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Structural studies of the exopolysaccharide from *Lactobacillus plantarum* C88 using NMR spectroscopy and the program CASPER

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ABSTRACT

Some lactic acid bacteria, such as those of the *Lactobacillus* genus, have the ability to produce exopolysaccharides (EPSs) that confer favorable physicochemical properties to food and/or beneficial physiological effects on human health. In particular, the EPS of *L. plantarum* C88 has recently demonstrated *in vitro* antioxidant activity and, herein, its structure has been investigated using NMR spectroscopy and the computer program CASPER. The pentasaccharide repeating unit of the *O*-deacetylated EPS consists of a trisaccharide backbone, $\rightarrow 4$)- α -D-Galp-(1 $\rightarrow$ 2)- α -D-Glcp-(1 $\rightarrow$ 3)- β -D-Glcp-(1 $\rightarrow$, with terminal D-Glc and D-Gal residues (1.0 and 0.8 equivalents per repeating unit, respectively) extending from O3 and O6, respectively, of the $\rightarrow 4$)- α -D-Galp-(1 $\rightarrow$ residue. In the native EPS an *O*-acetyl group is present, 0.85 equivalents per repeating unit, at O2 of the α -linked galactose residue; thus the repeating unit of the EPS has the following structure: $\rightarrow 4$)[β -D-Glcp-(1 $\rightarrow$ 3)][β -D-Galp-(1 $\rightarrow$ 6)] α -D-Galp2Ac-(1 $\rightarrow$ 2)- α -D-Glcp-(1 $\rightarrow$ 3)- β -D-Glcp-(1 $\rightarrow$. These structural features, and the chain length ($\sim 10^3$ repeating units on average), are expected to play an important role in defining the physicochemical properties of the polymer.

INTRODUCTION

Many strains of lactic acid bacteria (LAB) are able to produce exopolysaccharides (EPSs) on the bacterial cell wall to form a capsule or to be secreted into the surrounding growth medium to form loose slime.¹ EPSs produced by LAB have received increasing attention mainly because of the generally-regarded-as-safe (GRAS) status of the EPS-producing LAB and the remarkable physical and physiological functions of the EPSs.^{2,3} These natural biopolymers, which often are composed of oligosaccharide repeating units of sugar molecules such as glucose, galactose, or

rhamnose, etc., have been widely used as viscosifying, bioflocculating, stabilizing, gelling and emulsifying agents in the food industry.^{4,5} Some EPSs produced by LAB can be employed as natural texturizers in dairy processing and help protect the bacteria against harsh environmental conditions; resistance to gastrointestinal acids and bile salts is, for instance, a prerequisite for probiotic activity.⁶ Important health benefits such as immune stimulation, antitumor, cholesterol-lowering activity and antioxidant activities of fermented dairy products prepared with EPS-producing LAB or EPS itself have been investigated.⁷⁻⁹

Among EPS-producing LAB, *Lactobacillus plantarum* has frequently been isolated from food products, and it is also one of the most studied LAB species, particularly because some strains are considered to be probiotics due to several of their properties.¹⁰ *L. plantarum* 70810 isolated from Chinese Paocai was reported to produce an EPS with a high yield (0.859 g·L⁻¹), which could be used as a potential biosorbent for the removal of heavy metal from environment.¹¹ The EPS produced by *L. plantarum* KF5 was composed of mannose, glucose and galactose in an approximate ratio of 1:5:7, and the physicochemical characteristics of KF5 EPS were shown to differ from other commercially available gums, which imparted it potential applications in food industry.¹² *L. plantarum* EP56 was shown to produce two EPSs, one with low-molecular-mass that was released into the culture medium and another one with high-molecular-mass loosely, partially and temporarily linked to cells.¹³ The structure and biocompatibility of α -D-glucan (dextran) from probiotic *L. plantarum* DM5 and its unique physical and rheological properties facilitated its application in the food industry as a viscosifying and gelling agent.¹⁴

Previously, an EPS-producing strain of *L. plantarum* (C88) was isolated from traditional dairy Tofu in Inner Mongolia of China, and it showed prominent *in vitro* scavenging activity against hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, as well as *in vivo* antioxidant

activity as tested in a mice model.¹⁵ Recently, the EPS produced by *L. plantarum* C88 was confirmed to be involved in the antioxidant activity of this strain, since the purified EPS exhibited strong *in vitro* radical scavenging activity and antioxidant activity against H₂O₂-induced injury in Caco-2 cells.¹⁶ Here, we report the structural determination of the EPS produced by *L. plantarum* C88.

