ASSESSMENT ON THE FERMENTABILITY OF XYLOOLIGOSACCHARIDES FROM RICE HUSKS

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Rice husks were subjected to processing with hot, compressed water under selected conditions to cause the partial breakdown of xylan into soluble products (mainly xylooligosaccharides, XOS). The reaction media were subjected to membrane processing, endoxylanase treatment and ion exchange to obtain purified XOS concentrates. Human fecal slurry cultures with XOS were carried out to assess their bifidogenic activity to stimulate the production of Short Chain Fatty Acids (SCFA) and lactic acid. Results were compared with data obtained in similar cultures containing the prebiotic inulin and the non-prebiotic glucose. The experimental results confirmed the ability of XOS concentrates to act as fermentable carbohydrates for the human colonic microbiota, producing a rapid decrease of pH, comparable to that promoted by glucose and more pronounced than that occurring with inulin. XOS having different DP were degraded at different rates. The experimental results confirmed the ability of rice husk's XOS concentrates for supporting the growth of bifidobacteria and for acting as carbon sources, leading mainly to the generation of acetic and lactic acids.

Keywords: Rice husks; Xylooligosaccharides; Prebiotic; Intestinal microbiota; qPCR; Short chain fatty acids

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INTRODUCTION

The human colon microbiota play an important role in the maintenance of health, as alterations in intestinal microbiota composition and/or metabolic activity are thought to be the basis of the development of some diseases in humans (Van der Meulen et al. 2006). Therefore, the modulation of the complex microbial gut ecosystem is a point of interest in order to improve the host's health. This modulation can be achieved by intake of prebiotics (substrates improving the host health by selectively stimulating the growth and/or metabolic activity of one or a limited number of beneficial bacteria in the colon), probiotics (living microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance), or mixtures of prebiotics and probiotics (synbiotics) (Grootaert et al. 2009). Prebiotics could influence many aspects of bowel function through fermentation (Hernot et al. 2009); in this way particular interest has been directed towards assessing their ability to promote changes in the colonic microbiota

that may be related with some favourable effects. Traditionally, bifidobacteria and lactobacilli have been considered the main microbial targets of prebiotics (Wang 2009).

The major products resulting from prebiotic carbohydrate fermentation in the large bowel are short chain fatty acids (SCFA) (mainly acetate, propionate and butyrate), which can provide energy to the colon epithelium, stimulate the gut immune system, promote the synthesis of vitamins K and B, improve the colonisation resistance against pathogens, decrease pH, and stimulate absorption of some minerals (Pan et al. 2009; Van de Wiele et al. 2004). Prebiotic fermentation could have a strong influence on the gut microbiota, and its effects vary depending on individuals, substrate availability, and chemical composition and structure (Hernot et al. 2009).

In order to be considered as prebiotic, an ingredient must neither be hydrolyzed nor absorbed in the upper part of the gastrointestinal tract (Gibson et al. 1995). The best-known prebiotics to date are Non Digestible Oligosaccharides (NDOs) (Mandalari et al. 2008), including fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and lactulose. From a nutritional point of view, xylooligosaccharides (XOS) behave as NDOs, since they are not degradable by the low-pH gastric fluid or by human and animal digestive enzymes, and will therefore reach the large bowel intact (Van Laere et al. 2000). XOS have been classified as "emerging prebiotics" (Gibson et al. 2004). XOS can be manufactured from selected lignocellulosic materials (such as barley husks, rice husks, hardwoods or corn cobs) containing substituted xylan, which is made up of a 1-4-linked β–D-xylopyranose backbone substituted with arabinosyl and 4-O-methyl ether uronyl moieties, and esterified acids (Saha 2003).

The polymeric xylan backbone can be broken down by hydrolytic reactions (in aqueous media, eventually catalyzed by externally added acids or enzymes) to yield XOS. Interest has been paid to the partial hydrolysis of xylan contained in a number of lignocellulosic feedstocks using hot, compressed water (hydrothermal or autohydrolysis processing). This method enables high XOS yields, but the reaction is not selective, and undesired compounds (for example, coming from extractives, acid-soluble lignin, reaction products of proteins and saccharide degradation) are also present in the reaction media. Because of this, when XOS are to be used in food applications, the reaction media must be subjected to further processing, in order to remove, at least partly, the undesired components before utilization (Gullón et al. 2009).

Several strategies have been proposed in the literature for refining crude auto-hydrolysis liquors, including vacuum evaporation, solvent extraction, solvent precipitation (Vegas et al. 2004, 2005), adsorption (Yuan et al. 2004; Izumi et al. 2004a, b), flocculation (Yuan et al. 2004), ion exchange (Vegas et al. 2004, 2005; Yuan et al. 2004), chromatographic separation (Kabel et al. 2002a; Endo and Kuroda, 2000), and membrane processing (Yuan et al. 2004; Izumi et al. 2004a; Swennen et al. 2005; Vegas et al. 2006; Nabarlatz et al. 2007).

The purpose of this work was to investigate the capability of XOS generated by autohydrolysis of rice husks to stimulate growth of bifidobacteria and metabolic activity of intestinal microbiota by using *in vitro* fecal slurry cultures.

EXPERIMENTAL

Autohydrolysis

Rice husks were obtained in a local factory (Galicia, Spain), air-dried, homogenized in a single lot to avoid compositional differences among aliquots, and stored until use.

Autohydrolysis (Fig. 1) was carried out by mixing distilled water with rice husks at a liquid to solid mass ratio of 8 g water/g oven dry solid, in a stirred stainless steel reactor (Parr Instrument Company). Temperature was monitored using an inner thermocouple, and controlled by a Proportional Integral Derivate module. Reaction was carried out under selected conditions (185 °C for 20 min) selected on the basis a previous kinetic study (Vila et al., 2002) and confirmed for the specific feedstock lot. After cooling, the autohydrolysis liquors were recovered by filtration, analyzed, and processed as described in the next sections.

