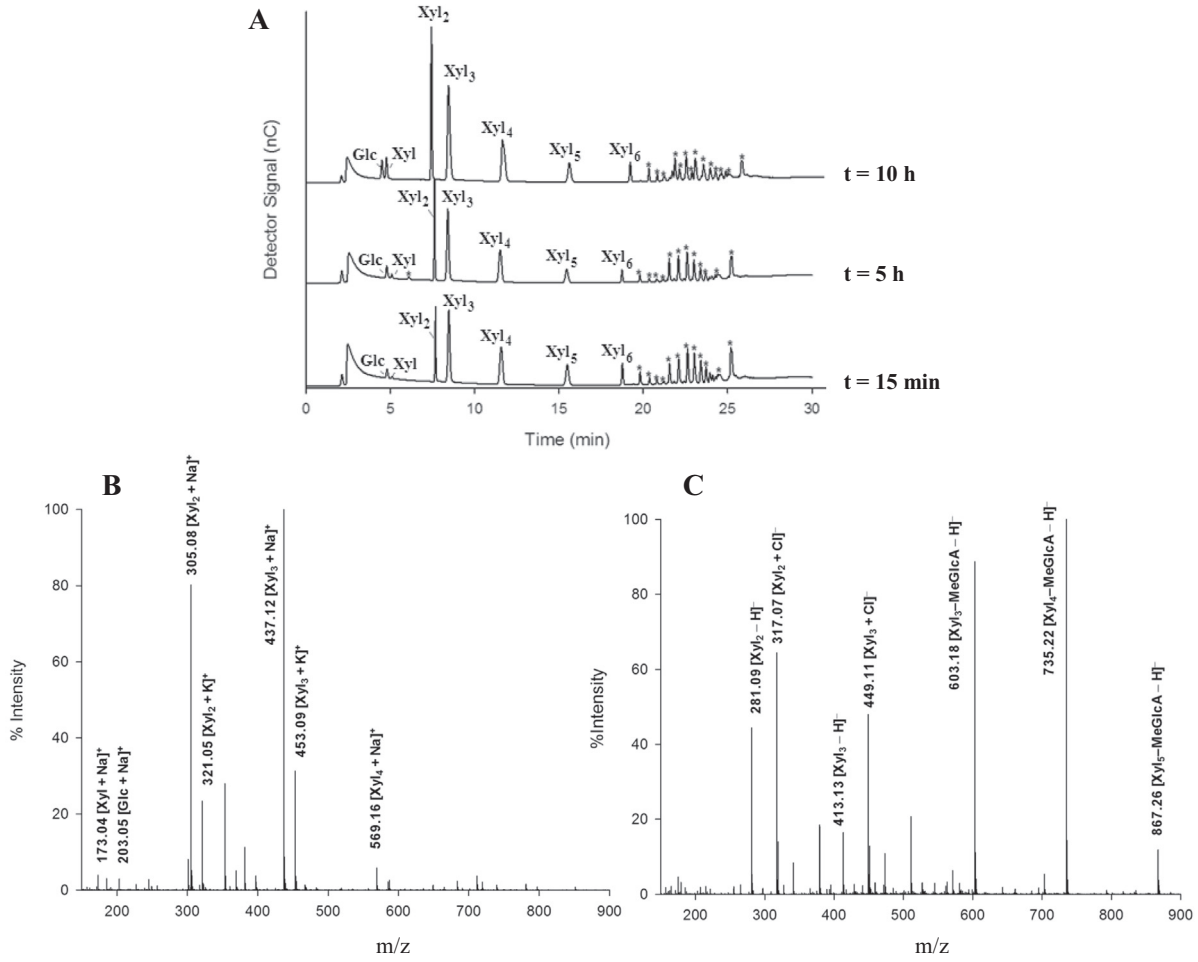

Fig. 2. Hydrolysis of 2% (w/v) birchwood xylan with XynM. A) HPAEC-PAD chromatograms from 15 min, 5 h and 72 h-reaction mixtures. B) ESI/MS spectra recorded in the positive ion mode; C) ESI/MS spectra recorded in the negative ion mode. Glc: glucose; Xyl: xylose; Xyl_2: xylobiose Xyl_3: xylotriose; Xyl_4: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

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	21.8
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.

children has been previously reported and has been 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 for the maintenance of the initial population (23%) in the same time period, and with 400 g/l it increased 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.

