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Article

Prebiotic Activity of Poly- and Oligosaccharides Obtained from *Plantago major* L. Leaves

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Abstract: The aim of the present study was to evaluate the prebiotic potential of *Plantago major* L. leaves water-extractable polysaccharide (PWP) and its lower molecular fractions. The structure of PWP was investigated by high pressure anion exchange chromatography (HPAEC), size exclusion chromatography coupled with multi-angle laser light scattering detector (SEC-MALLS) and Fourier-transform infrared (FTIR) spectroscopy. The chemical composition and monosaccharide analyses showed that galacturonic acid was the main monosaccharide of PWP followed by glucose, arabinose, galactose, rhamnose and xylose. FTIR study indicated a strong characteristic absorption peak at 1550 cm⁻¹ corresponding to the vibration of COO⁻ group of galacturonic acid. The PWP was subjected to hydrolysis using commercial enzymes to obtain *P. major* low molecular fraction (PLM) which was successively separated by size exclusion chromatography on Biogel P2. PWP and PLM were examined for in vitro prebiotic activity using various assays. Results gave evidence for changes in optical density of the bacteria cells and pH of the growth medium. A heterofermentative process with a lactate/acetate ratio ranged from 1:1 to 1:5 was observed. The ability of PLM to stimulate the production of certain probiotic bacteria glycohydrolases and to be fermented by *Lactobacillus* sp. strains was successfully proved.

Keywords: *Plantago major* L.; polysaccharides; oligosaccharides; enzymatic hydrolysis; prebiotic activity

1. Introduction

Plant derived polysaccharides are major components in the human diet and although widely regarded as primary sources of energy, they revealed numerous more complex biological properties [1]. They have beneficial effects on reducing the risk factors for some chronic diseases, including cardiovascular diseases, certain types of cancer and diabetes, also explored such as immunomodulating

and wound healing agents, plasma substitutes and scaffolds in tissue engineering [2–5]. The variety in the biological behavior of plant polysaccharides is due to diverse nature of their structure characteristics as molecular weight, the presence of different monosaccharides as building blocks, variations in sequence, linkage, branching, and distribution of side chains [2,4]. Furthermore, the possibility of modulating their structure make them particularly attractive. In recent years, lower molecular fractions of plant polysaccharides, and especially non-digestible oligosaccharides, gained attention regarded as functional foods with prebiotic activities [1,6,7].

Prebiotics, according to the International Scientific Association for Probiotics and Prebiotics, are defined as substrates that are selectively utilized by host microorganisms conferring health benefits [8]. The physiological benefits include stimulation of the intestinal microbiota with production of short-chain fatty acids (lactate, acetate, propionate, butyrate) and reduction of intestinal pH; inhibition of pathogen growth in the gastrointestinal tract; decreased insulin response and glucose uptake; reduction of blood lipid levels; enhancement of mineral bioavailability, etc. [9,10]. Investigations on the precise mode of action of prebiotics are still being conducted. Nowadays, only several types of oligosaccharides with prebiotic properties, such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS), are commercially available but there is an increasing interest in the development of “second generation” novel prebiotics with added functionality and lower cost [9,11]. The improved functional properties include ability for modulating microbiota, protection of colonic cells against pathogens and toxins, stimulation of apoptosis of human colonic adenocarcinoma cells, synergistic empowerment of immunomodulation caused by GOS and FOS, dermatological applications, and others [12]. On this point, pectic oligosaccharides (POS), have been considered as promising prebiotic agents [7,13]. POS are derived from the parent compound, “pectin,” which is a complex of plant cell-wall matrix polysaccharides consisting of homogalacturonan, rhamnogalacturonan I and II in the backbone with arabinan, galactan, arabinogalactan and xylogalacturonan side-chains attached to rhamnose in rhamnogalacturonan regions [14,15]. Given the complexity and the heterogeneity of the pectin polymer, POS can vary significantly in their molecular weight and monosaccharide composition [14]. In this respect, the major advantage of POS is that regarding the different structural blocks comprising pectins, a variety of POS as rhamnogalacturonan oligosaccharides, galacturonan oligosaccharides, arabinogalactan oligosaccharides, galactooligosaccharides, arabinooligosaccharides and xylooligosaccharides can be obtained [11,14].

Mostly, three different pathways are applied for the production of POS: (1) extraction from plants; (2) depolymerization of polysaccharides; (3) synthesis from mono- and disaccharides [1,16,17]. The process of depolymerization is considered as one of the most effective pathways because a wide variety of oligomers can be produced from one polymer [16]. Depolymerization strategies as physical, chemical and enzymatic hydrolysis have been developed [1,16,18]. Unlike the non-specific chemical and physical treatments, enzyme-catalyzed degradation is the major pathway to obtain more defined oligosaccharides with desired molecular weights and minimum adverse chemical modifications in the end products [7,9,18,19]. Nonetheless, the evidence for the relation between the chemical composition and prebiotic properties of these bioactive oligosaccharides is still not well established and requires further investigations to evaluate their potential [11,15,20].

Plantago major L. is a perennial herb from *Plantaginaceae* family. It is well known as a functional food source and medicinal plant related to the diverse content of biological active substances as polysaccharides, flavonoids, phenolic acids, iridoids and vitamins [21–23]. Cell wall pectic type polysaccharides have been previously isolated from *P. major* leaves and identified as rhamnogalacturonan and arabinogalactan type II [24,25]. Immunological activity of the isolated polysaccharides was proven [26,27]. To best of our knowledge, no study has been performed to investigate the prebiotic potential of *P. major* polysaccharides and lower molecular fractions (including oligosaccharides). The aim of the present study was to evaluate the relation structure-prebiotic activity of water-extractable polysaccharides from Bulgarian *P. major* leaves (PWPs) and the enzymatically obtained *P. major* low molecular weight fraction (PLM). The prebiotic potential of PWPs and PLM

was estimated by evaluating the growth, the synthesis of metabolites (lactate, acetate, ethanol) and the production of specific glycohydrolases of four different strains lactic acid bacteria: *Lactobacillus acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27.

2. Materials and Methods

2.1. Plant Material and Chemicals

Plantago major L. mature leaves were collected from Thracian valley floristic region, Bulgaria (42°08' N, 24°44' E), in the vegetative season of 2018. Acetic acid and L/D-lactic acid assay kits were purchased from Megazyme, Ireland. Enzymes used were as follows: hemicellulase "Amano-90" (Amano Pharmaceutical Co., Ltd., Nagoya 460-8630, Japan), endo-hemicellulase (Bakezyme HSP600, DSM) and endo-1,4- β -xylanase M1 *Trichoderma viride* (Megazyme, Ireland). All other chemicals were from Sigma-Aldrich (St. Louis, MO 63178, USA) and of analytical grade.

2.2. Extraction and Purification of *P. major* Water-Extractable Polysaccharide (PWPs)

As described in Figure 1, the extraction of PWPs was performed with some modifications according to an adapted method described in literature [24,25,28]. Briefly, fresh sliced leaves of *P. major* were treated with 96% ethanol (1:12, w/v) for 1 h at 70 °C and then filtered. The alcohol-insoluble part was washed successively with 96% ethanol, chloroform-methanol solution (1:1, v/v) and acetone [28]. The residue was extracted with distilled water (1:25, v/v) at 80 °C for 2 h with continuous stirring. The obtained extract was filtered and precipitated by adding two volumes of 96% ice-cold ethanol. The precipitate was recovered by centrifugation (5000 rpm, 15 min, 4 °C), resuspended in distilled water (200 mL) and precipitated again with two volumes of 96% ethanol. The last steps were repeated two times. The final precipitate was washed twice with acetone and then dried one night at room temperature.