Processing of Autohydrolysis Liquors

Membrane processing

The experimental setup (Fig. 1) used in this work was described by Gullón et al. (2008). Full recycle experiments were carried out at different transmembrane pressure (TMP) in order to determine the optimal operational conditions for the assayed membrane. The permeate flux was measured at TMP in the range 3 to 15 bar. Successive experiments were carried out from lower to higher pressures. Once the stable flux was reached at a given TMP, the pressure was increased to the next (higher) considered value. When the new stationary permeate flux was achieved, TMP was decreased to the previously tested value to assess irreversible fouling, and TMP was then increased to the next value. This set of experiments enabled the selection of the optimal TMP. Autohydrolysis liquors were submitted at Continuous Diafiltration (CD) at the TMP selected from the above experiments, by adding water to the feed tank at the same rate as the permeate was obtained, thus keeping the feed volume constant during the course of processing. The permeate volume was measured in terms of "diafiltration volumes" DV (one DV corresponds to the initial retentate volume). CD was continued up to reach 3 DV. Along CD, samples of the feed and permeate were taken and assayed by the same methods employed for autohydrolysis liquors. Additionally, the permeate flux and total cumulative permeate volume were measured. Diafiltered liquors were processed in the same membrane set-up operating in concentration mode. The degree of concentration was measured in terms of the Volume Concentration Ratio (VCR, defined as the ratio between the initial feed volume and the volume of retentate remaining at the considered time). Samples of permeate and concentrate were withdrawn at various VCR and assayed by the same methods employed for autohydrolysis liquors. Retentate was further subjected to ion exchange (leading to the fraction denoted XOS 1) and enzymatic reaction followed by ion exchange (leading to the fraction denoted XOS 2), and these samples were assayed as carbon sources (Fig. 1). Membrane fouling was assessed by measuring water permeability before and after experiments. To assess irreversible fouling, membranes were rinsed with water after each experiment, and the water permeability was measured again. Fouled membranes were cleaned with a caustic

detergent solution (1% Ultrasil 11, from Henkel Ecolab) at 50 °C for 30 min operating in full recycle mode, and then operated with fresh water to check the flux recovery.

Endoxylanase treatment

Retentate from the membrane concentration step was treated with a commercial endoxylanase (Pentopan Mono BG) kindly provided by Novozymes-Spain, in order to decrease the average molecular mass of xylan-hydrolysis products. The endoxylanase activity of the commercial concentrate (3500 XU/g) was measured by the Megazyme assay (Megazyme International Ireland Ltd.), based on the depolymerization of Remazol Brilliant Blue (RBB). The operational conditions were selected according to Vegas et al. (2008a) with the following values: 350 XU/kg liquor, pH 6, 50 °C, 48 h. Products from enzymatic reaction were subjected to ion exchange treatment (see Fig. 1).

Ion exchange processing

XOS concentrates were treated with Amberlite IRA 96 (a weak anion-exchange resin in OH- form) for further refining. Liquors and resin were contacted overnight with gentle agitation at room temperature using a mass ratio of liquor to resin of 15 g/g (see Fig. 1). The resulting solutions were freeze-dried and employed in fermentation experiments (samples XOS 1 and XOS 2, see Fig. 1).

Fecal Cultures

Faecal sample preparation

Fecal samples were obtained from three healthy adult donors (two women and one man, aged 25 to 40 years). None of the volunteers had taken antibiotics during the 6 months previous to the study. Samples were collected, kept in an anaerobic cabinet and used within a maximum of 15 min after collection. A 1/10 w/v dilution in pre-reduced phosphate buffered saline solution (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2PO_4) pH 7.3 was prepared and homogenized in a Lab-Blender 400 stomacher (Seward Medical) for 2 min. The fecal homogenates of each donor were stabilized by keeping at 37 °C overnight under anaerobic conditions (10% v/v H_2, 10% CO_2, and 80% N_2) in a chamber Mac 500 (Don Whitley Scientific) (Kabel et al. 2002c).

Faecal batch culture fermentations

Three independent trials, each corresponding to faecal batch fermentations made in independent duplicate experiments using samples of each of the three donors, were carried out in the carbohydrate-free basal medium (CFBM) described by Al-Tamimi et al. (2006) supplemented with the selected carbon source. In each batch, CFBM (54 mL) per carbohydrate source) was added into a glass flask with 0.6 g of the considered carbon source: XOS 1, XOS 2, the prebiotic inulin (Sigma-Aldrich), or the non-prebiotic glucose (Sigma-Aldrich). An additional flask (with no carbon source added) was prepared as the negative control. After complete dissolution, mixtures were sterilized by filtration through a 0.45 μm membrane. Flasks were kept at 37 °C overnight under anaerobic conditions, and then 6 mL of stabilized faecal homogenates were added (final concentration of each carbohydrate after inoculum addition, 1% w/v). The various media were distributed into 15 glass tubes (4 mL per tube). Fermentations were carried out at 37

°C under anaerobic conditions, and tubes were withdrawn at different times of incubation (0, 6, 9, 24, and 48 h). Cultures were centrifuged at each time (13200 rpm, 10 min), and pellets and supernatants were collected for further analysis (see below).

Analytical Methods

Analysis of the raw material

Rice husks samples were subjected to moisture determination (method ISO 638), to ash determination (method ISO 776), and to quantitative acid hydrolysis (method TAPPI T13m). The HPLC analysis of liquors was carried out using a 1100 series Hewlett Packard chromatograph fitted with a refractive index detector (temperature, 50 °C). The analysis conditions were: Aminex HPX-87H column (Bio-Rad, Life Science Group, Hercules, CA), mobile phase 0.003 M H_2SO_4, flow 0.6 mL/min. The results allowed the determination of the sample contents of glucan (cellulose and starch, based on the glucose present in liquors), hemicellulosic polysaccharide constituents, and acetyl groups. The oven-dry weight of the solid phase from quantitative hydrolysis measured the content of Klason lignin after correction for ashes. Uronic acids were determined by the method of Blumenkrantz and Asboe-Hansen (1973), using galacturonic acid as a standard for quantification. Starch was determined enzymatically (Boheringer-Mannheim kit reference no. 10207748035).