MATERIALS AND METHODS

Strain and culture media. *L. plantarum* C88 obtained from our laboratory collection was maintained at -80 °C in de Man Rogosa Sharpe (MRS) medium plus 20% glycerol.¹⁷ The strain was sub-cultured at least twice in MRS broth and transferred in semi-defined medium (SDM) using 1% (v/v) inoculation prior to experimental use.¹⁸

Isolation and purification of the EPS. After bacterial growth in SDM broth supplemented with 2% of glucose for 20 h at 37 °C, trichloroacetic acid (TCA) was added to the culture (1 L) to a final concentration of 4% (w/v), and the mixture was stirred for 30 min. Cells and precipitated proteins were removed by centrifugation (15 min, 10000×g, 4 °C), and the polysaccharide was precipitated from the supernatant by gradual addition of pre-chilled ethanol (4 °C) with 2 volumes of the supernatant. The pellet obtained by centrifugation (20 min, 10000×g, 4 °C) was dissolved in deionized water and extensively dialyzed (MW cut-off 3500 Da, Spectra/Por® 6, Spectrum, TX, U.S.A.) against water overnight at 4 °C. The dialyzed polysaccharide solution was then lyophilized to obtain the crude EPS (250 mg). A part of the crude EPS material (100 mg) was redissolved in water and subjected to ion exchange chromatography on a DEAE-Cellulose DE52 (Sigma) column (3.5 × 30 cm). Elution was performed with water at a flow rate of 0.5 mL·min⁻¹, and fractions (3.0 mL) were collected and

monitored for carbohydrates (phenol/sulfuric acid test: Absorbance at 490 nm). The fractions containing the EPS were pooled, desalted by dialysis against water for 48 h at 4 °C, and lyophilized.

***O*-deacetylation of the EPS.** The EPS (4 mg) was treated with 2 mL of 0.1 M NaOH for 19 h at 25 °C. The resulting solution was neutralized by addition of a cation exchange resin (Dowex 50H⁺), stirred for 10 min, filtered and freeze-dried. The product was purified by size exclusion chromatography on a SuperdexTM Peptide 10/300 GL (TricornTM) column (GE Healthcare) eluted with 1% 1-butanol in water at a flow rate of 1 mL·min⁻¹ with an ÄKTATM purifier system (GE Healthcare), to yield 2.2 mg of the *O*-deacetylated EPS. RI and UV detection at 214 nm were used to monitor elution.

Preparation of (+)-2-butyl glycosides. The EPS (1.8 mg, pH 7) was *O*-deacetylated by keeping the sample at 70 °C until disappearance of the ¹H NMR resonances of the methyl protons of the *O*-acetyl groups (~2 h). The *O*-deacetylated PS was then hydrolyzed with TFA (1.0 mL, 120 °C) for 30 min and the (+)-2-butyl glycosides prepared as previously described (the total butanolysis time was 40 h).¹⁹

NMR spectroscopy. The NMR spectra were recorded at 70 °C either on a Bruker Avance III 700 MHz spectrometer with dual receivers or on a Bruker Avance 500 MHz spectrometer, both equipped with 5 mm TCI (¹H/¹³C/¹⁵N) Z-Gradient (53.0 G cm⁻¹) CryoProbes. The chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-²H₄)-propanoate (TSP, δ_{H} 0.00) or external 1,4-dioxane in D₂O (δ_{C} 67.40) as references.

