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Manufacture and Properties of Bifidogenic Saccharides Derived from Wood Mannan

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ABSTRACT: *Pinus pinaster* wood samples were subjected to double hydrothermal processing. The liquors coming from the second stage, containing soluble saccharides of polymeric or oligomeric nature from hemicelluloses (POHs), were subjected to membrane processing (operating in discontinuous diafiltration) for refining and fractionation. Refined POH fractions were characterized by matrix-assisted laser desorption/ionization time of flight mass spectrometry and chromatographic techniques. The most complex POH component was made up of 14 hexoses and contained 4 acetyl groups. The fermentability of purified POHs by human fecal inocula was assessed by measuring both carbon source consumption and formation of short-chain fatty acids. The bifidogenic ability of POHs was confirmed by fluorescence in situ hybridization. The stimulatory effects on the bifidobacterial population reached by POHs were of the same order as those obtained with commercial fructooligosaccharides.

KEYWORDS: *autohydrolysis, FISH, fractionation, glucomannans, Pinus pinaster, prebiotics, refining, structure*

INTRODUCTION

The importance of human intestinal microbiota in maintaining host health is well-known. In the past few decades, the consumer's awareness for healthier foods has increased, boosting the interest in new prebiotics. A dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.[1] Nondigestible, fermentable carbohydrates have attracted increasing attention as prebiotics, derived from their ability to improve the bowel health of both humans and animals. These carbohydrates must resist hydrolytic digestion while being susceptible to fermentation in the large bowel, yielding short-chain fatty acids (SCFAs) and increasing the populations of beneficial bacteria (such as bifidobacteria and lactobacilli).

Nondigestible oligosaccharides (NDOs) have been the focus of interest in the development of new prebiotic ingredients from traditional and nontraditional sources, including wood. Whereas the prebiotic potential of hardwood-derived xylooligosaccharides has been assessed in depth, the prebiotic properties of saccharides derived from softwood hemicelluloses (mainly made up of glucomannans) remain almost unexplored in the scientific literature.

Pinus pinaster is the most widespread conifer species in Spain. The hemicellulosic fraction of *P. pinaster* wood is made up of a number of polysaccharides, glucomannans substituted with acetyl and galactosyl moieties being the major component. Among other polysaccharides, the presence of arabinoglucuronoxylans in pine hemicelluloses has been reported.[2] The backbone of pine mannans [including glucomannans (GMs) and galactoglucomannans (GGMs), which differ in the abundance of galactosyl substituents] is made up of randomly distributed D-glucose and D-mannose structural units linked by β (1 → 4) glycosidic bonds, which can be substituted with irregularly distributed acetyl groups.[3]

In pioneering works, GMs were isolated from *Pinus banksiana*[4] and *Pinus strobus*,[5] and the dependence of the polymer structure on the isolation procedure was pointed out. Partial hydrolysis of the polymers with formic acid led to a number of oligosaccharides keeping the general structure of the polymer.[5] Additionally, the acetylation pattern of pine GMs was established.[6]

Recently, the structure of an industrial byproduct from the fiberboad industry containing soluble products derived from loblolly pine GGMs was converted into oligomeric compounds by partial hydrolysis with trifluoracetic acid and purified by ethanol precipitation. The composition of the resulting products was characterized by a number of chromatographic, spectrometric, and spectroscopic techniques. The refined product ("temulose brown sugar") contained GGM-derived saccharides made up of a β-1,4-linked backbone of mannose and glucose residues, with occasional α-1,6-branching by single galactosyl units and possible mono- or disubstitution by acetyl groups.[7] It can be noted that the biological activity of other prebiotic oligosaccharides (for example, acetylated xylooligosaccharides from *Eucalyptus globulus* wood) lies in the presence of β (1 → 4) glycosidic bonds linking the structural units, which are responsible for their nondigestible character.

According to the literature, soluble saccharides of polymeric or oligomeric nature from pine hemicelluloses (which account for fragments of the various hemicellulosic polymers, including mannans and heteroxylan) can be produced by treatments with steam or compressed water. Hot water extraction has been employed in the isolation of pine glucomannan for structural studies and proposed as a processing stage prior to *kraft* pulping in a biorefinery approach.[8] In a recent study, the

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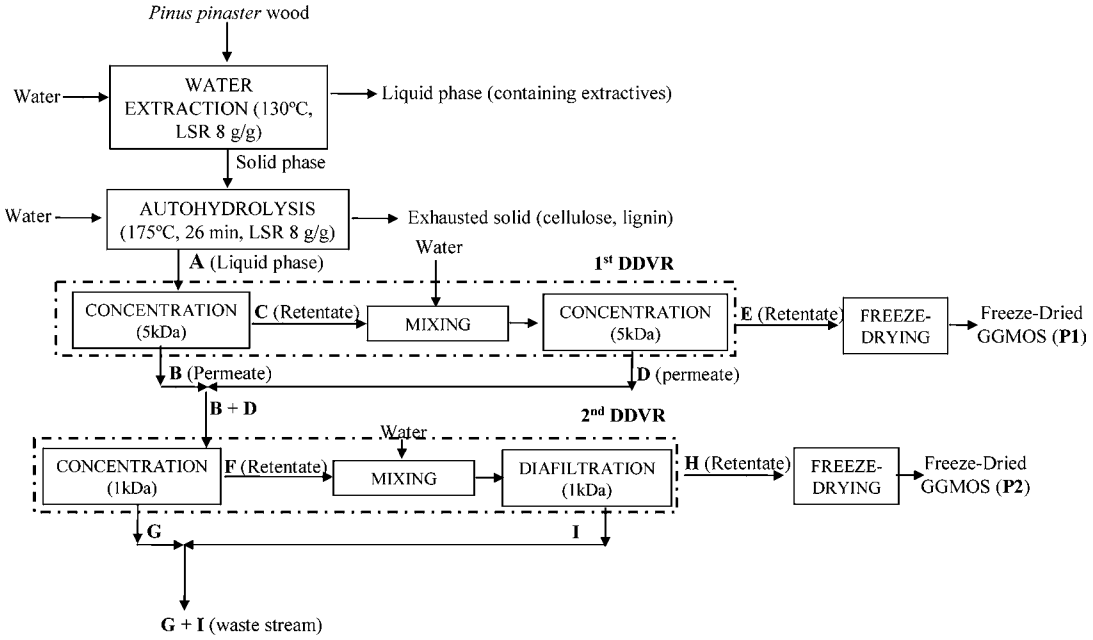

Figure 1. Scheme of the process employed for obtaining concentrates P1 and P2.