Analysis of autohydrolysis liquors

Samples of liquors were filtered through 0.45 μm cellulose acetate membranes and assayed by HPLC for glucose, xylose, arabinose, and acetic acid as described above. A second sample of liquors was subjected to quantitative posthydrolysis (with 4% H_2SO_4 at 121 °C for 20 min) before duplicate HPLC analysis. The increase in the concentrations of xylose and glucose caused by posthydrolysis provided a measure of the concentrations of oligomers (xylooligomers and glucooligomers) present in the media. In the same way, the increase in acetic acid concentration indicated the amount of acetyl groups (AcO) linked to oligosaccharides, and the increase in arabinose concentration measured the amount of arabinosyl moieties (ArO) linked to oligosaccharides. Uronyl substituents (UA) were determined by the same method already cited for the raw material, using galacturonic acid as a standard for quantification. All analyses were done in triplicate.

Determination of carbohydrates and fermentation products in batch cultures

Supernatants from the faecal batch cultures were filtered through 0.20 μm cellulose acetate membranes. One aliquot was assayed by HPLC for monosaccharides, SCFA, and lactate quantification, and another aliquot was subjected to acid posthydrolysis to quantify the consumption of total oligosaccharides using the method and conditions described above.

Quantification of bifidobacteria in batch cultures by quantitative PCR

The quantification of the *Bifidobacterium* population in faecal batch fermentations was performed by quantitative PCR (qPCR) using previously described genus-specific primers (Gueimonde et al. 2004). DNA isolation was performed with the QIAamp® DNA stool Mini kit (Qiagen, GmbH, Hilden, Germany). All reactions were

performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA), and amplifications were carried out in a 7500 Fast Real Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). One μL of purified DNA was used as template in the 25 μL PCR reaction. Thermal cycling consisted of an initial cycle of 95 °C 10 min followed by 40 cycles of 95 °C 15 seconds and 60 °C 1 min. Standard curves were made with the strain *Bifidobacterium longum* NCIMB8809, which was grown overnight in MRSC (MRS broth, Difco, Becton, Dickinson & Co, supplemented with 0.25% L-cysteine, Sigma) under anaerobic conditions. Standard curves were obtained by plotting the cycle threshold (C_t) values obtained for the standard culture as a linear function of the base-10 logarithm of the initial number of cells in the culture determined by plate counting. The numbers of *Bifidobacterium* cells in fecal samples was determined by comparing the C_t values obtained with the standard curve. Samples were analyzed in duplicate in at least two independent PCR runs for each faecal slurry trial.

Statistical Analysis

Data for the degree of XOS consumption were statistically analyzed for each donor independently at 6 and 48 h of incubation by means of one-way ANOVA tests using the SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL). The substrate was employed as factor, with two categories: XOS 1 and XOS 2.

RESULTS AND DISCUSSION

Raw Material Composition and Characterization of Autohydrolysis Liquors

Table 1 shows compositional data for the rice husk samples employed in this study. The most important results for the objectives of this work are the contents of xylan and xylan substituents (arabinose, uronic acid units, and acetyl groups), which accounted jointly for 21.9 weight (wt) % of the oven-dried (o.d.) sample. Upon autohydrolysis, xylan is broken down in fragments of lower molecular weight, and part of the substituents are split off from the backbone at different rates, owing to the different susceptibility of the corresponding bonds to the hydrolysis reaction.

Table 1. Rice Husks Composition

Component	Content (g/100g oven-dry rice husks)
Cellulose	22.7
Starch*	2.9
Xylan	17.5
Araban	1.7
Acetyl groups	1.6
Klason lignin	33.9
Uronic acids	1.1
Ash	12.6
Other	6.1
Moisture (water)	9.0

*The starch was determined using an enzymatic kit.

Cellulose and Klason lignin, which suffer little alteration upon mild autohydrolysis processing, accounted jointly for 56.6 wt% of the o.d. feedstock, whereas starch accounted for 2.9% of the sample dry weight. Ashes and other non-saccharide components (which corresponded mainly to proteins, extractives and acid soluble lignin) accounted jointly for 18.7 wt % of the sample (o.d. basis).

Autohydrolysis was carried out under optimized conditions (185 °C for 20 min), in order to cause the dissolution of xylan (which was converted into smaller polymers and oligomeric fragments), while keeping a low xylose concentration in the medium. It can be noted that harsher operational conditions would result in increased xylose production from XOS (resulting in decreased yields of these compounds), and in the generation of detrimental xylose-degradation products such as furfural (Vila et al. 2002). The reaction liquors (stream A in Fig. 1) contained water, volatile components (VC) generated from the feedstock (mainly acetic acid) and non-volatile components (NVC, mainly XOS and monosaccharides). Table 2 lists data concerning the composition of stream A.