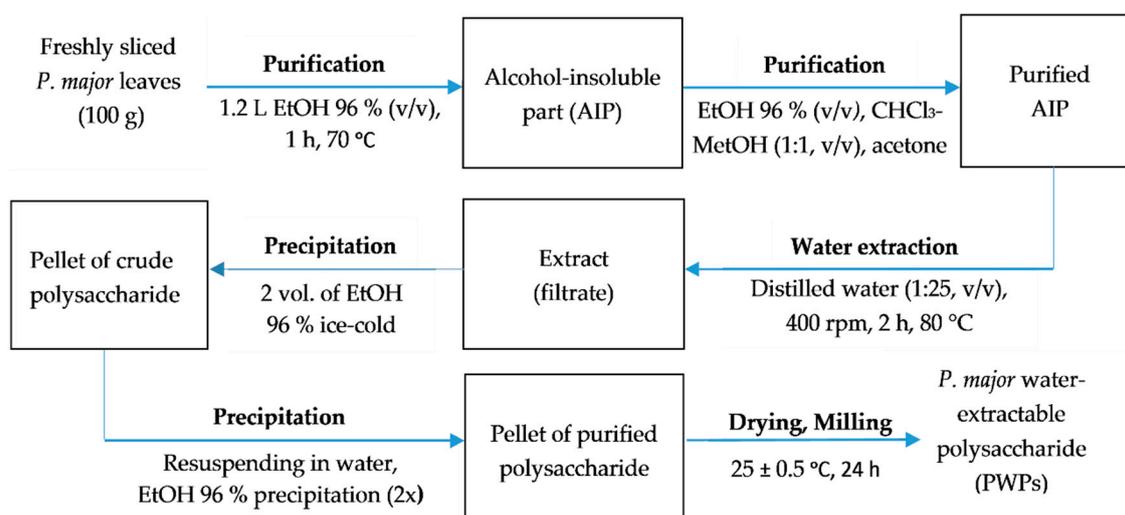


Figure 1. Extraction process of *P. major* water-extractable polysaccharide (PWPs).

2.3. Chemical Composition of PWPs

The neutral sugars content of PWPs was estimated by a colorimetric phenol-sulfuric acid method [29] using glucose as a standard. Uronic acid content was quantified by the method of Blumenkrantz and Asboe-Hansen [30], calibrated against a standard of galacturonic acid. Protein assay followed the Bradford method using a bovine serum albumin as a standard [31]. Total phenolic compounds were determined by the Folin–Ciocalteu procedure using gallic acid as a standard [32]. Finally, the total foliar chlorophyll was estimated by the noninvasive method of Richardson et al. [33].

2.4. FTIR Spectroscopy of PWP

Fourier-transform infrared (FTIR) measurements were carried out using a VERTEX 70 FTIR instrument. PWP was analyzed on ATR A225 diamond. The IR spectra (50 scans) were recorded at room temperature (referenced against air) with the wavenumber range of 500–4000 cm^{-1} . Spectra were processed with OPUS 7.2 software.

2.5. Monosaccharide Composition Analysis of PWP by HPAEC

PWP (10 mg) was mixed with 1 mL trifluoroacetic acid (2 M) in a glass tube, heated at 120 °C for 90 min and stirred periodically. After hydrolysis, pH was adjusted to 7 by addition of 200 μL ammonium hydroxide (28%, *w/v*). The solution was centrifuged at 14,000 rpm for 15 min at 25 °C and the supernatant was filtered through 0.2 μm membrane filter. Monosaccharide composition was analyzed by high pressure anion exchange chromatography (HPAEC) with an ICS 3000 (Dionex, Mundelein, IL 60060, USA) equipped with pulsed amperometric detection (PAD) and AS 50 autosampler. Twenty five μL of the sample was injected in the system and eluted into a guard CarboPacTM PA1-column (4 mm \times 50 mm) and an analytical CarboPacTM PA1-column (4 mm \times 250 mm). Before each injection, columns were equilibrated by running 15 min with 18 mM NaOH. The sample was eluted isocratically with 18 mM NaOH for 25 min, followed by a linear gradient between 0 to 1 M sodium acetate in 200 mM NaOH for 20 min to elute acidic monosaccharides. The columns were then washed with 200 mM NaOH for 15 min by keeping the eluent flow constant at 1 mL/min and thermostated at 25 °C. Results were analyzed with Dionex Chromeleon 6.80 software (Dionex Corporation, Sunnyvale, CA, USA) using the standards monosaccharides (L-Rha, L-Ara, D-Gal, D-Glc, D-Man, D-Xyl, D-Fru, D-GalA and D-GlcA).

2.6. Determination of Molecular Weight of PWP

The mass average molar mass (M_w) and number average molar mass (M_n) were evaluated by high pressure size exclusion chromatography (HPSEC) coupled with three detectors: Multi-angle laser light scattering detector (MALLS, Mini-DAWN, Wyatt Technology Corp., Santa Barbara, CA, USA), Differential refractive index (DRI) detector (RID-10 A, Shimadzu, Duisburg, Germany) and UV-vis detector (SPD-20A, Shimadzu, Duisburg, Germany). The HPSEC line consisted of an SB-G guard column and three columns in series (SB-806 HQ, SB-804 HQ and SB-803 HQ). The system was eluted with NaNO_3 0.1 M and NaN_3 0.5 mM, filtered through a 0.2 μm , 47 mm membrane filter (Anotop 47, Whatman, Maidstone, England), and carefully degassed. PWP (10 mg) was previously solubilized in 10 mL of the elution phase under stirring for 24 h and then filtered through a 0.2 μm syringe filter (Anotop 10, Whatman, Maidstone, UK). The solution was injected through a 100 mL full loop and the elution was performed with a flow rate of 0.5 mL/min. Data were evaluated using ASTRA software.

2.7. Enzymatic Hydrolysis of PWP

PWP (1%, *w/v*) was solubilized in 50 mM sodium acetate buffer (pH 5.5) and subsequently hydrolyzed in the presence of an enzyme mixture composed of hemicellulase (10 U/mL), endo-hemicellulase (10 U/mL) and endo-1,4- β -xylanase (10 U/mL). The enzyme reaction was conducted for 24 h at 40 °C in continuously shaking at 120 rpm. The samples were inactivated (100 °C, 10 min), centrifuged to remove enzymes, coagulated with two volumes of 96% ice-cold ethanol and centrifuged (7000 rpm, 10 min) to remove the non-hydrolyzed PWP. Finally, the supernatant containing low molecular weight fractions was concentrated under vacuum and then lyophilized. The hydrolysis yield (%) was calculated by the following formula:

$$\text{Hydrolysis yield (\%)} = \frac{100 \times (a - b)}{a},$$

where a is the initial weight of PWP and b is the weight of the non-hydrolyzed PWP.

The obtained hydrolysate was indicated as *Plantago major* low molecular weight fraction (PLM).