optimal conditions for producing soluble saccharides from *P. pinaster* wood hemicelluloses by processing with hot, compressed water (autohydrolysis or hydrothermal treatments) were identified.[9] Under suitable conditions, soluble saccharides were obtained at high yield and then saccharified with externally added acid to yield a mixture of hemicellulosic sugars. According to the kinetic principles governing the hydrolytic breakdown of hemicelluloses, and as has been reported for xylan-containing materials,[10,11] both the molar mass distribution and the substitution pattern of soluble products derived from hemicelluloses depend on the severity of the hydrothermal processing. Besides hemicellulose-derived poly- and oligosaccharides, the reaction liquor from hydrothermal processing contains monosaccharides and nonsaccharide compounds, which should be removed (at least in part) to improve the purity of the target compounds, particularly when food applications are envisaged. This goal can be reached by membrane processing: ultrafiltration (UF) allows the separation of suspended particles and high molar mass compounds, whereas nanofiltration (NF) can be useful for the simultaneous concentration, fractionation, and refining of the target products, particularly by removing undesired compounds of low molecular mass.[12]

The applications of mannans in the food industry are well known, and their description is out of the scope of this study. Focusing on the potential of wood-derived saccharides of polymeric or oligomeric nature from hemicelluloses (POHs; mainly made up of GGM-derived saccharides) as food additives, prebiotic properties of this type of product have been claimed in a recent patent[13] and assayed as a dietary supplement for dogs.[14] Additionally, results on the fermentation of the same products with dog feces have been reported.[15]

In this work, POHs (including soluble saccharides from mannans and xylans) were manufactured by autohydrolysis of *P. pinaster* wood and refined using membranes. The purification effects reached by treatments were measured, and refined POHs were assayed for prebiotic properties (including generation of SCFAs and bifidogenic potential) by in vitro fermentations.

MATERIALS AND METHODS

Raw Materials. *P. pinaster* wood samples (kindly provided by Orember-Finsa, Ourense, Spain) were air-dried, milled in a 40 kW mill (ATI, La Coruña, Spain) to pass an 8 mm screen, and homogenized in a single lot.

Autohydrolysis Conditions. Milled wood was subjected to two sequential aqueous treatments (Figure 1). In the first one, performed to remove water-soluble extractives, *P. pinaster* wood was mixed with distilled water (at a liquid to solid mass ratio of 8 g of water/g of oven-dried wood) in a stirred, stainless steel reactor (Parr Instrument Co.). The reactor was heated to 130 °C (time needed for heating starting from 60 °C, 15.4 min), and then it was immediately cooled to 40 °C by internal refigeration. Solids were separated by filtration, washed, air-dried, and subjected to a second aqueous treatment (in the same reactor using the same liquid to solid ratio) at 175 °C for 26 min.[9] Once the reaction time had elapsed, the reactor was cooled, and the liquor was recovered by filtration, analyzed, and processed.

Processing of Autohydrolysis Liquors: Membrane Processing. The autohydrolysis liquor was treated in a stirred Amicon cell (Millipore). Ultrafiltration and nanofiltration were carried out using regenerated cellulose membranes of 5 and 1 kDa cutoff (Millipore) with a filtration area of 41.8×10^{-4} m^2. The operating pressure was fixed at 4 bar, as enhanced oligosaccharide retention under these conditions was observed in preliminary experiments (data not shown). Pressure was provided by a compressed nitrogen gas cylinder, measured using a gauge attached to the nitrogen line, and controlled by a regulator. The solution was stirred continuously with a magnetic bar at about 110 rpm, avoiding the formation of a deep vortex. All experiments were carried out at room temperature.[16]

The processing scheme followed in this work is shown in Figure 1. The autohydrolysis liquor (stream A) was filtered using a 5 kDa cutoff membrane operating in concentration mode to achieve the desired volume reduction to separate the high molecular fraction as a retentate. For further purification, water was added to the retentate to restore the initial volume, and the resulting solution was concentrated (discontinuous diafiltration) using the same membrane. The refined retentate, containing POHs of higher molecular mass (stream E), yielded concentrate P1 after freeze-drying. The two permeates were mixed (to yield stream B + D) and subjected to concentration with the 1 kDa membrane to remove undesired low molecular mass compounds. The retentate from this stage was supplemented with water (up to the initial volume) and filtered through the same

membrane to reach the desired concentration degree to obtain a retentate rich in POHs of lower molecular mass (stream H, which yielded concentrate P2 after freeze-drying). The different streams were assayed for composition. Concentrates P1 and P2 were used as substrates for in vitro fermentations.

Fermentation of POH Concentrates. *Fecal Inocula.* Fecal samples were obtained from three healthy human volunteers, who usually ingested a normal diet, presented no digestive diseases, and did not receive antibiotics for at least 3 months. Feces were collected in sterile vials, kept in an anaerobic cabinet, and used within a maximum of 2 h after collection. Fecal inocula (FI) were prepared by dilution in a reduced physiological salt solution (RPS; cysteine−HCl, 0.5 g/L, and NaCl, 8.5 g/L) at 100 g of feces/L of RPS and pH 6.8, following the methodology described elsewhere.[16]

Fermentation Media. The nutrient base medium used in fermentations was modified according to Jaskari et al.:[17] a solution containing 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, MD), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, U.K.), 5.0 g/L yeast nitrogen base (YNB; Difco, Detroit, MI), 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany), 1.0% (v/v) salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2{\cdot}6H_2O$, 10.0 g/L $CaCl_2{\cdot}2H_2O$), trace mineral solution, 0.2% (v/v) salt solution B (200.0 g/L $K_2HPO_4{\cdot}3H_2O$), and 0.2% (v/v) 0.5 g/L resazurin solution was prepared in distilled water. The final pH of the medium was adjusted to 6.8. After deoxygenation, 8.0 mL aliquots were dispensed into airtight anaerobic culture tubes, which were sealed with aluminum caps before autoclave sterilization. Stock solutions of YNB, POH concentrates, and glucose (Sigma, St. Louis, MO) were sterilized through 0.2 μm Chromafils filters (Macherey-Nagel, Düren, Germany) into sterile airtight serum bottles. All additions and inoculations were carried out inside an anaerobic cabinet (5% H_2, 10% CO_2, and 85% N_2).