The ¹H decoupled ¹³C NMR spectrum of the (+)-2-butyl glycosides mixture from the EPS of *L. Plantarum* C88 was acquired on a 500 MHz spectrometer, whereas the ¹H and multiplicity-edited ¹H, ¹³C-HSQC NMR spectra were recorded on a 700 MHz spectrometer. The NMR spectra

of the *O*-deacetylated EPS (2.2 mg) were recorded in D₂O solution (0.55 mL) at a ¹H frequency of 500 MHz, with exception of the ¹H,¹³C-H2BC and PANSY (¹³C and ¹H,¹H-TOCSY) spectra that were recorded at the higher magnetic field. The NMR spectra of the native EPS (4.3 mg) were recorded in 25 mM phosphate buffer in D₂O (0.55 mL, pD 5) on a 700 MHz spectrometer.

The ¹H,¹H-TOCSY spectra²⁰ were obtained employing echo/antiecho gradient selection, an MLEV-17 spin-lock of 10 kHz and mixing times of 20, 40, 60 and 100 ms. The multiplicity-edited ¹H,¹³C-HSQC experiments²¹ were acquired employing the echo/antiecho method; adiabatic pulses^{22,23} were used for ¹³C inversion (20% smoothed CHIRP, 60 kHz, 500 μs) and refocusing (composite smoothed CHIRP, 60 kHz, 2.0 ms). The ¹H,¹³C-H2BC spectra²⁴ were recorded with a constant-time delay of 22 ms. The gradient selected ¹H,¹³C-HMBC experiments²⁵ were carried out with an evolution times of 60-65 ms, and the correlations to the carbonyl carbon in the native EPS were determined from the aliased spectrum. The gradient selected ¹H,¹H-NOESY and ¹H,¹³C-HSQC-NOESY experiments²⁶ were recorded with mixing times of 100 ms. During the PANSY experiment²⁷ a ¹H decoupled ¹³C NMR spectrum was recorded on a parallel receiver during the 120 ms of a DIPSI spin-lock of a ¹H,¹H-TOCSY experiment. The 2D spectrum of the PANSY experiment was recorded with the States-TPPI method over a spectral width of 3 ppm with 2k × 256 data points in *F2* and *F1*, respectively, and 16 scans per increment. The ¹³C spectrum of the PANSY experiment was recorded with 9k data points over a spectral region of 240 ppm. Zero-filling to 65k points, linear prediction to 36k points and an exponential window function (line broadening factor of 3 Hz) were applied prior to Fourier transformation. The 1D diffusion-filtered ¹H NMR spectrum of the native EPS was recorded using the 1D stimulated spin-echo pulse sequence with bipolar gradients and LED

(ledbpgp2s1d).²⁸ Diffusion encoded sinusoidal gradients pulses ($\delta/2$) of 1.5 ms and strength 30% of the maximum were used, and the diffusion time was set to 100 ms.

RESULTS AND DISCUSSION

The ^1H NMR spectrum of the native EPS of *L. plantarum* C88 showed a resonance at 2.211 ppm (3H, singlet) that disappeared after treatment with aqueous 0.1 M NaOH, indicating the presence of an *O*-acetyl group. The monosaccharide components of the EPS, and their absolute configuration, were determined using a methodology previously developed in our laboratory.^{19,29} In this method, the pre-hydrolyzate of the *O*-deacetylated EPS was subjected to (+)-2-butanolysis and the unassigned NMR data, from both 1D ^{13}C and 2D multiplicity-edited $^1\text{H},^{13}\text{C}$ -HSQC spectra, were used as input information in the ‘component analysis’ module of the web interface of the CASPER program^{30,31}([Table S1 in the Supporting Information](#)). The results (calculation time ~ 3 s) revealed two possible monosaccharide components: D-Glc and D-Gal ([Table S2 in the Supporting Information](#)). The resonances from the different pyranose and/or furanose forms of each monosaccharide derivative were identified by comparison of the anomeric region of the ^1H NMR spectrum of the (+)-2-butyl glycosides obtained from the EPS of *L. plantarum* C88 ([Fig. 1a](#)) with those of D-Glc ([Fig. 1b](#)) and D-Gal ([Fig. 1c](#)). In addition, the anomeric region of $^1\text{H},^{13}\text{C}$ -HSQC spectrum of the (+)-2-butyl glycosides of the hydrolyzate of the *L. plantarum* C88 EPS ([Fig. 1d](#)) is a perfect match to the overlay of the $^1\text{H},^{13}\text{C}$ -HSQC spectra of D-Glc (gray color in [Fig. 1e](#)) and D-Gal (black color in [Fig. 1e](#)). Integration of the respective resonances in the ^1H NMR spectrum revealed a ratio of D-Glc and D-Gal of 2:1, respectively, that is consistent with previous reports.¹⁶