2.8. Fractionation and Purification of *P. major* Low Molecular Weight Fraction (PLM)

2.8.1. Estimation of the Mw of PLM by HPSEC

Molecular weight analysis of PLM was performed by size exclusion chromatography (SEC) on an Agilent 1100 Series high performance liquid chromatograph with a RID (Refractive Index Detector) cell. The fractionation was carried out in two columns TSK G5000PWXL and TSK G3000PWXL (Tosoh Bioscience) coupled in series. Isocratic elution with sodium nitrate (NaNO_3 , 0.1 M) was applied at 1 mL/min. A standard range of pullulan (Sigma Aldrich) at 10 g/L of different molar masses from 1.3 kDa to 800 kDa was used. PLM was dissolved at 10 g/L in NaNO_3 buffer (0.1 M) and then filtered through 0.45 μm before injection.

2.8.2. Purification of Oligosaccharides by Gel Exclusion Chromatography

A liquid chromatography system Äkta purifier (GE Healthcare, Marlborough, MA 01752, USA) equipped with Biogel P2 column (2.6×80 cm) was used for separation and estimation of the degree of polymerization (dp) of each oligosaccharide from PLM fraction (nominal exclusion limit from 100 to 1800 daltons). The elution was performed at a flow rate of 0.6 mL/min with ammonium formate buffer 0.1 M (pH 6). The sample injection volume was 2 mL at concentration of 20 mg/mL. The column was thermostated at 25 °C and average dp were estimated by oligosaccharides calibration according to Kothari et al. [34].

2.9. Study of Prebiotic Potential

2.9.1. Bacterial Strains and Culture Conditions

Four *Lactobacillus* strains were used for evaluation of the prebiotic potential of PWP and PLM. The probiotic strains of *Lactobacillus acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 were obtained from the bacterial culture collection of the department of Biochemistry and Microbiology, Plovdiv University, Bulgaria. The strains were routinely cultivated overnight in de Man, Rogosa and Sharpe (MRS) medium (Merck) at 37 °C. Overnight grown cells of *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 were washed twice in 0.85% NaCl (*w/v*) saline solution, and 10% (*v/v*) of bacterial suspension were used to inoculate modified MRS (mMRS) broth medium containing: 1% meat extract, 1% peptone, 0.5% yeast extract, 0.1% ammonium hydrogen citrate, 0.5% sodium acetate trihydrate, 0.2% $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$, 0.01% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.05% $\text{MnSO}_4 \times 4\text{H}_2\text{O}$, 0.1% Tween 80, pH –6.8. As a carbon source was added 1% of the previously sterilized samples (PWP or PLM) and glucose as a standard. The anaerobic fermentations with the *Lactobacillus* strains were performed in 50 mL PS bottles at 37 °C for 20 h under non-pH-controlled conditions.

2.9.2. Bacterial Growth

Bacterial growth was measured by a turbidimetric method at 600 nm and calibrated against a cell dry weight standard curve using a UV/Vis spectrophotometer, Beckman Coulter, Brea, CA, USA. To evaluate the pH directly in the bacterial growth culture, a pH microelectrode (Consort C6010, Belgium) was used. Growth of each strain was monitored by measuring the OD and pH of the cultures at 0 h, 3 h, 6 h, 10 h, 14 h and 20 h. The results were calculated from duplicate samples of two separate anaerobic fermentations.

After fermentation, cells were collected by centrifugation at 9000 rpm, 15 min, 4 °C and used for further enzymatic assays. The separated supernatants were deproteinized and used for metabolite assays.

2.9.3. Analysis of Metabolites

L/D-Lactic acid and acetic acid were assayed enzymatically with commercially available kits (Megazyme, Ireland). Respectively, L-lactate dehydrogenase and D-lactate dehydrogenase were used for determination of L/D-lactic acid, and acetyl-CoA synthetase, citrate synthase and malate dehydrogenase were used for acetic acid determination. Calculations were made by using the Megazyme Mega-Calc™. Ethanol was quantified by HPLC system Konik-Tech, with RI Detector Shodex R1-101 and Tracer Excel ODSB (150 × 0.4 mm) column, mobile phase water, flow rate 0.3 mL/min and temperature 30 °C.

2.9.4. Enzymatic Activity

The bacteria cells were washed twice with 50 mM sodium acetate buffer (pH 6.5) and suspended in 1 mL lysis buffer (50 mM sodium acetate buffer pH 7.5, 300 mM sodium chloride and 2% glycerol). The sonication of bacteria cells were performed with an UP 50 H Ultrasonic Processor (Hielscher Ultrasound Technology, Germany) for 15 cycles and 50% amplitude. Each cycle was with a duration of 5 Sec and 2 min break between cycles on ice. After sonication, the lysates were centrifuged (1,200 rpm, 10 min, 4 °C) and the supernatants were collected for evaluation of α -glucosidase, α -galactosidase, and β -xylosidase activity in lactobacilli strains.

α -glucosidase activity was measured according to the procedure described by Dewi et al. [35]. The amount of *p*-nitrophenol (*p*NP) released by the degradation of the substrate *p*NP- α -D-glucopyranoside (Sigma-Aldrich) was estimated. Briefly, to 100 μ L of the bacterial lysate were added 250 μ L of 5 mM *p*NP- α -D-glucopyranoside (pH 6.8), 150 μ L water and then incubated for 10 min at 37 °C. The enzyme process was stopped by adding 2 mL 1 M Na₂CO₃. Finally, the amount of *p*NP was determined by measuring the absorbance at 405 nm.

α -galactosidase activity assay followed the method of Petek et al. [36]. The amount of *p*NP released by the degradation of *p*NP- α -D-galactopyranoside (Sigma-Aldrich) was quantified. The reaction mixture containing 100 μ L bacterial lysate, 100 μ L 9.9 mM *p*NP- α -D-galactopyranoside (pH 6.5) and 300 μ L water, was incubated for 5 min at 37 °C. The enzyme process was stopped by the addition of 1 mL borate buffer (pH 9.8). The absorbance of the released *p*NP was measured at 405 nm.

β -xylosidase activity was estimated by the method of Lasrado and Gudipati [37] as the amount of *p*NP released by substrate degradation of *p*NP- β -D-xylopyranoside (Sigma-Aldrich). To 100 μ L of the bacterial lysate were added 900 μ L 5 mM *p*NP- β -D-xylopyranoside (pH 5.7) and 100 μ L water. The mixture was incubated for 30 min at 30 °C. The enzyme process was stopped by adding 100 μ L saturated sodium tetraborate solution. The amount of *p*NP was quantified at 405 nm.

2.10. Statistical Analysis

For each experiment, data were systematized and analysed using Microsoft Excel statistical package and expressed as mean values \pm SD. Three-way ANOVA was used to analyse the statistical significance of individual factors (with different number of levels) and their interactions on dependant variables. Tukey's HSD (Honestly Significant Difference) tests for post-hoc analysis were used in each of the ANOVA tests to conclude whether all the mean differences of the dependent variables were significant. Statistical analyses and modelling were performed using IBM SPSS Statistics Version 25 software. The calculations are given in the Supplementary Materials (Tables S1–S3).