Before inoculation, both YNB and carbohydrate solutions were aseptically added to the anaerobic culture tubes with nutrient base medium (using syringes and needles under anaerobic conditions in an anaerobic cabinet), to achieve final concentrations of 5 and 10.0 g/L, respectively. The tubes with the fermentation media were inoculated with 0.2 mL of fecal slurry dilution (2%, v/v) prepared in duplicate as described above and incubated at 37 °C for 48 h without shaking. Cells were harvested from samples by centrifugation, and supernatants were filtered for HPLC analysis. The pH of the tube contents was measured with a standard pH meter.

Quantitation of Bifidobacteria in Batch Cultures by Fluorescent in Situ Hybridization (FISH). Quantitation of bifidobacteria in fecal batch fermentations was performed by FISH[16] using *Bif*164 probes targeting specific regions of the 16S rRNA gene labeled with fluorescent dye Cy3 (Sigma).

At fixed fermentation times, samples were centrifuged at 15000*g* for 2 min to separate particulate matter and fixed overnight in paraformaldehyde (4 g of paraformaldehyde/100 g of solution) at 4 °C (volume ratio of sample to paraformaldehyde solution, 1:3). Cells were washed with phosphate-buffered saline (0.1 M, pH 7.0), resuspended in 150 μL of phosphate-buffered saline plus 150 μL of ethanol, and stored at −20 °C for at least 1 h before further processing. In 1.5 mL microcentrifuge tubes, 200 μL of filtered hybridization buffer (40 mmol/L Tris−HCl, pH 7.2, 1.8 mol/L NaCl, and a 20 mL/L solution containing 100 g of sodium dodecyl sulfate/L), 64 μL of deionized distilled water, and 16 μL of the fixed cells were added. In a 0.5 mL microcentrifuge tube, 5 μL of the probe (50 ng/μL) was mixed with 45 μL of the above hybridization solution, and the mixture was shaken and incubated overnight at 50 °C.

In a 10 mL centrifuge tube, 5.0 mL of prewarmed hybridization buffer (20 mmol/L Tris−HCl, pH 7.2, 0.9 mol/L NaCl) and 20 μL of 4′,6-diamidino-2-phenylindole (500 ng/mL) were added. The mixture was returned to the oven at the adequate temperature for 30 min. The washing mixture was filtered through a 0.20 μm Nuclepore Policarbonate filter (Whatman, Kent, U.K.) under vacuum. After addition of 5 μL of poly(vinyl alcohol) mounting medium with 1,4-diazabicyclo[2.2.2]octane antifade reagent (Sigma), the filters were placed on slides and covered with a coverslip. The slides were stored in the dark at 4 °C for a maximum of 3 days and were examined during this period using an epifluorescence microscope (Olympus BX41) equipped with Fluor 100 lenses. FISH counts were made in duplicate.

Analytical Methods. Samples of liquors were filtered through 0.45 μm cellulose acetate membranes, neutralized with barium carbonate, and assayed by HPLC for glucose, xylose, mannose, galactose, and arabinose using a 1100 series Hewlett-Packard chromatograph fitted with a refractive index detector operated at 50 °C and a 300 × 7.8 mm CARBOsep CHO 682 column (Transgenomic, Glasgow, U.K.) operating at 80 °C. Distilled water was used as the mobile phase (flow rate 0.4 mL/min). Acetic acid was determined by HPLC using a 300 × 7.8 mm Aminex HPX-87H column (BioRad, Hercules, CA) operated at 60 °C (mobile phase 0.003 M H_2SO_4, flow rate 0.6 mL/min).

The concentrations of POHs and linked acetyl groups were determined from the concentrations of monosaccharides and acetic acid present in liquors previously subjected to a quantitative posthydrolysis (treatment with 4% sulfuric acid at 121 °C for 20 min). Before analysis on a CARBOsep CHO 682 column, posthydrolysis samples were neutralized with barium carbonate. Uronyl substituents were determined by a previous method[18] using galacturonic acid as a standard for quantitation. All analyses were made in triplicate. The content of nonvolatile compounds (NVCs) in the liquors was measured by oven-drying at 105 °C until constant mass was achieved.

Supernatants from the anaerobic culture tubes inoculated with FI were filtered through 0.20 μm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (lactic, acetic, formic, propionic, and butyric acids) by HPLC-RI using an Aminex HPX-87H column (BioRad) using the method described above. Monosaccharides and total oligosaccharides were determined by HPLC using a CARBOsep CHO 682 column (Transgenomic) using the method described above.

High-Performance Size Exclusion Chromatography (HPSEC). The molecular mass distribution of the target products was determined by HPSEC using two 300 × 7.8 mm TSKGel G3000PWXL columns in series (Tosoh Bioscience, Stuttgart, Germany), in combination with a 40 × 6 mm PWX-guard column, operating at 30 °C with a refractive index detector. The mobile phase was distilled water (flow rate 0.6 mL/min). Dextrans (1000−80000 g/mol) from Fluka (parent company of Sigma-Aldrich) were used as calibration standards.

High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Samples were analyzed by HPAEC-PAD using an ICS3000 chromatographic system (Dionex, Sunnyvale, CA), equipped with a 250 × 2 mm CarboPac PA-1 column in combination with a 25 × 2 mm CarboPac PA guard column and an ISC3000 PAD detector. A flow rate of 1 mL/min was used with mobile phases A (distilled water), B (0.2 M NaOH), and C (2 M NaOAc in 0.2 NaOH). Elution was done with the following program: 88% A/12% B isocratic for 20 min, then to 50% A/50% B in 5 min (concave gradient), then to 50% A/48% B/2% C in 25 min (concave gradient), then to 50% A/45.5% B/4.5% C in 10 min (concave gradient), then to 50% A/43% B/7% C in 25 min (linear gradient), and then to 50% A/50% C in 10 min (linear gradient). Before the next analysis, the column was equilibrated with the initial mobile phase concentration for 20 min. Galactomannan-derived and mannan-derived oligosaccharides (degree of polymerization (DP) from 2 to 6) from Megazyme (Wicklow, Ireland) were used as standards.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). MALDI- TOF analyses were performed using an Ultraflex workstation (Bruker Daltonics) equipped with a 337 nm nitrogen laser. Measurements were performed in the positive mode. After a delayed extraction time of 150 ns, ions were accelerated with a 25 kV voltage. Data were collected from 100 laser shots using the lowest energy necessary to obtain sufficient spectral intensity. The mass spectrometer was calibrated with a mixture of galactomannan-derived and mannan-derived oligosaccharides (DP = 2−6) from Megazyme.

For sample preparation, 1 μL of sample solution (1 mg/mL) was mixed with 1 μL of matrix (10 mg/mL 2,5-dihydroxybenzoic acid from Bruker Daltonics, Bremen, Germany) in 30% acetonitrile (v/v), directly applied on the MS target plate, and dried under a stream of warm air.