The structural analysis of the *O*-deacetylated EPS of *L. plantarum* C88 was carried out by submitting selected unassigned NMR data to the ‘determine structure’ module of the CASPER program (cf. [Table S3 in the Supporting Information](#)). Thus, $^3J_{H1,H2}$ (two of 2-7 Hz and three > 7 Hz) and $^1J_{C1,H1}$ (three < 169 Hz and two > 169 Hz) couplings were extracted from 1D 1H ([Fig. 2a](#)) and 2D coupled multiplicity-edited $^1H,^{13}C$ -HSQC NMR spectra, respectively. ^{13}C chemical shifts were obtained from a 1D ^{13}C NMR spectrum ([Fig. 2b](#)), and 1H - ^{13}C correlations from a 2D multiplicity-edited $^1H,^{13}C$ -HSQC spectrum ([Fig. 2d-e](#)) were used to correlate the ^{13}C resonances to their directly attached protons. Additional data from a 2D $^1H,^1H$ -TOCSY spectrum ([Fig. 2c](#)) was also used to assist in the assignment of the respective 1H spin systems of each monosaccharide component. One should note that the ^{13}C and $^1H,^1H$ -TOCSY spectra were obtained simultaneously using the parallel detection technique (PANSY),²⁷ thus reducing the total acquisition time of both spectra to ~3 h. Since five anomeric resonances corresponding to hexopyranosyl residues were found in the anomeric region of the multiplicity-edited $^1H,^{13}C$ -HSQC spectrum ([Fig. 2e](#)), and the component analysis revealed a ratio of D-Glc:D-Gal of 2:1, the number of equivalents per repeating unit of these monosaccharides was anticipated to be 3 and 1.5, respectively. Thus, the structural information was given as follow: three D-Glcp and two D-Galp, each with all the linkage positions marked as unknown. The calculation (~3 min) returned a list of ten possible structures ranked according to the deviation between predicted and experimental 1H and ^{13}C chemical shifts ([Table S4 in the Supporting Information](#)); a significant score difference was observed between the structures ranked in the first and second positions ([Fig. 3](#)). The CASPER report for the top-ranked structure, including its 1H and ^{13}C chemical shifts assignments, is shown in the [Supporting Information](#). The different residues in the *O*-deacetylated EPS were named **A-E** in order of decreasing chemical shifts of their anomeric