3. Results and Discussion

3.1. Chemical Composition of PWP

The chemical composition (carbohydrates, proteins, phenolic compounds, chlorophyll) and extraction yield of PWP were shown in Table 1. *P. major* L. leaves are generally considered as a rich source of polyphenols and chlorophyll [21,22,38–40]. The initial purification of leaf mass using a series of different solvents (ethanol, chloroform/methanol and acetone) allowed producing a colorless polysaccharide with chlorophyll and polyphenol content less than 0.5%. The extraction yield of

PWPs was estimated at 19 g/kg ($1.92\% \pm 0.11$) based on fresh leaves. It is important to mention that Olennikov et al. [25] obtained similar values for polysaccharide content of *P. major* fresh leaves (1.5–3.3%) using a water extraction. The main compounds of PWPs determined by colorimetric assays were uronic acids ($59.34\% \pm 1.05$) followed by neutral sugars ($39.14\% \pm 0.22$). The presented results are similar to those previously reported in our study [41] and to those found in literature [24,25] where pectic substances were confirmed. Finally, the protein content ($0.51\% \pm 0.06$) was lower than the value reported by Samuelsen et al. [24] (1.8%) for water-soluble polysaccharide of *P. major* leaves proving that our extraction process of PWPs was more efficient.

Table 1. Chemical composition of PWPs extracted from *P. major* L. leaves.

Extraction Yield (%, w/w)	Neutral Sugar (%, w/w)	Uronic Acid (%, w/w)	Protein (%, w/w)	Total Phenolic Compounds (%, w/w)	Total Chlorophyll (%, w/w)
1.92 ± 0.11	39.14 ± 0.22	59.34 ± 1.05	0.51 ± 0.06	0.34 ± 0.03	0.47 ± 0.09

All analyses were conducted in triplicate, and data are expressed as mean values \pm SD.

3.2. FTIR Spectroscopy of PWPs

FTIR spectrum of PWPs is shown in Figure 2. The analysis indicated a broad absorption peak at 3285 cm^{-1} corresponding to the hydroxyl (O-H) stretching vibration of polysaccharide as well as water absorption [42]. The displayed weak absorption band at 2936 cm^{-1} suggested the asymmetric vibration of (C-H) group [43]. The strong characteristic absorption peak at 1550 cm^{-1} was attributed to the vibration of COO^- group of the galacturonic acid and those at 1399 cm^{-1} could correspond to ester carbonyl groups of the carboxylic function of GalA [42]. The signal obtained at 1017 cm^{-1} resulted from the presence of carbohydrate (C-O) functions [44]. Consequently, the results from FTIR assumed a rich polygalacturonic acid pectin type structure of PWPs.

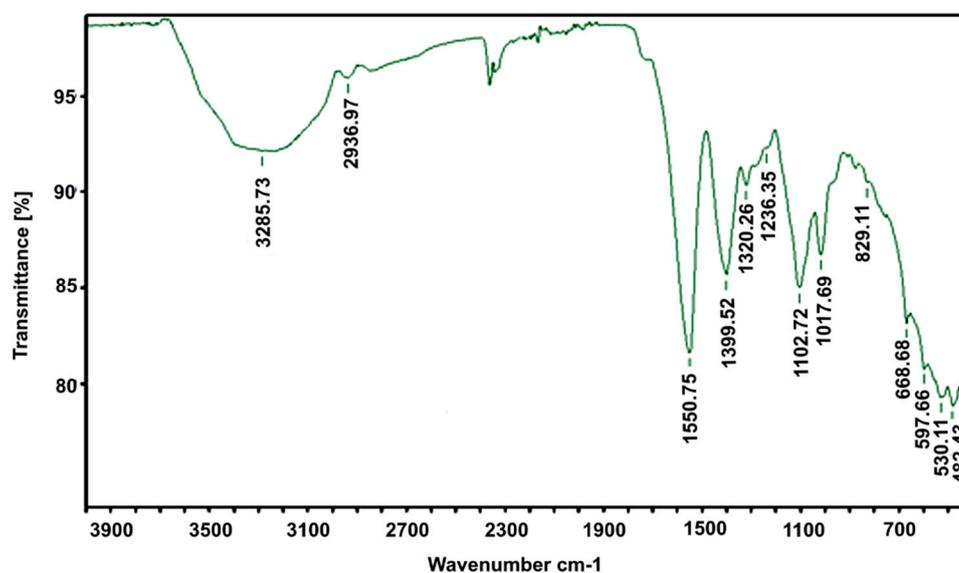


Figure 2. FTIR spectrum of PWPs extracted from *P. major* L. leaves.

3.3. Monosaccharide Composition of PWPs

To confirm the pectic type nature of PWPs observed in FTIR, monosaccharide estimation was carried out using HPAEC (Table 2). The analysis showed that the main constituent of PWPs was galacturonic acid (GalA) (55.38%), followed by glucose (Glc) (21.50%), arabinose (Ara) (9.88%), galactose (Gal) (8.02%), rhamnose (Rha) (3.17%) and xylose (Xyl) (2.05%). The presented monosaccharide composition

assumed that PWP was composed of a pectic type polysaccharide and some neutral fractions, as previously described in literature [24,25]. The molar ratio of 17.58 GalA/Rha and the almost equal content of Ara and Gal suggested a not highly branched rhamnogalacturonan backbone with side chains of arabinogalactans. In their work, Samuelsen et al. [24] reported that crude water-extractable polysaccharides from *P. major* leaves had a content of 36.0% and 73.4% GalA, and 26.2% and 8.45% Glc, using an extraction temperature of 50 °C and 100 °C respectively. They particularly showed that the Rha content did not depend on temperature and was estimated at around 2%. More, after further fractionation of the crude polysaccharides, Samuelsen et al. [24] separated two acidic fractions defined as rhamnogalacturonan and acidic arabinogalactan type II, and a neutral fraction composed of high amounts of glucose and mannose. A fraction of 1,4-linked xylose units was also identified [24].

Table 2. Characterization of PWPs extracted from *P. major* L. leaves.

Mw ^(a) (kDa)	Mn ^(b) (kDa)	Polydispersity Mw/Mn	Monosaccharides ^(c) (mol, %)						
			Rha	Ara	Gal	Glc	Xyl	GalA	GalA/Rha
66.00 ± 2.36	38.30 ± 3.68	1.72 ± 0.18	3.17	9.88	8.02	21.50	2.05	55.38	17.47

^(a) Mw: Mass-average molecular mass was measured by SEC-MALLS-DRI. Each assay was conducted in duplicate, and data are expressed as means (±SEM). ^(b) Mn: number-average molecular mass was measured by SEC-MALLS-DRI. Each assay was conducted in duplicate, and data are expressed as means (±SEM). ^(c) Monosaccharides composition was measured by HPAEC. Rha: Rhamnose; Ara: Arabinose; Gal: Galactose; Glc: Glucose; Xyl: Xylose; GalA: Galacturonic acid. All analyses were run in duplicate and the relative standard deviations were <5%.

3.4. Molecular Weight of PWPs

The molecular weight distribution was evaluated using SEC-MALLS analysis. As shown in Table 2, PWPs was characterized by a weight-average molecular mass (Mw) of 66 kDa and number-average molecular mass (Mn) of 38.3 kDa. The value of polydispersity index (Mw/Mn = 1.72) assumed a slight heterogeneous nature for PWPs. According to literature, the heterogeneity of PWPs could confirm the presence of a pectin type polysaccharide as it was largely described in literature about pectins [45]. In their study on *Plantago major* leaves, Samuelsen et al. [24,26] defined a pectic fraction from water-extractable polysaccharide with Mw of 46–48 kDa and Mw/Mn = 1.4–1.9, and also an arabinogalactan II fraction with Mw of 77–80 kDa and Mw/Mn = 1.1–1.2. Biringanine et al. [46] reported a pectic fraction from *P. palmata* leaves with Mw of about 1200 kDa, Mw/Mn = 1.05, and similar monosaccharide composition to those extracted from *P. major* leaves. Finally, a lot of gel chromatography studies on *P. media* and *P. depressa* leaves established that all polysaccharide fractions were heterogeneous and did not have a common characteristic Mw distribution. They contained three groups of polymers, respectively with Mw > 150 kDa, Mw = 50–110 kDa, and Mw = 8–20 kDa [47].