RESULTS AND DISCUSSION

Autohydrolysis of *P. pinaster* wood. *P. pinaster* wood contains water-soluble extractives, which are transferred to the liquid phase along the autohydrolysis process. As this type of extractable material hinders the subsequent POH purification and fractionation, wood was first subjected to an aqueous extraction under mild conditions to leave hemicelluloses unaltered in the solid phase, and the resulting solids were employed as a substrate for autohydrolysis (performed under conditions leading to hemicellulose solubilization). According to the literature,[9] aqueous extraction was carried out in nonisothermal mode up to 130 °C, whereas autohydrolysis was performed at 175 °C for 26 min (conditions that allowed a high yield of POHs with minimal generation of sugars and sugar-degradation compounds such as furfural and hydroxymethylfurfural). The reaction liquors contained water, volatile compounds (VCs) generated from the feedstock (mainly acetic acid), and NVCs, including POHs, sugars, and nonsaccharide compounds. Table 1 shows the composition of autohydrolysis liquors: POHs accounted for 0.776 g/g of NVCs, whereas monosaccharides accounted for 0.180 g/g of NVCs. POHs included compounds made up of glucosyl units, xylosyl units, galactosyl units, arabinosyl units, and mannosyl units, with uronic acid and acetyl group substituents (see footnote *a* of Table 1 for nomenclature). On the other hand, the limited concentrations of monosaccharides and the high conversion of hemicelluloses into POHs (89.6%) confirmed the suitability of the autohydrolysis conditions for the purposes of this study.

Table 1. Composition of Streams A, E, and H in Figure 1[a]

component	component concn (kg of component/kg of NVCs)		
	stream A	stream E	stream H
glucose	0.006	0.000	0.002
xylose	0.064	0.002	0.003
galactose	0.026	0.001	0.002
arabinose	0.070	0.004	0.006
mannose	0.014	0.000	0.001
acetic acid	0.012	0.001	0.001
GlPOHs (as glucose)	0.092	0.137	0.135
XyPOHs (as xylose)	0.113	0.033	0.100
GaPOHs (as galactose)	0.103	0.107	0.145
ArPOHs (as arabinose)	0.007	0.003	0.005
MaPOHs (as mannose)	0.376	0.600	0.560
AcPOHs (as acetic acid)	0.055	0.075	0.075
UAPOHs (as galacturonic acid)	0.030	0.013	0.023
ONVCs (by difference)	0.044	0.026	0.000

[a]Nomenclature: AcPOHs, acetyl group substituents in hemicellulose-derived products; ArPOHs, arabinosyl units in hemicellulose-derived products; GaPOHs, galactosyl units in hemicellulose-derived products; GlPOHs, glucosyl units in hemicellulose-derived products; MaPOHs, mannosyl units in hemicellulose-derived products; UAPOHs, uronic acid groups in hemicellulose-derived products; XyPOHs, xylosyl units in hemicellulose-derived products. Volumetric concentrations of NVCs in streams A, E, and H were 23.7, 39.0, and 29.7 g/L, respectively.

The fraction denoted as ONVCs (other nonvolatile compounds; mass fraction 0.044 g/g of NVCs) accounted for undesired, nonsaccharide compounds.

Membrane Processing of Autohydrolysis Liquors. Membranes are potentially suitable for POH refining, operating in ultra- and/or nanofiltration. Components with a molecular size larger than that of the membrane pore can be separated from other components that can permeate the membrane, enabling the fractionation of components. The application of membrane technology for processing autohydrolysis liquors from a number of raw materials has been considered in the literature.[11,19]

Figure 1 shows the processing scheme followed in this work, which was carried out with a dead-end filtration device operating in ultra- and nanofiltration in discontinuous diafiltration with reduction of volume (DDRV). Using this approach (also named "intermittent feed diafiltration"), the sample is first concentrated to a predetermined volume and then diluted back to its original volume with water. Finally, a postconcentration step is applied to achieve the final volume.

The first DDRV was carried out using a membrane with a 5 kDa cutoff and intended the removal of sugars and low molecular mass ONVCs, which are unwanted for prebiotic applications. The degree of concentration achieved in each stage was measured in terms of the volume concentration ratio (VCR), defined as

$$\mathrm{VCR} = \left(\frac{V_f}{V_r}\right) = \left(\frac{V_f}{V_f - V_p}\right)$$

where V_f is the volume of the feed, V_r is the volume of the retentate, and V_p is the volume of the permeate. The first DDRV included two stages, which were performed to achieve VCR = 9. The retentate, containing POHs of higher DP, was freeze-dried (to yield concentrate P1), whereas the permeates were mixed and processed in a second DDVR step (performed with the 1 kDa membrane) to remove low molecular mass, undesired compounds (particularly sugars). The second DDRV again included two stages, which were performed to reach VCRs of 9 and 7.65, respectively. The retained solution was freeze-dried to yield concentrate P2, which is expected to have a composition closely related to that of P1, except for the lower DP distribution.

First Discontinuous Diafiltration with Reduction Volume. The autohydrolysis liquors (stream A) (Figure 1) were subjected to ultrafiltration in concentration mode through a 5 kDa membrane to yield the retentante C, which was diluted with water and concentrated again under the same conditions. The resulting permeates (streams B and D) (Figure 1) were mixed to yield the stream denoted B + C, whereas concentrate E (which yielded P1 after freeze-drying) presented the composition listed in Table 1.

In comparison with stream A, stream E was almost free from monosaccharides (with about 99% removal), whereas the recoveries of POH components (denoted as shown in footnote *a* of Table 1) were 26.1% for GlPOHs, 5.06% for XyPOHs, 18.2% for GaPOHs, 6.7% for ArPOHs, 27.8% for MaPOHs, 23.6% for AcPOHs, and 7.81% for UAPOHs. Overall, the recovery of the target compounds in stream E was 21.7% of the amount present in stream A, yielding concentrate P1 of high purity (with 0.97 g of target compounds/g of NVCs).