proton resonances (cf. Fig. 2a) and, according to assignments predicted by CASPER, residues **A**, **C** and **D** were assigned to glucosyl residues whereas residue **B** and **E** were assigned to galactosyl residues. This is consistent with the correlation patterns, from the anomeric protons, observed in the ^1H , ^1H -TOCSY spectrum (Fig. 2c), where protons up to H6 can be traced in residues with the *gluco*-configuration (**A**, **C** and **D**) and only those up to H4 can be identified in residues **B** and **E** (which is typical for hexopyranosyl residues with the *galacto*-configuration, and attributed to their small $^3J_{\text{H4,H5}}$ coupling). The configurations of the anomeric centers were confirmed by analysis of the $^1J_{\text{C1,H1}}$ couplings, residues **A** and **B** are α -linked since they both have $^1J_{\text{C1,H1}}$ couplings of 171 Hz whereas the remaining residues (**C**, **D** and **E**) are β -linked since they have $^1J_{\text{C1,H1}}$ couplings of 159-163 Hz. The ^1H and ^{13}C chemical shifts assignments of the *O*-deacetylated EPS of *L. plantarum* are compiled in Table 1, and they were compared to those of the corresponding monosaccharides.³² The glycosylation shifts³³ are then consistent with the glycosylation patterns of the candidate structure suggested by CASPER: residue **A** is $\rightarrow 2)$ - α -D-Glcp-(1 $\rightarrow$ since $\Delta\delta_{\text{C2,A}}$ is 5.20, residue **B** is $\rightarrow 3,4,6)$ - α -D-Galp-(1 $\rightarrow$ since $\Delta\delta_{\text{C3,B}}$, $\Delta\delta_{\text{C4,B}}$ and $\Delta\delta_{\text{C6,B}}$ are 9.45, 7.31 and 8.02, respectively, and residue **C** is $\rightarrow 3)$ - β -D-Glcp-(1 $\rightarrow$ since $\Delta\delta_{\text{C3,B}}$ is 6.80. Residues **D** and **E** do not show any significant ^{13}C glycosylation shifts for the C2 – C6 atoms and thus are terminal β -D-Glcp-(1 $\rightarrow$ and β -D-Galp-(1 $\rightarrow$ residues, respectively (Table 1). Furthermore, the structure on the top of the list of Fig. 3 could readily be confirmed as the correct structure using additional 2D NMR spectra, such as ^1H , ^{13}C -H2BC (Fig. 4 top), ^1H , ^{13}C -HMBC (Fig. 4 bottom) and ^1H , ^1H -NOESY spectra. The inter-residue correlations from anomeric atoms observed in the ^1H , ^1H -NOESY and ^1H , ^{13}C -HMBC spectra are compiled in Table 1.

Additional resonances, attributed to a minor component, were also observed in the NMR spectra (denoted with primed characters in Fig. 2). In particular, a spin system similar to residue

B, but with slightly altered chemical shifts, was identified using ^1H , ^1H -TOCSY, ^1H , ^1H -NOESY and multiplicity-edited ^1H , ^{13}C -HSQC spectra. The ^1H and ^{13}C chemical shifts of residue **B'** are shown in Table 1. Since significant ^{13}C glycosylation shifts were observed only at C3 and C4 ($\Delta\delta_{\text{C}}$ 9.45 and 6.91, respectively), residue **B'** is consistent with a 3,4-di-substituted D-Galp residue. This was also supported by correlations observed in the ^1H , ^1H -NOESY spectrum from the resonances at 4.698 (attributed to H1 in residue **D'**) and 4.882 ppm (attributed to H1 in residue **C'**) to H3 and H4 in residue **B'**, respectively. Thus, residues **D'** and **C'** are linked to O3 and O4 of residue **B'**, respectively. Furthermore, two additional cross-peaks were observed in the multiplicity-edited ^1H , ^{13}C -HSQC spectrum at $\delta_{\text{H}}/\delta_{\text{C}}$ 5.499/97.32 and 3.706/76.81, and attributed to H1/C1 and H2/C2 of residue **A'**, respectively. Therefore, residue **B** and **B'** only differ in the presence or absence, respectively, of a terminal galactosyl residue (**E**) at O6. The experimental ^1H and ^{13}C chemical shifts of the minor component structure (Table 1) are in very good agreement with those predicted using the CASPER program (cf. Table S5 and Fig. S1 in the Supporting Information). Integration of the H5 resonance of residue **B'** in the ^1H NMR spectrum revealed that this monosaccharide component represents ~0.2 equivalents per repeating unit, and thus residue **E** represents 0.8 equivalents. This is consistent with amounts of D-Glc and D-Gal of 3 and 1.8 equivalents per repeating unit, respectively, which are similar to the values determined in the component analysis (3 and 1.5 equivalents per repeating unit, respectively).