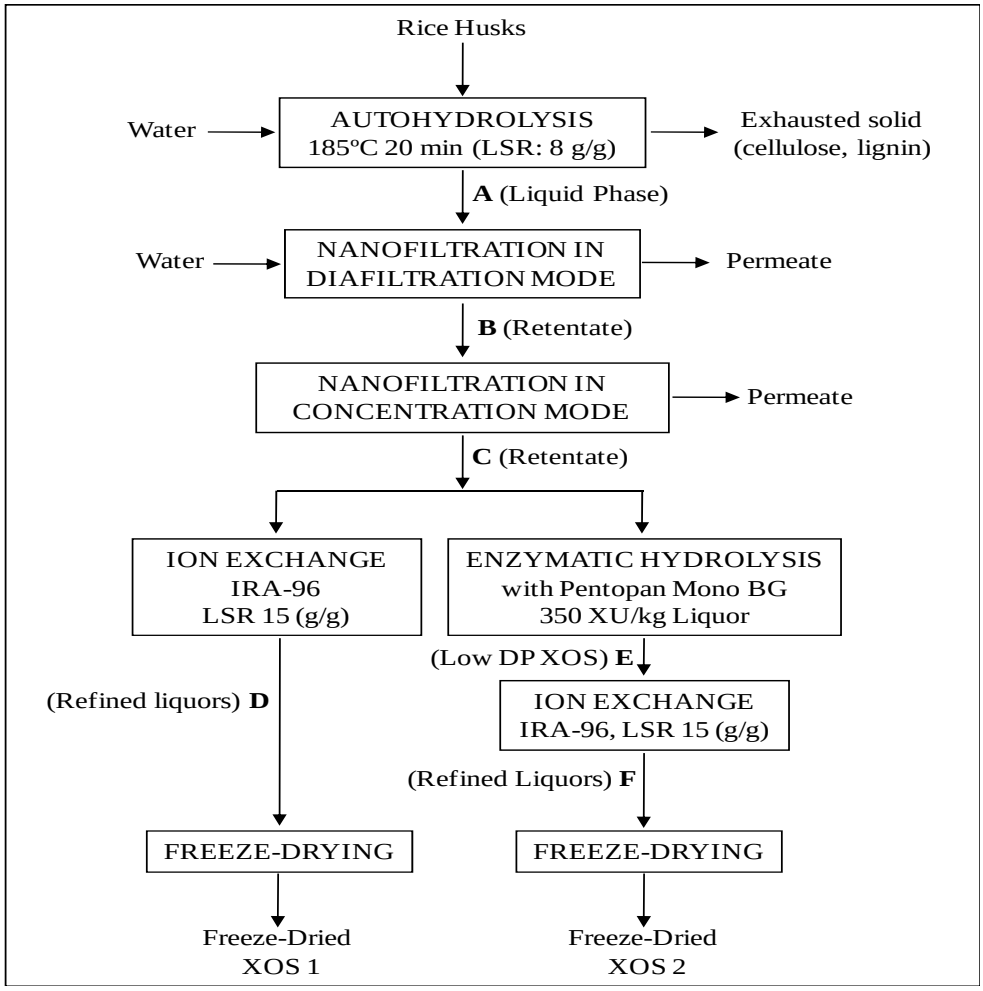

Fig. 1. Scheme of the process employed in this work for obtaining purified xylooligosaccharide concentrates

Table 2. Composition of Streams A, B, C and D in Figure 1 (expressed as kg of component/kg of NVC) *

Component	Stream A	Stream B	Stream C	Stream D
Glucose	0.0075	0.0013	0.005	0.001
Xylose	0.0302	0.0044	0.008	0.001
Arabinose	0.0328	0.0037	0.006	0.001
Acetic acid	0.0363	0.0018	0.002	0.001
GlucOS (as glucose)	0.1536	0.2117	0.205	0.248
XOS (as xylose)	0.4271	0.5568	0.549	0.611
ArO (as arabinose)	0.0222	0.0281	0.023	0.026
AcO (as acetic acid)	0.0329	0.0427	0.044	0.047
UA (as galacturonic acid)	0.0420	0.0644	0.046	0.056
ONVC (by difference)	0.2517	0.0870	0.118	0.009

* Volumetric Concentrations of NVC in Streams A to D: 24.46, 15.95, 77.46 and 63.02 g/L, respectively

The properties of XOS obtained by autohydrolysis (including molar mass distribution and type, number, and distribution of substituents) depend on both the type of raw material employed and the reaction conditions. The data in Table 2 confirm the presence of acetyl groups in XOS, whereas the fraction denoted "other non-volatile compounds" (ONVC) (mass fraction, 0.252 g/g NVC) corresponds to undesired, non-saccharide compounds, the proportion of which should be decreased in further refining treatments. The experimental results (and particularly, the limited concentrations of monosaccharides and the high concentration of XOS, indicative of a high degree of xylan conversion into the target compounds) confirmed the suitability of the autohydrolysis conditions for the objectives of this study. Considering the composition of raw liquors, the joint contribution of oligomeric saccharides (denoted TotalOS, which include XOS, XOS substituents and glucooligosaccharides, here denoted GlucOS), corresponded to a mass ratio of 0.678 g/g NVC. In order to produce food-grade XOS (whose usual commercial purity lies in the range 75-95%), the autohydrolysis liquors have to be refined. Refining involves the selective removal of undesired compounds (usually, monosaccharides and non-saccharide compounds) to obtain a concentrate with a XOS content as high as possible (Moure et al. 2006). For this purpose, several separation methods (individually or in combination) could be suitable, including solvent extraction, solvent precipitation, chromatographic separation, ion exchange, and membrane processing (Vegas et al. 2008b). Membrane technologies have been employed for oligosaccharide refining (Goulas et al. 2002; Nabarlatz et al. 2007; Swennen et al. 2005). Diafiltration and/or concentration through nanofiltration (NF) membranes enables an efficient purification of macro-microsolutes at an economically acceptable permeate flux (Li et al. 2004).

Membrane Processing of Autohydrolysis Liquors

Preliminary NF assays were carried out, operating in full recycle mode (data not shown) to assess the effects of TMP along the whole experimental range (3 to 15 bar). On the basis of this information, the TMP selected for operation in concentration mode was 13.8 bar.

CD of autohydrolysis liquors was performed up to 3 DV to remove undesired, low-molecular solutes from autohydrolysis liquors. Table 2 lists compositional data concerning the diafiltered solution (stream B in Fig. 1). The recovery percentages of the various solutes in stream B with respect to stream A were 7.3 to 11.0% for monosaccharides and 3.2% for acetic acid, in comparison with 89.8% for GlucOS, 85.0% for XOS, 82.3% for arabinooligosaccharides (ArO), 84.7% for acetyl groups linked to oligosaccharides (AcO), and 87.6% for uronic acids (UA). In summary, CD enabled: a) an almost complete removal of monosacccharides; b) a marked drop of the ONVC mass fraction (from 0.2517 to 0.0870 g/g NVC in stream B); and c) an increase in the mass fraction of total oligosaccharides (TotalOS, which include XOS, GlucOS, and oligosaccharide substituents, from 0.678 to 0.896 g/g NVC).