Second Discontinuous Diafiltration with Reduction Volume. The second DDRV involved the processing of the B

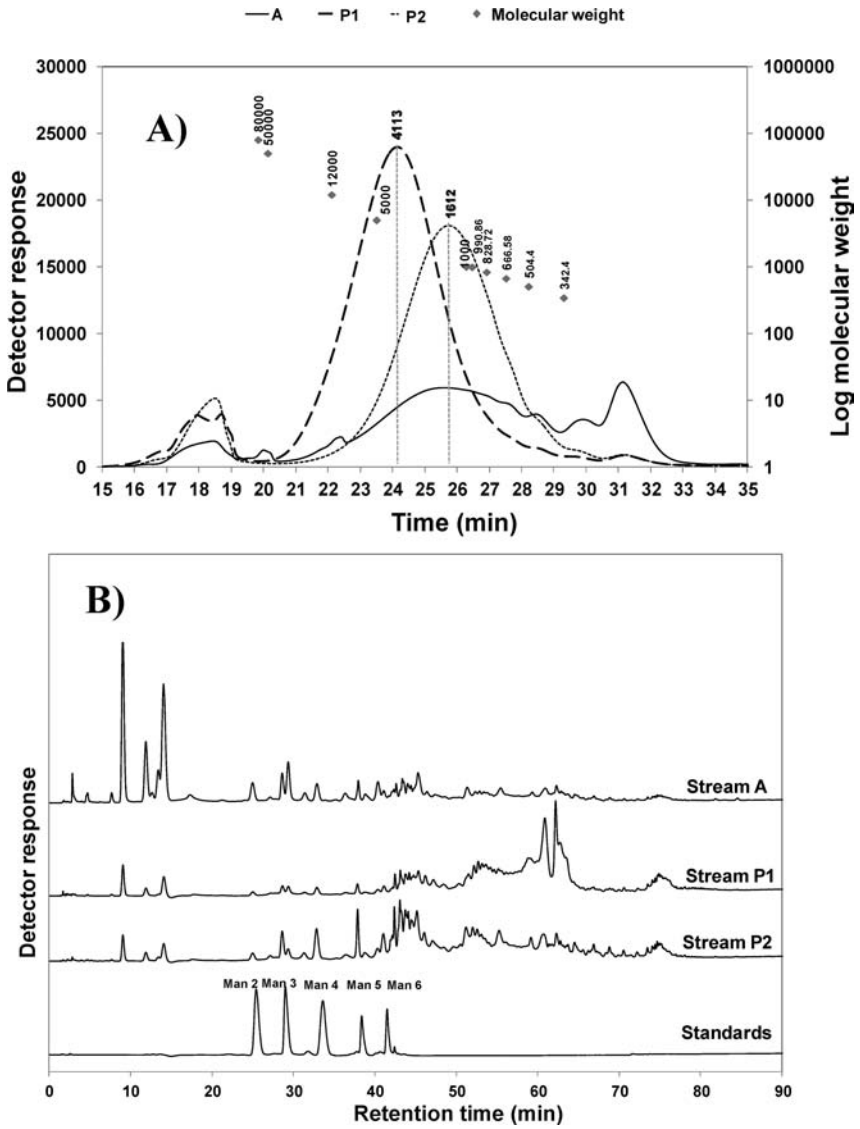

Figure 2. Elution profiles of streams A, P1, and P2: (A) HPSEC, (B) HPAEC-PAD.

+ D stream with the 1 kDa cutoff membrane (Figure 1), following the same sequence concentration−diafiltration described in the previous section. With respect to stream A, the mixed B + D stream contained 89.5% of the total monosaccharides, 64.9% of GlPOHs, 100% of XyPOHs, 83.1% of GaPOHs, 88.0% of ArPOHs, 74.1% of MaPOHs, 65.1% of AcPOHs, and 87.2% of UAPOHs, whereas the recovery of ONVCs was close to 100%. Overall, the percentage of target compounds recovered in streams B + D (including GlPOHs, XyPOHs, GaPOHs, ArPOHs, MaPOHs, AcPOHs, and UAPOHs) accounted for 77.6% of the amount present in stream A. The second DDVR yielded retentate H and permeates G and I. Concentrate P2 was obtained by freeze-drying stream H.

The composition of stream H (Table 1) enabled the calculation of the following recoveries with respect to stream A: 2.1% for total monosaccharides, 40.8% for GlPOHs, 24.6% for XyPOHs, 39.1% for GaPOHs, 19.1% for ArPOHs, 41.2% for MaPOHs, 37.8% for AcPOHs, and 21.8% for UAPOHs. The overall recovery of target compounds (including GlPOHs, XyPOHs, GaPOHs, ArPOHs, MaPOHs, AcPOHs, and UAPOHs) in stream H accounted for 37.3% of the amount present in stream A, yielding concentrate P2 of high purity, with nearly 1 g of target compounds/g of NVCs.

The above results confirm that the scheme depicted in Figure 1 allowed the manufacture of refined products of high purities (which compare favorably with the reported purities for commercial oligosaccharides[3]) at high yield: if the two fractions P1 and P2 were combined, 59% of the target compounds present in stream A were recovered, whereas the rest (together with monosaccharides and nonsaccharide compounds) appear in the waste stream G + I. This latter solution could be subjected to further processing to recover other products (such as monosaccharides and/or extractive-derived compounds).

Chemical Characterization of POHs. The elucidation of the structural features of soluble saccharides is essential to understand their biological properties (including the potential prebiotic effect).[20] In this work, the type and relative proportions of structural units and substituents making part of the POHs are shown in Table 1. Looking for additional information on the molar mass distribution and structural features of POH, additional experimental work was carried out using HPSEC, HPAEC-PAD, and MALDI-TOF-MS.

Table 2. Degree of Consumption (%) of POHs along in Vitro Fermentations Using Fecal Inocula from Three Human Donors[a]

	donor 1				donor 2				donor 3			
time (h)	GlPOHs	XyPOHs	GaPOHs	MaPOHs	GlPOHs	XyPOHs	GaPOHs	MaPOHs	GlPOHs	XyPOHs	GaPOHs	MaPOHs
					Degree of Consumption of Fraction P1							
7	17.5	62.8	59.8	24.7	36.9	87.2	76.5	36.4	37.4	83.2	88.3	27.0
11	55.6	70.3	76.0	79.2	67.2	93.7	81.2	78.8	81.0	85.9	92.8	77.8
29	87.8	79.3	86.5	94.3	100.0	100.0	89.5	100.0	100.0	100.0	100.0	100.0
45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
					Degree of Consumption of Fraction P2							
7	19.9	77.8	73.5	17.6	22.0	76.4	82.8	11.7	23.0	79.6	69.8	7.6
11	47.2	83.6	85.0	73.6	82.0	93.0	93.4	87.2	84.4	89.8	92.2	78.6
29	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]Nomenclature as in Table 1.