The ^1H and ^{13}C NMR spectra of the native EPS (Fig. 5a and 5b, respectively) revealed a complex material with a single peak in the region for methyl groups of *O*-acetyl moieties (δ_{H} 2.211 and δ_{C} 21.58, respectively). The location of the *O*-acetyl group was determined using the CASPER program. In this regard, the structural information available from the *O*-deacetylated polysaccharide was submitted as input information to the ‘determine structure’ module of the

program (this is, monosaccharide components, sequence and linkage positions), in addition to ^1H - ^{13}C correlations from a multiplicity-edited ^1H , ^{13}C -HSQC spectrum of the native EPS (Fig. 5c-d and Table S6 in the Supporting Information). In the latter, only those cross-peaks of minimum relative intensity of 10% (with respect to the strongest cross-peak) were considered in the peak picking process. The calculation (~3 s) returned a list of ten possible structures (Table S7 in the Supporting Information) with a large score difference between the first and second top-ranked structures (1.00 and 1.64, respectively). The candidate structure then contains a 2-*O*-acetylated 3,4,6-tri-substituted α -D-Galp residue (**B**). The ^1H and ^{13}C chemical shifts assignments of the native EPS (compiled in Table 2) were carried out in a straightforward manner using the predictions made by CASPER (Table S8 in the Supporting Information) and data from 2D NMR experiments. From comparison of the multiplicity-edited ^1H , ^{13}C -HSQC spectrum of the native EPS (Fig. 5c-d) with that of the *O*-deacetylated EPS (Fig. 2d-e) it is evident that only those correlations from residue **B** show significant chemical shifts differences that could be attributed to perturbations due to *O*-acetylation.^{34,35} Since residue **B** is a 3,4,6-tri-substituted galactosyl residue, the only possible *O*-acetylation position in this residue is at O2. This was confirmed by an ^1H , ^{13}C -HMBC correlation, in the aliased NMR spectrum, from the resonance at 173.95 ppm (CO) to the protons at 2.211 (*O*-acetyl methyl protons) and 5.358 ppm (H2 in residue **B**).

In addition to the major component, two minor sets of resonances were also identified in the NMR spectra of the native EPS. One set of signals is consistent with the chemical shifts of the *O*-deacetylated material (Table 1), and the integration of the resonance at 5.493 ppm (H1 in residue **A** in the *O*-deacetylated material) in the ^1H NMR spectrum revealed that this component represents 15% of the repeating units in the polymer. The second set of signals is similar to that of the major component, but with slightly altered chemical shifts (denoted with double primed

characters in Fig. 5d). For instance, most of the proton resonances in residue **A''** were traced using ^1H , ^1H -TOCSY spectra, and they were found at 5.683 (H1), 3.711 (H2), 3.915 (H3), 3.503 (H4), 4.091 (H5) ppm. Furthermore, two inter-residue correlations were observed in the ^1H , ^1H -NOESY spectrum from the anomeric proton in residue **A''** to H1 in residue **B''** (5.380 ppm) and H3 in residue **C''** (3.734 ppm). The ^{13}C chemical shifts assignments of C1 (δ_{C} 95.27) and C2 (δ_{C} 75.09) in residue **A''**, C1 in residue **B''** (δ_{C} 93.92) and C3 in residue **C''** (δ_{C} 80.37) were obtained from the multiplicity-edited ^1H , ^{13}C -HSQC spectrum. Additionally, a correlation was observed in the ^1H , ^{13}C -HSQC-NOESY spectrum from H1 in residue **A''** to C3 in residue **C''**. Integration of the resonance at 5.683 ppm (H1 in residue **A''**) in the ^1H NMR spectrum revealed that this minor component also represents 0.15 equivalents per repeating unit, indicating that this may be an *O*-acetylated repeating unit in which one of the neighboring repeating units does not carry an *O*-acetyl group. Integration of the methyl protons resonances of the *O*-acetyl group (2.211 ppm) with respect to those of the anomeric resonances of the three populations of $\rightarrow 2)\text{-}\alpha\text{-D-Glcp-(1}\rightarrow$ residues (5.683, 5.652 and 5.493 ppm) established that the *O*-acetyl group represents 2.6 equivalents per repeating unit. Therefore, the structure of the native EPS of *L. plantarum* C88 is as shown in Fig. 6. The average molecular weight of a previously reported *L. plantarum* C88 EPS preparation was estimated to be 1.15×10^6 Da;¹⁶ this data suggests that the number of repeating units in the EPS is on the order of 10^3 on average.