3.5. Enzymatic Hydrolysis of PWPs

Based on weighing method, the yield of the enzyme hydrolysate from *P. major* water-extractable polysaccharide (indicated as PLM) was estimated at 22.35% ± 1.07. HPAEC analysis of PLM showed that the main monosaccharide was galacturonic acid (62.07%), followed by arabinose (11.96%), galactose (10.83%), rhamnose (5.25%), glucose (3.13%) and xylose (1.38%). The conducted analysis confirmed that PLM was a pectic fraction composed of oligo-rhamnogalacturonan backbone (molar ratio of 11.82 GalA/Rha) with side chains of arabinogalactans (molar ratio of 1.09 Ara/Gal). The average molecular weight of PLM was estimated at 1.69 kDa by using SEC analysis performed in two columns TSK G5000PWXL and TSK G3000PWXL coupled in series. The obtained result proved the efficient production of low molecular weight fractions of PWPs using our enzymatic process with a mixture of hemicellulases and endo-1,4-β-xylanase. Finally, to estimate the distribution of oligosaccharide families in PLM an exclusion size fractionation onto Biogel P2 was used.

As observed in Figure 3, different families of oligosaccharides were fractionated from dp 2 to dp 10 according to Mw calibration [34].

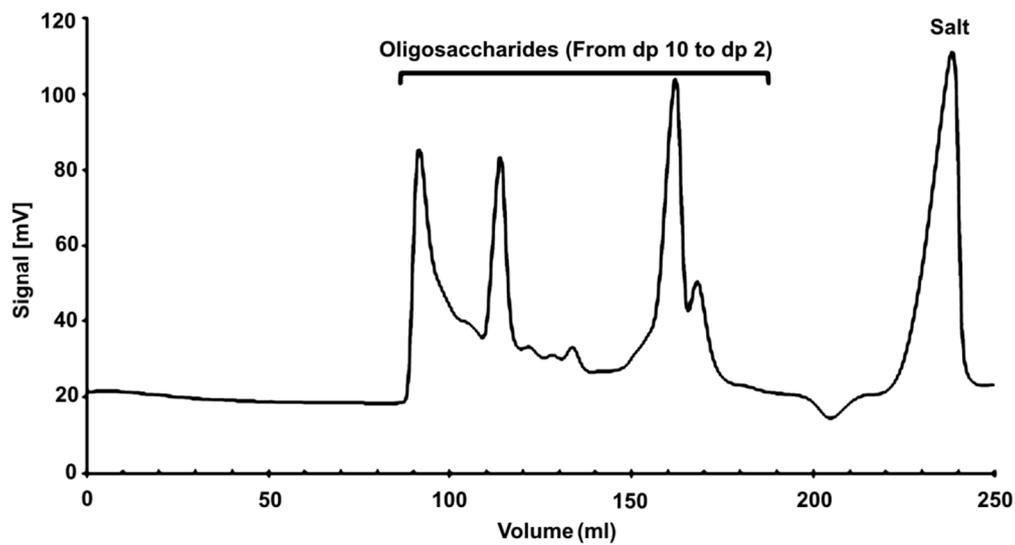


Figure 3. Biogel P2 chromatogram of *P. major* low molecular fraction (PLM).

3.6. Prebiotic Potential

The growth kinetics of *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 were evaluated in terms of OD 600 nm during 20 h of fermentation. The polysaccharide (PWPs and PLM) utilization was compared with those of glucose and was shown on Figure 4.

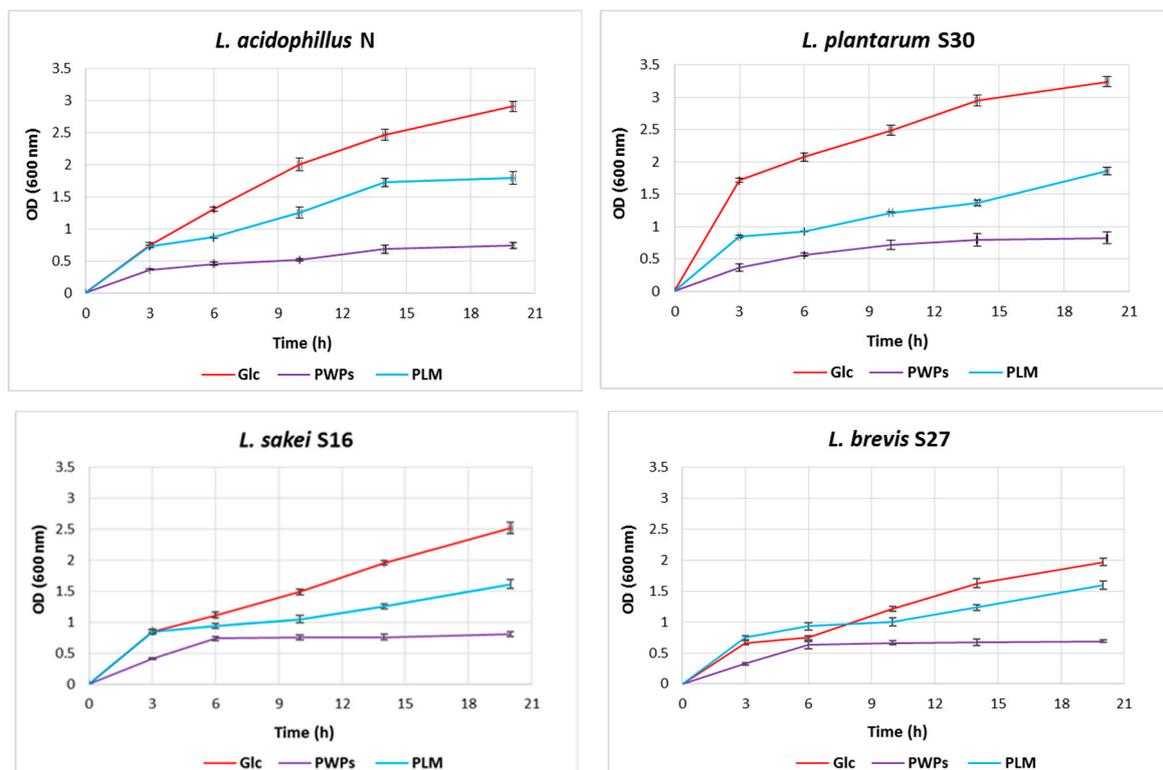


Figure 4. Optical density value (OD) of *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 strains in the presence of glucose (Glc), PWPs and PLM. All analyses were conducted in triplicate, and data are expressed as mean values \pm SD. Three-way ANOVA test: Sig. ($p < 0.05$), partial η^2 (0.822–0.998), $R^2 = 0.997$ ($R^2_{Adj.} = 0.996$).