NF in concentration mode was performed until reaching VCR = 5, in order to concentrate the liquors from the CD step and looking for additional purification effects. Table 2 lists compositional data of the retentate (stream C in Fig. 1). The recovery percentages were 94.4% for GlucOS, 95.8% for XOS, 76.4% for ArO, 94.4% for AcO, and 88% for UA. No removal of ONVC was observed in the concentration stage, whereas the saccharide losses resulted in an increased ONVC mass fraction. Considering the overall CD-concentration processing, the volumetric concentrations of TotalOS increased by a factor of 4.03 (from 16.58 g/L in stream A to 66.86 g/L in stream C). The percentages of recovery in concentrate were 11.8 to 16.4% for monosaccharides, in comparison with 84.5% for GlucOS, 81.4% for XOS, 62.8% for ArO, 80.0% for AcO, and 77.1% for UA. On the other hand, just 29.8% of the initial ONVC mass was kept in the retentate. One aliquot of stream C was subjected to ion exchange and an another was subjected to xylanase treatment for DP reduction and ion exchange (see below), to obtain samples XOS 1 and XOS 2 (see Fig. 1), which were used as carbon sources in fermentation assays.

Enzymatic Processing

During the course of autohydrolysis treatments, the xylan chains are broken down randomly, leading to hydrolysis products having a wide DP distribution (Kabel et al. 2002b). In order to obtain short-chain XOS (the preferred ones for food applications), an aliquot of diafiltered and concentrated liquors (stream C in Fig. 1) was treated with commercial endoxylanases under conditions selected from the literature (as described in the Methods section). Endoxylanase treatment resulted in the generation of short-chain oligomers (mainly with DP in the range 2 to 4) from xylan-derived products of higher molecular weight (see Fig. 2). As the compositional data reported in this work are based on results from total hydrolysis, and considering that the addition of the enzyme concentrate resulted in just in hydrolysis reactions (Vegas et al., 2008a) it can be assumed that the composition of the hydrolyzed concentrates (stream E in Fig. 1) was essentially the same as the composition of stream C in Fig. 1 (except for the DP distribution).

Ion Exchange Processing

Samples of non-hydrolyzed concentrates and concentrates subjected to enzymatic hydrolysis were subjected to ion exchange with Amberlite IRA-96 in order to remove ONVC components (stream D and F, in Fig. 1). Compositional data of stream D are given in Table 2.

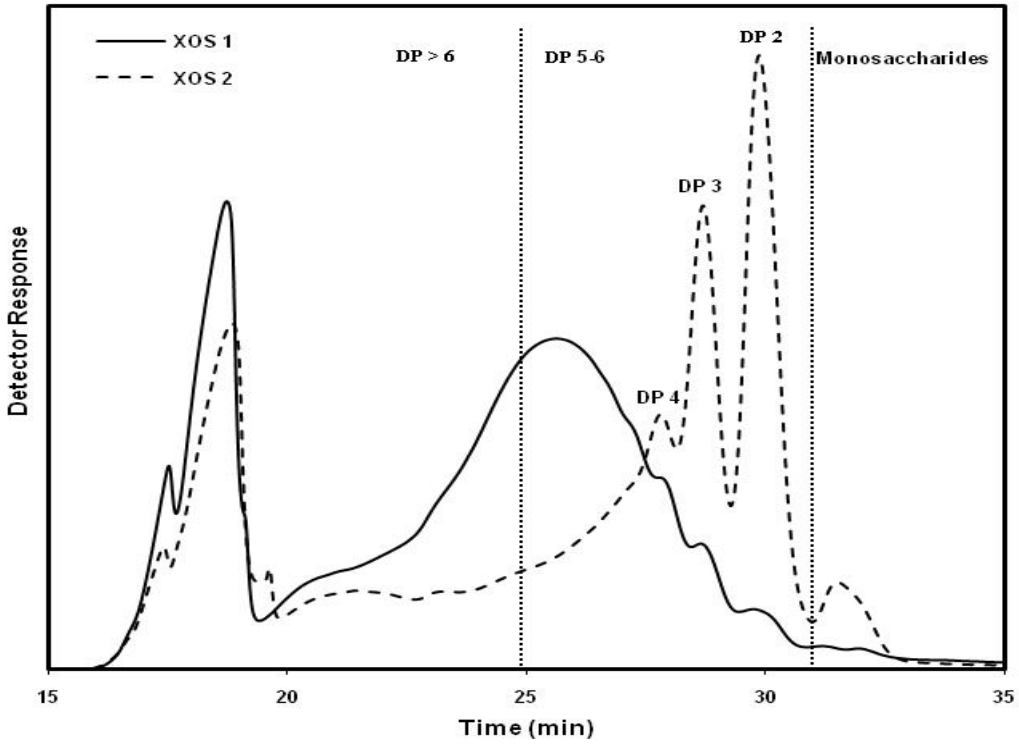

Fig. 2. HPSEC profiles of samples XOS 1 and XOS 2 (see Fig. 1)

These results in Table 2 show that 94.7% of ONVC in stream C were removed in this stage, leading to a marked drop in its mass fraction (from 0.118 to 0.009 g/g NVC). On the other hand, the recovery percentages of the various oligosaccharides in stream D respect to stream C were 83.6% for GlucOS, 76.8% for XOS, 82.6% for ArO, 77.3% for AcO and 74.6% for UA. The composition of stream D was the same as the composition of stream F in terms of target compound concentration.

Considering the overall CD-concentration-ion exchange process, the volumetric concentration of TotalOS increased from 16.6 g/L in stream A up to 62.3 g/L in stream D. In parallel, the mass fraction of the target compounds increased by 45.9%, resulting in concentrates, and TotalOS accounted for 98.9% of the mass of the non-volatile solutes present in stream D. This result confirmed the almost complete removal of non-saccharide compounds from the refined liquors. The joint contribution of XOS and XOS substituents accounted for 74.9% of the mass of NVC, and the rest (up to 90.2%) corresponded to GlucOS.