Figure 2A shows the HPSEC chromatograms determined for streams A, P1, and P2. Although the elution profiles are complex (owing to the multiple saccharides in solution, which include backbones made up of hexoses or pentoses with different substitution patterns), the elution profiles showed the refining effects caused by the membrane processing. For example, the removal of monosaccharides and nonsaccharide compounds from stream A can be observed in the profiles determined for P1 and P2 through the different sizes of peaks eluted after 29 min and in the range of 17−20 min, respectively. On the other hand, the effects of the fractionation by the 5 and 1 kDa membranes can be seen from the fairly symmetrical elution chromatograms determined for the retentates, with a higher average DP for P1 than for P2.

HPAEC-PAD analysis of the samples (Figure 2B) showed the presence of linear oligosaccharides (up to DP 5) and some complex substituted oligomers. Again, the differences among the chromatograms obtained for the raw liquor and for purified products confirmed the refining effects caused by membrane processing, as well as the different DP distributions of P1 and P2. It can be noted that no conclusions on acetylation can be drawn from HPAEC-PAD data, as the samples are saponified by the alkaline mobile phase.

A deeper insight into the structure of POHs with medium to high DP was obtained from MALDI-TOF-MS data. Although compounds with m/z below 600 could not be analyzed (due to interference with matrix peaks), it can be noted that the presence of these compounds was already confirmed by HPAEC. The MALDI-TOF-MS spectra of the sodium adducts determined for concentrate P1 confirmed the presence of a wide range of POH components. The data of Table 1 suggest that hexoses correspond mainly to mannose. POH components made up of hexoses, mainly from GM or GGM breakdown, were substituted with one or more acetyl groups, giving a series of neutral (acetylated) oligomers [H_nAc_m] with DP in the range of 3−14. The most complex POH components were a DP = 13 saccharide bearing five acetyl groups and a DP = 14 saccharide bearing four acetyl groups. Other POH components are made up of pentoses, and (according to the compositional data in Table 1 and literature information) they come from heteroxylan (with a backbone made up of xylose units). POHs derived from xylan included a homologous series of compounds with DP = 5−7 and an *O*-methylglucuronylated DP = 3 oligomer. The general findings from the MALDI-TOF data are in agreement with reported data.[7]

Assessment of the Prebiotic Potential of POH Concentrates P1 and P2. In the past few decades, increasing interest has been paid to the prebiotic effect of various nondigestible oligosaccharides, including xylooligosaccharides,[11] pectooligosaccharides,[17] and mannooligosaccharides.[7] In several cases, the studies were based on in vitro fermentations with animal or human fecal inocula. However, scarce data have been reported on the in vitro fermentation of soluble saccharides derived from GMs or GGMs using human fecal bacteria. Albrecht et al.[20] studied the influence of structural characteristics of konjac glucomannan oligosaccharides on their in vitro fermentability by human gut microbiota. Previously, Matsuura et al.[21] found that konjac glucomannan was almost completely degraded by the simultaneous action of enzymes and intestinal anaerobic bacteria present in human feces, producing formic acid, acetic acid, propionic acid, and 1-butyric acid. The fermentation of soluble saccharides derived from pine wood hemicelluloses has been considered recently.[14,15] In this work, concentrates P1 and P2 (containing POHs derived from GGMs, GMs, and xylan) were tested for prebiotic potential by in vitro fermentation. On the basis of the predominance of mannan-derived saccharides, the contribution of xylan-derived saccharides to the prebiotic properties of concentrates P1 and P2 is considered of minor importance.

Experiments were performed using human inocula from three healthy donors according to the methods reported in the literature. For comparative purposes, additional results were determined for media containing for fructooligosaccharides (FOS) (selective control, selected as model NDOs due to their well-established prebiotic properties[22]) and for media without a carbon source (negative control).

The prebiotic potential of POHs was studied on the basis of the substrate assimilation, SCFA and lactate accumulation, pH shifts, and evolution of the bifidobacterial population along fermentation experiments.

Consumption of POHs in Fecal Cultures. The data concerning the consumption of P1 and P2 along fermentations are listed in Table 2. Both concentrates were extensively metabolized by the bacteria present in the intestinal microbiota, but differences in the degradation pattern between individuals were observed. The dynamics of POH consumption along the incubation time was in good agreement with the SCFA concentration profiles. During the first 7 h of incubation, saccharides consisting of XyPOHs and GaPOHs were preferentially consumed by cultures from the three donors, although the degrees of consumption were different: about 80% of the cited POH components were assimilated in experiments from donors 2 and 3, whereas slightly lower results were achieved in media from donor 1. The consumption of glucosyl

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Table 3. Concentrations (mM) of the Three Major SCFAs and Lactate and pH Values of Fecal Cultures[a]