The presented results revealed that the utilization of PWPs and PLM was strain specific and *Lactobacillus* strains utilized the tested substrates in the analogous manner. All strains showed about two times higher growth rates values when cultivated on PLM compared to PWPs. Furthermore, similarly to glucose, PLM had a long logarithmic rate phase of up to the 20th hour while PWPs was more rapidly depleted (by the 14th hour for *L. acidophilus* N and *L. plantarum* S30, and by 6th hour for *L. sakei* S16 and *L. brevis* S27). These results could confirm that the examined strains probably fermented only the hairy neutral regions of the pectin polysaccharide which were depleted till the 6–14 h. The short-chain oligosaccharides from PLM ($M_w < 3000$ Da, $DP < 20$) provided a longer term substrate utilization than the complex polysaccharide molecules from PWPs ($M_w = 66$ kDa) which needs firstly to be depolymerized by *Lactobacillus* strains before its further metabolism.

The presented results from the growth kinetics of *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 were statistically analysed using three-way ANOVA through tests of between-subject effects and Tukey's HSD test (Supplementary Materials: Table S1). The statistical significance of the individual factors (three independent variables: bacterial strain, carbon source and time) and their combinations on the response (dependant variable: OD 600) was evaluated. The estimated values of Sig. ($p < 0.05$) and Partial Eta² (0.822–0.998) from the tests of between-subject effects indicated that the results of the individual factors and their combination interactions were statistically significant. The coefficient of determination was close to 1 ($R^2 = 0.997$), which revealed that there was a considerable linear relationship between the factors and the dependant variable. Moreover, the value of R^2 showed that with 99% confidence, the change in the response could be explained with the independent variables of the model. The slight difference between R^2 and the adjusted coefficient of determination ($R^2_{Adj} = 0.996$) showed that some insignificant conditions were included in the model.

The results from the conducted Tukey's HSD tests for post-hoc analysis confirmed that in the different time points (0 h, 3 h, 6 h, 10 h, 14 h and 20 h) all the mean differences of the dependent variables were significant.

Evidence on a better consumption of a structurally different type short chains saccharides compared to longer ones could be found in literature [48–52]. In vitro fermentations of xyloglucans, dextrans and various types of glucose containing polysaccharides have shown that some intestinal bacteria, which were not able to grow on polysaccharides, were able to completely ferment their oligosaccharide derivatives with different degrees of polymerization [13,48–50]. In their study, Rossi et al. [52] observed a strain-dependent capability of *Bifidobacteria* to degrade fructans of different lengths. Similarly to our results, they reported higher fermentation rates of short chains fructans with a single uninterrupted exponential phase compared to long chain fructans which were gradually consumed. In the same way, an in vitro experimentation on *Bifidobacterium* strains showed a higher biomass yield during fermentation using short chains FOS in comparison with longer FOS [51]. Furthermore, in their study, Olano–Martin et al. [13] used a partial hydrolysis of pectin polysaccharide to improve their bifidogenic properties. They demonstrated that pectic oligosaccharides possess a better prebiotic activity than the complex pectin molecule, although their bifidogenic effect was lower compared to FOS.

The prebiotic activity of PWPs and PLM was confirmed by lower pH values and higher organic acid concentrations during the fermentation of *Lactobacillus* strains (Table 3). After 20 h of fermentation the bacteria acidified the initial pH of PWPs (pH = 6.55 at 0 h) and the initial pH of PLM (pH = 6.52 at 0 h) to pH 5.48 and pH 5.72, respectively. The lower pH microenvironment resulted by the production of organic acids. The microbial conversion of complex polysaccharides to monosaccharides involves various biochemical pathways and leads to the formation of fermentative end products as short-chain fatty acids (SCFAs), lactate and ethanol [53]. The major end products of the fermentation of PWPs and PLM were lactate, acetate and ethanol in different ratio. The expected easier fermentation of the low molecular weight fractions (PLM) compared to the native water-extractable polysaccharide from *P. major* leaves (PWPs) was proven. The major product from glucose fermentation was lactic acid while a heterofermentative process with a significant production of acetate was observed in PWPs and PLM fermentations. The lactate/acetate ratio ranged from 1:1 to 1:5 as the

highest amount of acetate was observed in *L. plantarum* S30. The L/D-lactic acid ratio varied from 1:1 to 2:1 in different strains. In agreement with our result, lactate and to a lower extent acetate, were the main end products of *L. plantarum* fermentation using glucose and FOS under anaerobic conditions [54]. Whereas, using *Amorphophallus konjac* glucomannans and their hydrolysates as substrates, other authors [55,56] found that acetic acid was the chief SCFA produced by *L. plantarum* in vitro fermentation. The utilization of the heterogenic PWP and PLM by *Lactobacillus* strains could be due to their heterofermentative pattern [57], whereas the observed strain-specificity might be result from the metabolism-specificity of the different monosaccharide units composing the structure of PWP and PLM [53].

Table 3. Values of pH and ratios of lactic acid, acetic acid and ethanol production by *L. acidophilus* N (*L. A. N*), *L. plantarum* S30 (*L. P. S30*), *L. sakei* S16 (*L. S. S16*) and *L. brevis* S27 (*L. B. S27*) strains in the presence of glucose, PWP and PLM.

Carbon Source	Strain	Time (h)	pH *	D-Lactic: L-Lactic Acid Massic Ratio **			Total Lactic Acid (a): Acetate (b): Ethanol (c) Massic Ratio **
Glucose	<i>L. A. N</i>	0	6.54 ± 0.02	0	:	0	0 : 0 : 0
		6	4.02 ± 0.05	1.04	:	1.00	23.10 : 1.00 : 0.11
		20	3.94 ± 0.06	1.00	:	1.31	29.73 : 1.00 : 0.23
	<i>L. P. S30</i>	0	6.54 ± 0.02	0	:	0	0 : 0 : 0
		6	4.20 ± 0.05	1.00	:	1.47	27.00 : 1.00 : 0.89
		20	4.06 ± 0.05	1.00	:	1.50	28.45 : 1.00 : 0.36
	<i>L. S. S16</i>	0	6.54 ± 0.02	0	:	0	0 : 0 : 0
		6	4.35 ± 0.04	1.16	:	1.00	20.43 : 1.00 : 0.24
		20	4.12 ± 0.05	2.03	:	1.00	23.81 : 1.00 : 0.38
	<i>L. B. S27</i>	0	6.54 ± 0.02	0	:	0	0 : 0 : 0
		6	4.00 ± 0.05	1.08	:	1.00	36.88 : 1.00 : 0.56
		20	3.84 ± 0.04	1.16	:	1.00	37.74 : 1.00 : 0.53
PWP	<i>L. A. N</i>	0	6.55 ± 0.03	0	:	0	0 : 0 : 0
		6	5.66 ± 0.01	1.35	:	1.00	1.00 : 1.26 : 0.02
		20	5.62 ± 0.02	1.30	:	1.00	1.00 : 1.76 : 0.03
	<i>L. P. S30</i>	0	6.55 ± 0.03	0	:	0	0 : 0 : 0
		6	5.80 ± 0.02	3.38	:	1.00	1.00 : 3.90 : 0.03
		20	5.71 ± 0.00	2.32	:	1.00	1.00 : 5.24 : 0.03
	<i>L. S. S16</i>	0	6.55 ± 0.03	0	:	0	0 : 0 : 0
		6	5.73 ± 0.01	1.00	:	1.26	1.00 : 1.25 : 0.01
		20	5.72 ± 0.02	1.00	:	1.30	1.00 : 1.41 : 0.02
	<i>L. B. S27</i>	0	6.55 ± 0.03	0	:	0	0 : 0 : 0
		6	5.62 ± 0.03	1.00	:	1.07	1.00 : 2.01 : 0.05
		20	5.63 ± 0.02	1.00	:	1.44	1.00 : 1.95 : 0.09
PLM	<i>L. A. N</i>	0	6.52 ± 0.02	0	:	0	0 : 0 : 0
		6	5.56 ± 0.04	1.72	:	1.00	1.00 : 0.94 : 0.06
		20	5.55 ± 0.01	1.28	:	1.00	1.00 : 1.77 : 0.10
	<i>L. P. S30</i>	0	6.52 ± 0.02	0	:	0	0 : 0 : 0
		6	5.63 ± 0.03	1.43	:	1.00	1.00 : 1.91 : 0.03
		20	5.61 ± 0.00	1.74	:	1.00	1.00 : 2.12 : 0.04
	<i>L. S. S16</i>	0	6.52 ± 0.02	0	:	0	0 : 0 : 0
		6	5.59 ± 0.02	1.40	:	1.00	1.00 : 2.38 : 0.01
		20	5.60 ± 0.01	1.06	:	1.00	1.00 : 2.59 : 0.04
	<i>L. B. S27</i>	0	6.52 ± 0.02	0	:	0	0 : 0 : 0
		6	5.57 ± 0.01	1.00	:	1.85	1.00 : 1.83 : 0.01
		20	5.48 ± 0.00	1.00	:	1.00	1.00 : 2.01 : 0.01