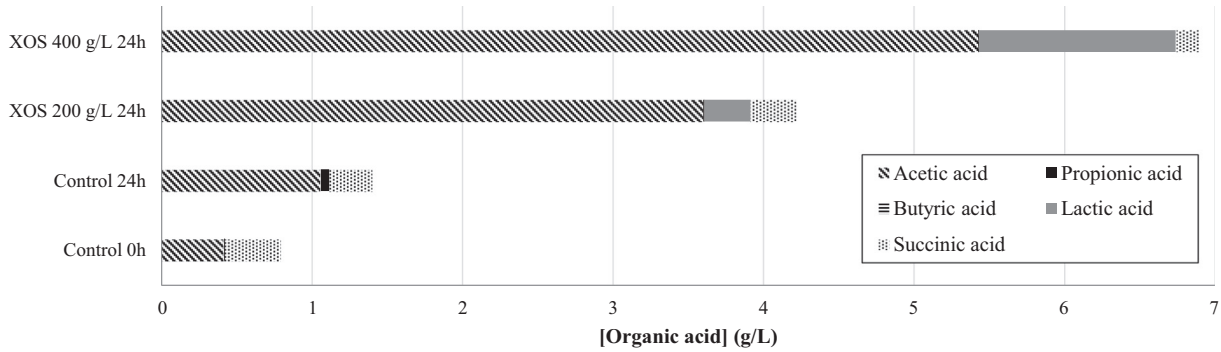

Fig. 3. Profile of SCFAs, lactic and succinic acid in the fermentations with XOS and controls.

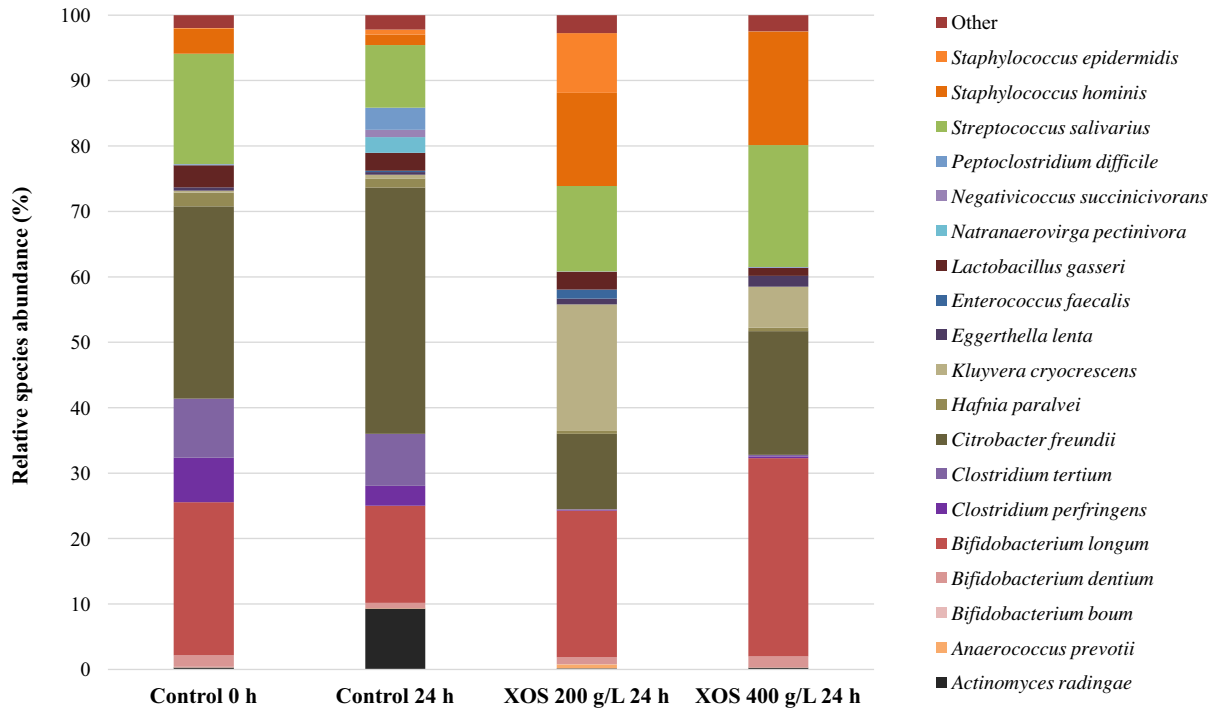

Fig. 4. Relative species abundance of bacterial microbiomes from fermentations of control faecal samples (0 and 24 h) and in 24 h-samples containing a mixture of XOS.

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

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

The presence of two clostridia in control samples is also notable (16% at 0 h and 12% after 24 h). One of them, *Clostridium perfringens*, is a normal member of the gut microbiota that has been related to several diseases when its population increases to pathogenic levels (Smedley, Fisher, Sayeed, Chakrabarti, & McClane, 2005). The data in Fig. 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, such 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 prevented the increase of enterobacteria while 400 g/l reduced their concentration 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 (Chapla et al., 2012; Li et al., 2015), while the number of *in vivo* reports is much more limited and restricted to the effect of commercial XOS mixtures on the adult population (Aachary & Prapulla, 2011; Lecerf et al., 2012). Fermentation of faecal 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 et al., 2004). However, the investigations based on the effect of XOS in faecal 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 a 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 effects of other prebiotics beyond GOS and FOS.

4. 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 faecal 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.

Author declaration

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.149.

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