The data in Table 2 show that the whole processing resulted in complete monosaccharide removal, with global recovery percentages of 70.6% for GlucOS, 62.6% for XOS, 51.9% for ArO, 61.9% for AcO, and 58.3% for UA. These data confirm that the CD-NF concentration-ion exchange scheme proposed in this work resulted in strong purification effects. Streams D and F were freeze-dried to yield the concentrates denoted XOS 1 (DP > 5-6) and XOS 2 (DP 2-4) in Fig. 1, which were used as carbon sources in fermentation assays.

Modulation of the Intestinal Microbiota by Purified XOS Mixtures

Fermentation experiments with human faecal inocula were performed using XOS concentrates as substrates. Results were compared with cultures using inulin and glucose as positive, selective, and non-selective controls, respectively. Glucose is not abundant in the colon since it is absorbed in the small intestine; therefore, its influence in the colon ecosystem could be considered negligible. However, the prebiotic effect of inulin has been widely studied (Meyer and Stasse-Wolthuis 2009). Faecal batch cultures without external carbon sources added were used as negative controls.

Changes in Bifidobacterium population levels in faecal cultures

qPCR was used to evaluate the bifidogenic effect of XOS 1 and XOS 2 concentrates in faecal batch cultures (Fig. 3). Clearly higher increases of bifidobacteria population levels were found along incubation in the presence of XOS mixtures, inulin, and glucose, than in negative control cultures, which was indicative of a stimulatory effect of these substrates on bifidobacteria. The degree of stimulation varied depending on the carbohydrate source and the individual, as it was previously established for other carbohydrates by several authors. It has been indicated that in general the higher initial level of faecal bifidobacteria resulted in the lower increase of these microorganisms (Matteuzzi et al. 2004; De Preter et al. 2008; Salazar et al. 2008, 2009; Cardelle-Cobas et al. 2009), and a great variability with respect to the stimulatory effect can be obtained within a human population. In the present study, at 6 hours of incubation the bifidogenic effect of XOS mixtures was more pronounced than that of inulin for cultures from the three individuals. However, at 24 h of incubation in faecal cultures from individuals 2 and 3, who presented lower initial bifidobacterial counts (Fig. 3), the stimulation of bifidobacterial growth obtained with XOS was intermediate between that obtained with inulin (the lowest) and glucose (the highest). In contrast, in cultures from individual 1, at 24 hours of incubation the bifidogenic effect of XOS, inulin, and glucose, was similar. The bifidogenic effect of XOS from different origins and obtained by different methods has been well established (Campbell et al. 1997; Hsu et al. 2004; Moura et al. 2007; van Craeyveld et al. 2008; Ohbuchi et al. 2009). It is also known that chemical structures and DP can affect fermentation and prebiotic properties of XOS (Moura et al. 2007; van Craeyveld et al. 2008); however, in our case no clear difference between XOS 1 and XOS 2 mixtures was found with respect to the bifidogenic effect.

Consumption of XOS in faecal cultures

The consumption of XOS in two points along incubation is summarized in Table 3. Results show that XOS were actively degraded by the intestinal microbiota, although degradation during early stages of fermentation varied notably among individuals. Consumption occurred fast during the first 6 h of incubation, which was coincident with the period of fastest growth of *Bifidobacterium* spp. At this time XOS consumption accounted for more than 34% (with the exception of donor 2 with XOS 1) and exceeded 69% after 48 h of incubation. It has been indicated that generally the lower the DP of oligosaccharides is, the faster its consumption by the intestinal microbiota (Hughes et al. 2007; Moura et al. 2008). Thus, as expected from their lower average DP (see Fig. 2), in most cases XOS 2 mixture was degraded significantly faster ($P < 0.01$) than XOS 1.

SCFA and lactate production in faecal cultures

Table 4 presents data concerning the production of SCFA (acetate, propionate, butyrate) and lactate as well as pH values along fermentations. The increase of total SCFA along incubation was considerably more pronounced in samples containing carbohydrates than in negative control cultures. This and the notable decrease of pH during incubation confirmed the suitability of carbohydrates assayed as fermentable substrates for the intestinal microbiota. A fast pH drop was observed in the first hours of fermentation, and the values of this parameter attained in XOS cultures at 6 hours of incubation were comparable to those obtained in medium with glucose, but lower than in cultures with inulin (Table 4). The concentrations of SCFA and lactate increased with the XOS consumption, confirming that most acids were produced from XOS. This finding is in agreement with the results reported by Kabel et al. (2002c) using mixed human fecal inocula.

Table 3. Consumption of Xylooligosaccharides in the Course of Fermentations *

	Degree of consumption (%)			
	6 h		48 h	
Donor	XOS 1	XOS 2	XOS 1	XOS 2
1	40.60 ± 0.13	51.52 ± 0.13 *	71.33 ± 0.60	79.26 ± 0.35 *
2	16.80 ± 0.81	36.90 ± 2.17 *	73.66 ± 0.58	76.66 ± 0.87
3	34.32 ± 0.28	45.98 ± 0.57 *	68.81 ± 0.27	77.16 ± 1.15 *

* Results are mean ± SD of two separate trials. Substrates were compared for each individual at two points of fermentation (ANOVA: $P < 0.01$). The consumption percentage of xylooligosaccharides was calculated taking into account the amount of XOS at 6 and 48 h with respect to the initial amount.

Notable variations in SCFA concentrations were evidenced among fecal cultures from different individuals. Because of this, data from each donor were considered separately. The highest total SCFA concentrations in cultures from the three donors at 48 h of fermentation were achieved in media containing inulin. At this time, levels of total SCFA attained with XOS were higher in cultures from individuals 2 and 3 than in cultures from individual 1; conversely, lactic acid production was more pronounced in cultures from individual 1 than in the others. Acetic acid was the most abundant SCFA formed.

Whereas a clear rise of acetic and lactic acids occurred during incubation with all substrates, the increase of both propionic and butyric acids was moderate in the case of XOS and glucose. This increase in acetic and lactic acids was directly related to the increase of bifidobacteria counts in fecal cultures with all substrates assayed, as determined by qPCR (Table 4 and Fig. 3). According to the faster degradation of XOS 2 indicated above, production of lactic, acetic and propionic acid from this substrate was in general slightly higher than from XOS 1.