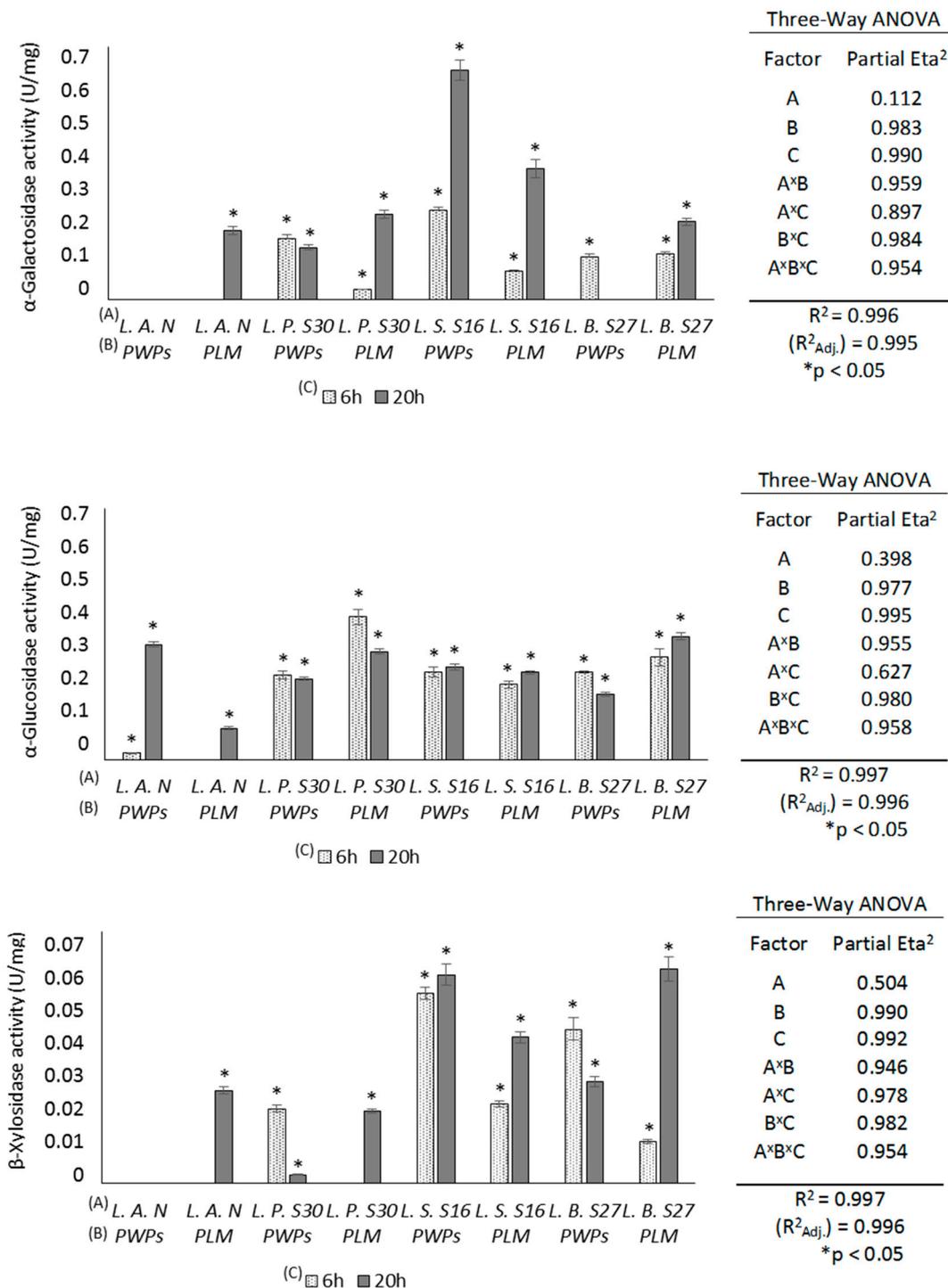
* All analyses were conducted in triplicate, and data are expressed as mean values ± SD. ** All analyses were conducted in triplicate, and data are expressed as ratio. (a) Three-way ANOVA test: sig. ($p < 0.05$), partial η^2 (0.972–0.999), $R^2 = 0.999$ (R^2 Adj. = 0.999). (b) Three-way ANOVA test: sig. ($p < 0.05$), partial η^2 (0.979–1.000), $R^2 = 0.999$ (R^2 Adj. = 0.999). (c) Three-way ANOVA test: sig. ($p < 0.05$), partial η^2 (0.644–0.984), $R^2 = 0.984$ (R^2 Adj. = 0.976).

A Three-Way ANOVA test was performed three times in order to evaluate statistically the obtained results for the production of total lactic acid, acetic acid and ethanol by the three tested bacterial strains.

In each of these three tests the independent variables (individual factors) were the same—carbon sources (glucose, PWP and PLM), bacterial strains (*L.A.N.*, *L.P* S30, *L.S.* S16 and *L.B.* S27) and time points (0 h, 6 h and 20 h). The three ANOVA tests differed in terms of the dependent variable (response)—total lactic acid, acetic acid and ethanol, respectively. The results showed that there was a statistically significant difference between the individual factors and their combination interactions for each of the three fermentation products ($p < 0.05$). The coefficient of determination R^2 (0.984–0.999) indicated a considerable linear relationship between the factors and the dependant variables. Moreover, Tukey's HSD tests for post-hoc analysis were carried out in each of the three different ANOVA tests, which confirmed that in the different time points (0 h, 6 h, 20 h) all the mean differences of the dependent variables were significant (Supplementary Materials: Table S2).