		donor 1						donor 2						donor 3					
carbon source	time (h)	pH	L	A	P	B	SCFAs	pH	L	A	P	B	SCFAs	pH	L	A	P	B	SCFAs
control	0	6.8	0.0	0.0	0.1	0.0	0.1	7.7	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.0	0.0	0.0	0.0
	4	6.8	0.1	0.0	2.3	0.4	2.7	7.7	0.0	2.8	0.8	0.3	3.9	6.8	0.0	0.0	0.5	0.0	0.5
	7	6.7	0.0	5.4	2.5	1.4	9.3	7.5	0.0	7.7	1.7	1.3	10.7	6.7	0.0	0.2	0.5	1.1	1.8
	11	6.7	0.0	12.2	1.8	3.4	17.3	6.5	0.0	17.3	3.9	4.0	25.2	6.7	0.0	9.7	0.0	2.9	12.7
	21	6.8	0.0	18.8	3.5	4.8	27.2	6.8	0.0	27.0	5.9	4.9	37.8	6.6	0.0	13.7	0.6	4.7	18.9
	29	6.7	0.0	22.4	3.8	5.9	32.2	6.5	0.0	31.1	8.7	5.0	44.8	6.7	0.0	13.9	1.2	5.5	20.6
	45	6.6	0.0	32.5	4.3	6.6	43.5	6.5	0.0	34.0	8.0	5.6	47.7	6.6	0.0	21.9	1.5	5.2	28.6
P1	0	6.8	0.0	0.0	0.1	0.0	0.1	7.7	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.2	0.0	0.0	0.0
	4	6.6	0.4	4.8	0.5	0.6	5.9	7.3	0.6	12.0	3.5	0.0	15.5	6.5	0.0	9.0	2.7	0.0	11.8
	7	6.2	0.1	23.4	1.4	3.6	28.4	6.0	0.0	31.6	10.0	4.1	45.7	6.3	0.7	33.7	8.8	3.9	46.5
	11	5.2	3.1	45.8	2.1	11.2	59.1	5.2	0.3	56.2	19.2	10.9	86.4	5.1	0.0	61.3	19.3	10.8	91.4
	21	4.8	3.6	51.8	4.2	19.3	75.3	4.7	0.0	66.5	22.9	16.6	106.1	4.9	0.3	63.0	21.2	14.8	99.0
	29	4.8	0.0	57.1	7.3	25.5	89.9	4.7	0.0	68.9	23.4	17.9	110.2	4.9	0.0	62.7	19.0	14.2	95.9
	45	4.8	0.0	59.2	10.9	28.6	98.8	4.7	0.0	69.4	23.2	18.3	111.0	4.8	0.0	73.2	21.7	17.0	111.9
P2	0	6.8	0.0	0.0	0.1	0.0	0.1	7.7	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.0	0.0	0.0	0.0
	4	6.6	0.4	0.0	0.2	0.0	0.2	7.2	2.1	11.9	0.7	1.2	13.8	6.4	3.6	13.5	1.0	1.1	15.6
	7	6.1	0.6	25.3	1.8	3.7	30.8	6.0	0.3	33.3	3.4	4.4	41.1	6.2	3.2	31.1	2.6	3.5	37.2
	11	5.4	1.0	50.0	3.5	12.0	65.5	5.2	0.0	59.7	9.9	13.0	82.6	5.1	0.0	63.7	8.3	16.0	87.9
	21	5.0	0.0	59.8	6.2	18.5	84.6	4.8	0.0	67.2	13.7	18.3	99.3	5.0	0.0	64.2	9.5	18.1	91.7
	29	5.0	0.0	59.7	8.0	18.4	86.2	4.6	0.0	61.3	13.0	18.1	92.4	5.0	0.0	65.7	9.9	18.7	94.3
	45	5.0	0.0	64.4	11.5	20.1	95.9	4.7	0.0	72.1	15.7	22.7	110.4	5.0	0.0	75.8	10.5	21.1	107.3
FOS	0	6.8	0.0	0.0	0.1	0.0	0.1	7.7	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.0	0.0	0.0	0.0
	4	6.8	1.0	3.7	0.7	0.6	5.0	7.7	1.0	8.2	2.8	0.0	10.9	6.8	1.6	11.1	0.7	1.2	13.0
	7	6.6	8.7	22.1	1.6	6.4	30.0	7.3	6.1	30.2	6.8	5.0	41.9	6.5	7.8	42.5	2.8	5.5	50.9
	11	6.0	25.9	33.5	2.4	17.2	53.1	5.6	8.4	43.8	10.3	12.7	66.8	5.8	7.8	65.8	3.9	16.8	86.5
	21	4.8	21.6	30.2	1.2	17.9	49.3	4.6	0.0	62.7	12.8	23.1	98.6	5.0	0.0	60.3	4.4	26.2	90.9
	29	4.8	0.0	29.9	3.3	20.9	54.1	4.6	0.0	62.7	14.1	22.9	99.7	5.0	0.0	62.6	4.2	24.7	91.6
	45	4.9	0.0	30.6	7.3	29.0	66.9	4.6	0.0	54.4	11.6	23.0	89.0	5.2	0.0	65.0	4.8	27.4	97.2

[a]Data obtained for media containing concentrates (P1 or P2) or FOS as the carbon source. Nomenclature: L, lactate; A, acetate; P, propionate; B, butyrate. Values are expressed as the mean of independent duplicate assays.

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and mannosyl units in POHs occurred principally in the period from 7 to 11 h for experiments from donors 1 and 3 using P1 as the carbon source, whereas in the fermentation using P2 as the substrate, the cultures from donors 2 and 3 resulted in the highest consumption of POHs made up of glucosyl and mannosyl units. In the fermentation using P1 as the carbon source and inocula from donor 2, consumption of glucosyl and mannosyl POHs took place mainly in the first 11 h. For donor 1 and fraction P2, a preferential assimilation of mannosyl units occurred in the period from 7 to 11 h. From the experimental data, it can be concluded that the major utilization of POH components took place during the first 11 h of incubation, causing a correspondent increase in the production of SCFAs (see below). Total consumption of the substrate was observed after 29 h of fermentation, except in the experiment using P1 as the carbon source and inocula from donors 1 and 2 (which needed 45 h to reach complete and incomplete substrate conversion, respectively). The experimental data did not show significant effects associated with the differences in molecular mass distribution between P1 and P2, as the expected influence on the assimilation kinetics was overcome by individual differences among donors and by the experimental error.

SCFA and Lactate Production in Fecal Cultures. Table 3 shows the concentrations of SCFAs and lactate along fermentations and the pH shifts resulting from the generation of acids. The type of metabolic products confirmed that POHs were metabolized by the various species present in the fecal microbiota.[23]

Both increases in SCFA concentration and pH drops along incubation were considerably more pronounced in samples containing carbohydrates than in negative control cultures. It can be noted that SCFA generation in cultures lacking a carbon source is due to degradation of protein by putrefactive bacteria present in the intestinal microbiota. Consequently, low SCFA generation in blanks is expected.[24]

Two stages can be distinguished in POH fermentations: in the first one (lasting about 11 h), pH dropped significantly due to the growth and/or metabolic activity of intestinal bacteria and then remained fairly constant to the end of the experiments (see Table 3).

In agreement with literature reports,[11] the SCFA concentration profiles were markedly different in either experiment using the same carbon source and inocula from different individuals or in cultures with different carbon sources inoculated with samples from the same donor (see Table 3). After 45 h of fermentation, the total SCFA concentrations in cultures from the three donors were similar in media made up of either P1 or P2, achieving concentrations higher than those determined in media containing FOS. At the same fermentation time, the concentrations of total SCFAs achieved in media containing P1 or P2 were higher in cultures inoculated with inocula from donors 2 and 3 than from donor 1.

Lactic acid, a typical metabolic product of bifidobacteria and lactic bacteria, was more abundant in cultures from individual 1 using P1 and FOS as carbon sources, whereas in experiments made with P2, the highest lactic acid production corresponded to cultures with inocula from individuals 2 and 3. In all cases, lactic acid reached low concentrations at the beginning of the fermentations and disappeared rapidly.