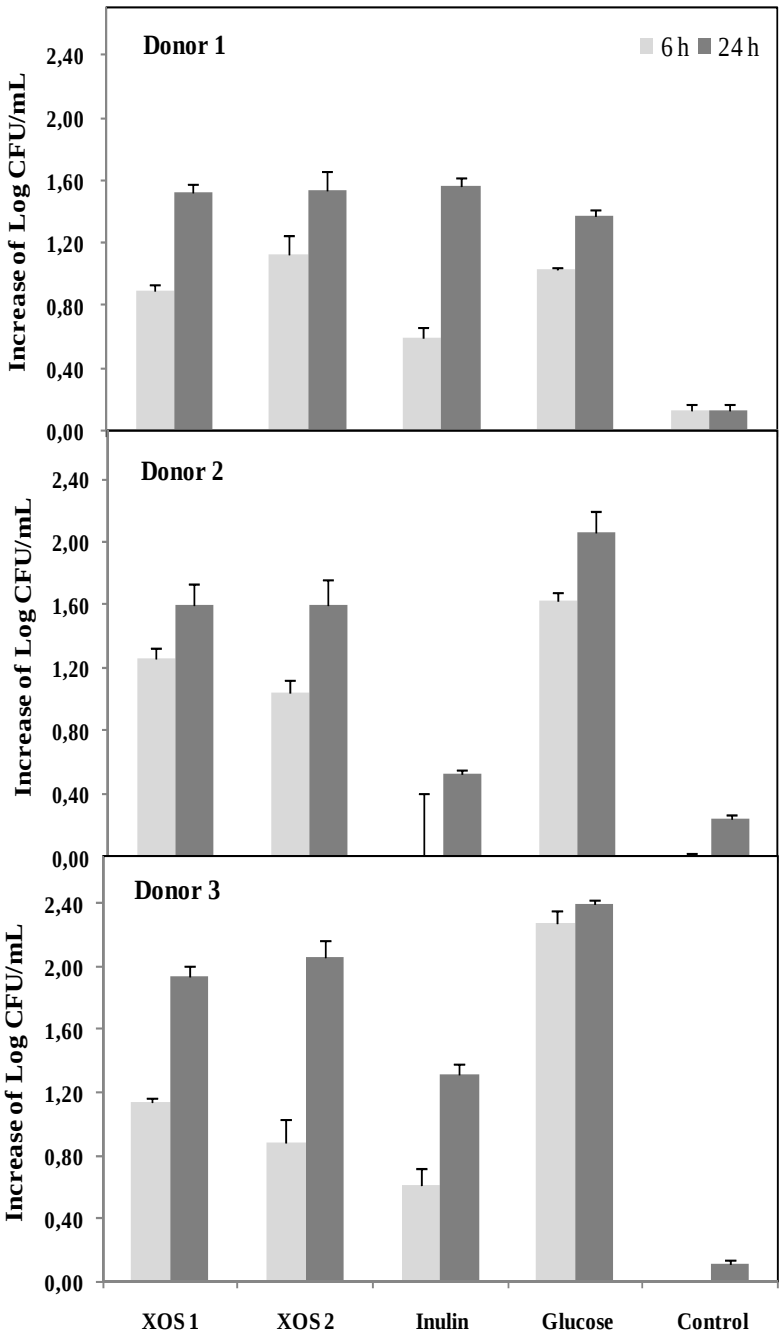

Fig. 3. Increase (with respect to time 0 h) of *Bifidobacterium* counts determined by qPCR in fecal slurry cultures from three donors using as carbon sources XOS 1, XOS 2, glucose or inulin. Data obtained after 6 h (soft grey bars) and 24 h (grey bars) of incubation. Control does not contain added carbohydrates. Initial *Bifidobacterium* counts were as follows: 8.97± 0.15 log CFU/g for donor 1, 8.48 ± 0.12 log CFU/g for donor 2, and 8.48 ± 0.09 log CFU/g for donor 3. Error bars indicate standard deviation.

Table 4. Concentrations (mM) of the Three Major SCFA and Lactate, and pH Values of Faecal Cultures from 3 Donors