Most of the *Lactobacillus* and *Bifidobacterium* strains possess large number of genes that encode carbohydrate active enzymes and can switch readily between different energy sources in the gut depending on availability. The variety of secreted enzymes allows a degradation of complex carbohydrate substrates [58,59]. Generally, bacteria synthesize degradative enzymes only when substrates for these enzymes are present in their environment. Moreover, the concentration of the produced enzymes in bacterial cells could be in relevance with the amount of the available substrate [60]. The investigation of the type and concentration of the produced glycohydrolases plays a crucial role in the detection of substrates nature used by bacteria during their fermentation [61]. The utilization of *P. major* poly- and oligosaccharides were evaluated by measuring the activity of α -galactosidase, α -glucosidase and β -xylosidase produced by probiotic bacteria during the fermentation process. Glucose was used as a control for glycohydrolase synthesis.

All *Lactobacillus* strains showed highest values for α -galactosidase activity (up to 0.64 U/mg), followed by α -glucosidase activity (up to 0.38 U/mg), and about 5–10 times lower values of β -xylosidase activity (up to 0.06 U/mg) (Figure 5). Regarding the dynamics of glycohydrolase secretion, all strains showed higher rates of α -galactosidase activity at the 20th hour (0.14–0.68 U/mg) compared to the 6th hour (0.08–0.24 U/mg). Except *L. acidophilus* N, which showed higher α -glucosidase activity at the end fermentation hour, all other *Lactobacillus* strains demonstrated similar values of α -glucosidase activity at the 6th (0.21–0.38 U/mg) and 20th hour (0.22–0.33 U/mg). As expected, no enzyme activity was detected in all investigated strains using glucose in the culture media (data not shown). Mainly, bacteria do not need the production of glycohydrolases when they use monosaccharides as carbon sources [60]. Similar results had Rossi et al. [52] which reported for 17 *Bifidobacterium* strains low or no enzyme activity when using glucose as energy source.



Factors for statistical analysis: (A) bacterial strains; (B) carbon source; (C) time.

Figure 5. α-Galactosidase, α-glucosidase and β-xylosidase activity of *L. acidophilus* N (*L. A. N*), *L. plantarum* S30 (*L. P. S30*), *L. sakei* S16 (*L. S. S16*) and *L. brevis* S27 (*L. B. S27*) strains in the presence of PWP5 and PLM. Each assay was conducted in triplicate, and data are expressed as mean values ± SD.

The detection of the three glycohydrolyse enzymes (α-glucosidase, α-galactosidase and β-xylosidase) in all investigated strains cultivated on media with *P. major* polysaccharides proved their strong capacity to produce a complex of enzymes able to hydrolyze different glycosidic linkages in plant polysaccharides. The dynamics of their secretion probably depend on the accessibility of

the glycosidic bonds in the structure of the polysaccharide fragments hydrolyzed by the particular enzymes. On the other hand, there is a strain specificity regarding the possibility of secretion of the studied enzymes. For example, the strain *L. sakei* S16 showed the highest α -galactosidase activity, reaching two to three times higher values than the other investigated strains. Except for *L. acidophilus*, all other strains showed almost identical values of α -glucosidase activity. The obtained results indicated, on the one hand, the presence of accessible α -glycosidic linkages in the polysaccharide fractions, and on the other hand, that the tested strains had affinity towards more easily digestible monosaccharide components for their subsequent utilization.

The established β -xylosidase activity in most of the tested strains is a proof of the branched structure of *P. major* heteropolysaccharide fractions and the ability of the strains to adapt to growth media with different carbohydrate substrates. β -xylosidase activity of *L. sakei* S16 and *L. brevis* S27 was higher compared to the other tested strains but it was 8–10 times lower than the values of α -glucosidase activity.

A three-way ANOVA test was performed three times in order to evaluate the statistical significance of the individual factors—carbon source (glucose, PWP and PLM), bacterial strains (*L. A. N*, *L. P. S30*, *L. S. S16* and *L. B. S27*) and time (6 h and 20 h) on the dependant variables (the enzyme activities: α -galactosidase, α -glucosidase and β -xylosidase activity). The results (Supplementary Materials: Table S3) showed that there was a statistically significant difference between all of the individual factors and their combination interactions ($p < 0.05$), with R^2 value close to 1 (0.996–0.997), indicating a considerable linear relationship between the factors and the dependant variable. Tukey's HSD tests for post-hoc analysis were also carried out in each of the three different ANOVA tests. The results showed that in the different time points (0 h, 6 h, 20 h) all the mean differences of the dependent variables were significant, except for 6 h and 20 h of the α -glucosidase activity and the values for all time points of β -xylosidase activity.

There are different mechanisms, which explain the fermentations of oligosaccharides by lactic acid bacteria: (i) splitting of oligosaccharides by extracellular enzymes with further transport and metabolism of monosaccharides, (ii) transport of oligosaccharides into the cell by specific oligosaccharide transport mechanisms for further hydrolysis by intracellular enzymes [62]. No specific trend was observed in the enzyme activity of *Lactobacillus* in the presence of PWP and PLM. It was probably due to the limited ability of the investigated strains to produce extracellular enzymes rather than intracellular enzymes which resulted in the predominant use of the final monosaccharide components. That hypothesis was confirmed by the results of the accumulated biomass. The amount of biomass was twice higher when using oligosaccharides compared to polysaccharides, but it was much less than that on glucose cultivation. In their work, Rossi et al. [52] also observed a strain specificity in extracellular enzyme production: some bacteria completely hydrolyzed FOS and inulin, while for others no extracellular hydrolytic activity against FOS and inulin was detected. Moreover, according to Madhukumar and Muralikrishna [62] the difference in the enzyme production could be due to the difference in degree of polymerization of oligosaccharides and their extent of purity.

4. Conclusions

The impact of non-digestible plant derived carbohydrates on human microbiota depends on the complex relationship between their chemical compositions, bacteria strain specificity and metabolism. The ability of pectin type water-extractable polysaccharides from *Plantago major* L. leaves, as well as their lower molecular weight hydrolysates, to stimulate the growth of some *Lactobacillus* strains was proven. The investigated probiotic bacteria utilized *P. major* carbohydrates fractions producing end metabolic products in different ratios. The obtained high amount of acetate and the established strain specificity of glycohydrolase enzymes induction are a prerequisite for an in-depth study of the prebiotic capacity of *P. major*. Furthermore, substantial in vitro and in vivo investigations on the correlation between the probiotic properties of lactobacilli and the prebiotic activity of *P. major* carbohydrates fractions could reveal their potential application as functional food with synbiotic characteristics.

Supplementary Materials: The statistical analyses are available online at <http://www.mdpi.com/2076-3417/10/8/2648/s1>, Table S1: tests of between-subject effects and Tukey's HSD test (Three-Way ANOVA), evaluating the statistical significance of the independent variables (bacterial strain, carbon source and time) and their combinations on the dependent variable (OD 600); Table S2. Tests of between-subject effects (three-way ANOVA), evaluating the statistical significance of the independent variables (bacterial strain, carbon source and time) and their combinations on the dependent variables (fermentation products: total lactic acid, acetic acid and ethanol); Table S3. Tests of between-subject effects (three-way ANOVA), evaluating the statistical significance of the independent variables (bacterial strain, carbon source and time) and their combinations on the dependent variables (enzyme activity: α -galactosidase, α -glucosidase and β -xylosidase activity).

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