Acetic acid was the major SCFA found in media from all individuals with all substrates. The highest acetate concentrations were achieved in media containing P2 as the carbon source, followed by the media made with P1 and FOS. Among donors, donor 3 achieved the highest acetate concentration for all substrates, followed by donor 2 and donor 1. Increased acetic acid production was directly related to increased bifidobacteria counts. The predominant formation of acetic acid is in agreement with the results reported by Brück et al.[25] for the fecal fermentation of beet fiber. The acetogenic potential of commercial xylooligosaccharides has been confirmed by Smiricky-Tjardes et al.[26] using in vitro fermentations with pig feces and in vivo experiments with rats. Acetate largely bypasses colonic and liver metabolism and is metabolized by peripheral tissues.[27]

Butyrate was produced at a higher concentration than propionate in experiments with samples from all donors and the two types of substrates (see Table 3). Butyrate is the main source of energy for colonocytes, and its increased production by gut bacteria has been linked to a reduced incidence of colon cancer.[28] Propionate is utilized primarily by the liver, and its role as a potential modulator of cholesterol synthesis has been proposed.[27] Enhanced generation of acids in intestinal fermentation might be desirable because acidic environments can inhibit the growth of potentially pathogenic microorganisms and putrefactive bacteria.[22]

Dynamics of the Bifidobacterium Population. FISH was used for evaluating the bifidogenic effect of the purified POH concentrates. As a reference for comparison, cultures containing FOS as the carbon source and blanks were also analyzed (Figure 3). As expected, the increases in bifidobacteria counts were higher for media containing POH concentrates and FOS than in negative controls, confirming the suitability of these substrates as carbon sources for the metabolism of bifidobacteria. The increase in growth depended on the carbon source, even though important differences among donors were also noticed. After 11 h of incubation, the increases in bifidobacteria counts determined for media contaning P1 or P2 were slightly less pronounced than those observed in cultures containing FOS. However, the bifidobacterial population increased after 29 h for the various donors and substrates ranked as follows: for donors 1 and 2, P2 > FOS > P1, and for donor 3, P1 > FOS > P2. In overall terms, it can be seen that the POH fractions obtained in this work caused stimulatory effects in the bifidobacterial population which were similar to those obtained with commercial FOS. Furthermore, the increase observed was greater than 1 log-fold (Figure 3), revealing modifications in the intestinal microbiota.[29] The experimental results achieved in this work are in agreement with literature reported for other NDOs. Faber et al.[14] investigated the prebiotic characteristics of molasses composed of mannoligosaccharides, xylooligosaccharides, and glucooligosaccharides using a canine fecal inoculum and concluded that these substrates were able to modify the *Bifidobacterium* population compared to a proven prebiotic such as FOS. Yoon et al.[30] reported on the effects of partially hydrolyzed galactomannan (guar gum) on the fecal microbiota of nine healthy human volunteers administered at a dose of 7 g of product/volunteer/day for 14 days and concluded that both the *Bifidobacterium* spp. counts and percentage of these species with respect to the total counts increased significantly after the administration period.

In summary, autohydrolysis of *P. pinaster* wood under the conditions employed in this work resulted in extensive hemicellulose solubilization, leading to a liquor containing hemicellulose-derived saccharides. The process depicted in Figure 1 enabled the selective removal of monosaccharides and

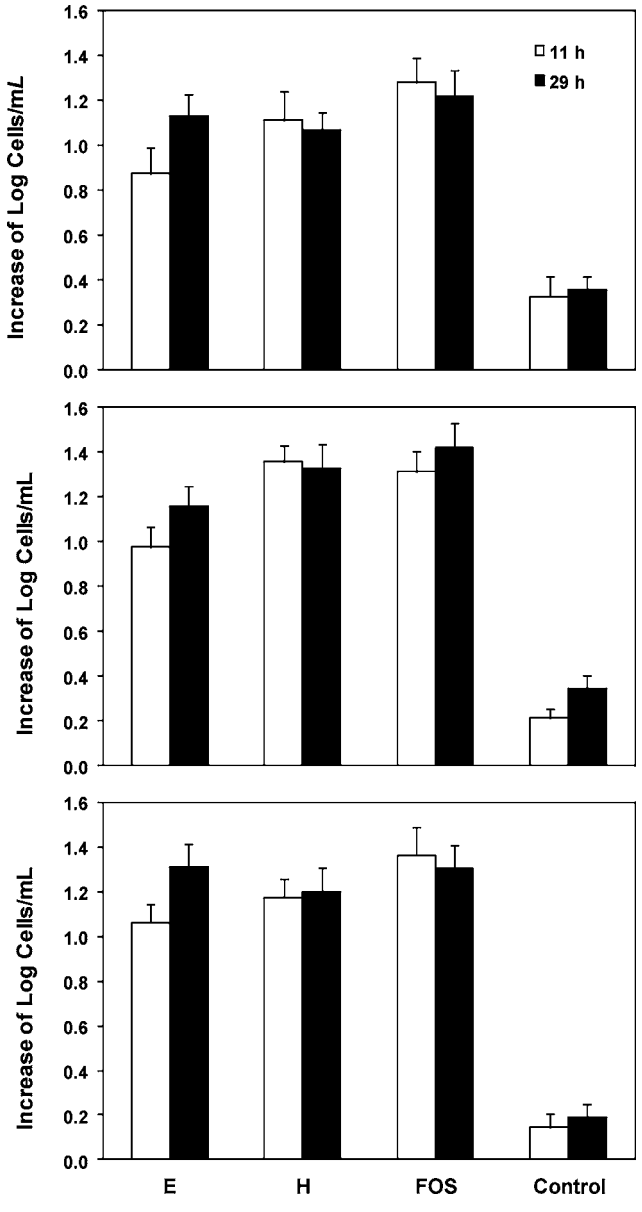

Figure 3. Increase (with respect to time 0 h) of *Bifidobacterium* counts determined by FISH in fecal cultures from three donors using P1, P2, or FOS as the carbon source. Controls did not contain added carbohydrates. Initial *Bifidobacterium* counts: 7.72 ± 0.13 log cells/mL for donor 1, 7.45 ± 0.11 log cells/mL for donor 2, and 7.44 ± 0.09 log cells/mL for donor 3. Error bars indicate standard deviations.

nonsaccharide compounds, leading to concentrates (P1 and P2) of high purity and different molar mass distributions. In vitro fermentation experiments confirmed the ability of P1 and P2 to support the growth of bifidobacteria. The prebiotic potential of both concentrates was confirmed by the production of SCFAs and lactate in the fermentation media, as well as by their bifidogenic capacity.

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ABBREVIATIONS

DDRV, discontinuous diafiltration with reduction of volume; DP, degree of polymerization; FISH, fluorescence in situ hybridization; HPAEC-PAD, high-performance anionic exchange chromatography with pulsed amperometric detection; HPLC-RI, high-performance liquid chromatography with refraction index detection; HPSEC, high-performance size exclusion chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; VCR, volume concentration ratio

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