Carbon source	Time (h)	Donor 1						Donor 2						Donor 3					
		pH	L	A	P	B	Total SCFA	pH	L	A	P	B	Total SCFA	pH	L	A	P	B	Total SCFA
Control	0	7.3	0.21	0.60	1.41	0.15	2.15	7.3	0.18	1.19	2.26	0.43	3.89	7.4	0.17	1.18	2.02	0.41	3.62
	6	7.1	0.87	3.84	2.26	0.52	6.63	6.9	0.00	8.15	1.91	1.65	11.72	7.0	0.26	4.55	1.99	0.75	7.29
	9	7.1	0.64	5.86	2.12	1.36	9.34	7.0	0.00	10.16	1.26	2.23	13.65	7.1	0.09	6.53	2.46	1.55	10.54
	24	7.1	0.00	10.44	2.82	2.87	16.14	7.1	0.00	12.75	1.96	3.00	17.70	7.1	0.00	10.03	2.56	2.40	15.00
	48	7.2	0.00	11.37	2.89	3.77	18.03	7.2	0.00	13.59	3.00	3.47	20.06	7.3	0.00	11.48	2.92	3.94	18.34
XOS 1	0	7.3	0.36	5.18	2.07	0.19	7.44	7.3	0.29	7.01	2.39	0.43	9.84	7.3	0.33	3.99	2.05	0.44	6.48
	6	5.1	16.59	16.50	2.77	1.29	20.56	4.9	10.14	23.64	3.87	2.72	30.22	5.3	9.66	21.16	3.87	1.83	26.86
	9	4.6	21.81	24.09	3.16	1.67	28.92	4.5	17.34	34.89	3.15	3.45	41.50	4.8	12.49	30.05	4.29	2.68	37.01
	24	4.2	31.81	37.53	3.21	2.18	42.92	4.2	24.40	47.63	3.42	4.34	55.39	4.6	13.16	42.58	4.62	4.56	51.76
	48	4.1	34.98	40.35	2.97	2.14	45.46	4.1	27.60	51.60	3.60	4.57	59.77	4.4	12.79	50.76	4.44	4.63	59.83
XOS 2	0	7.3	0.47	5.63	1.85	0.20	7.68	7.2	0.38	8.08	2.51	0.48	11.07	7.3	0.35	6.37	2.33	0.53	9.23
	6	4.8	20.39	21.77	2.85	1.52	26.14	4.9	11.32	25.85	4.14	2.90	32.89	5.1	13.02	24.12	3.60	1.89	29.61
	9	4.3	27.99	30.48	2.89	1.67	35.03	4.4	21.99	39.30	4.28	3.66	47.23	4.5	20.92	38.67	3.75	2.67	45.09
	24	4.1	36.39	40.81	3.03	1.81	45.65	4.1	28.74	50.24	4.51	4.29	59.05	4.2	24.51	49.66	3.99	3.66	57.31
	48	4.1	37.92	41.03	2.74	1.66	45.43	4.1	30.37	50.79	4.36	4.02	59.17	4.2	23.49	52.55	3.64	3.16	59.34
Inulin	0	7.3	0.28	1.79	2.06	0.20	4.05	7.2	0.19	1.72	2.29	0.36	4.38	7.4	0.21	1.61	2.06	0.41	4.08
	6	6.6	2.78	10.60	2.13	1.45	14.19	6.0	2.19	13.51	3.80	2.11	19.41	6.2	1.53	11.15	4.44	2.24	17.84
	9	6.1	2.69	15.63	2.36	3.01	21.00	5.0	5.46	30.07	5.60	4.00	39.66	5.7	2.94	17.11	8.29	3.60	29.00
	24	4.7	4.64	34.90	3.12	17.68	55.71	4.4	7.48	43.40	6.36	9.08	58.84	4.8	0.00	30.74	17.63	14.91	63.28
	48	4.5	10.30	40.69	2.60	23.41	66.70	4.4	10.12	49.46	6.14	17.75	73.34	4.6	0.00	31.66	18.53	14.92	65.12
Glucose	0	7.3	0.20	2.78	1.77	0.24	4.79	7.2	0.19	3.49	2.34	0.49	6.32	7.1	0.22	3.70	2.05	0.54	6.30
	6	4.8	15.65	18.32	2.65	1.03	22.00	4.5	8.10	17.38	1.86	1.49	20.73	4.7	13.45	27.70	3.08	1.63	32.41
	9	4.3	22.40	25.58	2.81	1.02	29.42	4.4	16.59	35.82	3.31	2.05	41.18	4.5	15.16	31.04	3.12	1.76	35.92
	24	4.0	32.51	28.27	2.58	1.19	32.05	4.3	18.37	38.33	3.44	2.14	43.91	4.3	19.02	37.19	3.16	2.18	42.52
	48	3.9	40.61	29.29	2.47	1.40	33.16	4.3	18.75	38.78	3.57	2.36	44.71	4.3	18.42	39.21	2.79	1.89	43.89

Data obtained for media containing purified XOS mixtures, inulin, and glucose as carbon sources . L= Lactate, A= Acetate, P= Propionate, B= Butyrate. Values are expressed as the mean of independent duplicate assays. The coefficient of variation (100*SD/mean) of SCFA data ranged from 0.07 to 26.56 % (donor 1), from 0.15 to 26.67 % (donor 2), and from 0.19 to 28.91(donor 3). The coefficient of variation (100*SD/mean) of pH data ranged from 0.41 to 3.14 %.

As previously indicated by other authors, the inulin resulted in higher butyrate concentrations as compared with XOS (Campbell et al. 1997). In spite of that, interesting differences were found comparing individuals at the end of fermentation; thus, cultures from individual 1, presenting the highest initial fecal levels of bifidobacteria, resulted in higher production of butyrate from inulin and lower from XOS as compared to the other individuals. Conversely, cultures from individuals 2 and 3 produced less butyrate from inulin but formed more butyrate from XOS than individual 1. A possible explanation for the different behavior among individuals is that the different initial counts and probably composition of their fecal microbiota could lead to complex metabolic interaction between gut microbial communities that could vary among donors. On the other hand, the marked pH drop in initial steps of fermentation in XOS cultures is probably due to a great stimulation of growth and/or metabolic activity of intestinal bacteria, which could have impaired at the same time the shift to butyrate production in these cultures, as indicated by Walker at al. (2005) when the pH of faecal cultures decreased to values lower than 6.0.

Finally, generation of high concentrations of acids during fermentation might be desirable because acidic environments can inhibit the growth of potentially pathogenic microorganisms and putrefactive bacteria (Gibson et al. 1995). The XOS concentrates produced in this work present a low degree of substitution, and the preference of bifidobacteria to ferment low-substituted XOS, both *in vitro* and *in vivo*, has been reported previously (Okazaki et al. 1990).

CONCLUSIONS

1. Xylooligosaccharide-containing liquors were obtained from rice huks by aqueous processing, using a reaction method suitable for achieving the partial breakdown of xylan into lower-DP products. Under the experimental conditions assayed, xylooligosaccharides (XO) were the major reaction products, accounting for more than 4% of the non-volatile solutes.
2. The reaction liquors were refined by membrane processing and ion exchange (with or without an intermediate xylanase treatment) to obtain two xylooligosaccharide (XO) concentrates of high purity (content of non-saccharide compounds, 0.009 g/g non-volatile solutes), but with different DP distribution, which were assayed as fermentation substrates.
3. XOS were rapidly degraded by the human intestinal microbiota, acting as fermentable substrates in fecal batch cultures.
4. Fermentation of XOS caused a rapid decrease of pH and an increase of the *Bifidobacterium* population, acetic and lactic acids being the main organic acids produced.
5. The XOS fermentation pattern was notably different from that of inulin, a commercial prebiotic.

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