The very good agreements between experimental and CASPER predicted ^1H and ^{13}C NMR chemical shifts in both the *O*-deacetylated and native EPSs are illustrated in the plots of Fig. 7. One should note that the ^1H and ^{13}C resonances of carbohydrates are usually poorly dispersed, and these plots only present a spectral region spanning 2.6 ppm in the case of δ_{H} and 50 ppm in the case of δ_{C} . At the $\alpha\text{-(1}\rightarrow 3)\text{-}$ linkage between residues **A** and **C** of the native EPS the ^{13}C

NMR chemical shifts appear at lower values (upfield) than those predicted by CASPER ($\Delta\delta_{C1,A} - 1.6$ and $\Delta\delta_{C3,C} - 2.7$, cf. [Fig 7 top right](#)); this indicates that reduced flexibility and steric crowding as well as a change in conformational preferences have occurred at this glycosidic linkage³³ compared to the constituent disaccharide.³⁶ The same phenomenon is observed in the case of H4 in residue **C** ($\Delta\delta_{H4,C} - 0.2$, cf. [Fig 7 bottom left](#)) and may be attributed to the close proximity of this proton to the methyl protons of the *O*-acetyl group. This was confirmed by a $^1H, ^1H$ -NOESY correlation observed from the methyl protons of the *O*-acetyl group to a proton at 3.453 ppm (H4 in residue **C**), and a correlation in the $^1H, ^{13}C$ -HSQC-NOESY spectrum from the same methyl protons to a carbon at 72.10 ppm (C4 in residue **C**).

In order to improve the technological and functional properties of this kind of biopolymers it is of key importance to have an understanding of the relationship between their structures and physicochemical characteristics. For instance, the viscosity imparted to an aqueous solution of an EPS can, among other things, be influenced by the stiffness of the polymer chain. Furthermore, it has been reported that monosaccharide residues connected via β -(1 $\rightarrow$ 4)-linkages may contribute to stiffer chains when compared to α -(1 $\rightarrow$ 4)- or β -(1 $\rightarrow$ 3)-linkages.^{4,37,38} In this regard, the structures ranked in the first and fourth positions in [Fig. 3](#) are positional isomers; in the former the β -D-Glcp-(1 $\rightarrow$ 4)-D-Galp moiety is part of the backbone whereas in the latter it constitutes a lateral chain. This illustrates the importance of a correct and unambiguous structural characterization, since these two polymers may display different dynamic behaviors. On the other hand, the presence of side-chains^{39,40} can decrease the polymer flexibility, and the removal of terminal linked galactosyl residues have been reported to increase it.³⁸ This may be relevant in the case of the EPS of *L. plantarum* C88, in which one of the two lateral chains (a terminal galactosyl residue) is absent in 20% of the EPS repeating units. Likewise, the *O*-deacetylation of

the native EPS seems to induce a conformational change at one of the glycosidic linkages, which could be associated to an increased flexibility of the polymer. Thus, it would be of interest to evaluate if changes in the bacterial growth conditions⁴¹ could lead to EPSs with different degrees of degalactosylation and/or *O*-deacetylation, and whether these changes may influence the viscosifying effect of the EPS.

CONCLUSIONS

These results demonstrate that the CASPER program is not only capable to deduce the structure of a polymer composed of regular monosaccharide components in rapid and efficient manner, but also determine the exact location of the *O*-acetyl group substituent. Once the NMR data was acquired and processed, the CASPER program was able to deduce the correct structure in a total time of ~3 min. The EPS of *L. plantarum* C88 is composed of a doubly branched pentasaccharide structure, in which one of the lateral chains (consisting of a terminal D-Galp residue) is partially absent. Further studies are then required to evaluate the connection between the growth conditions and the degree of 6-*O*-degalactosylation and/or 2-*O*-deacetylation of the 3,4,6-trisubstituted α -D-Galp2Ac residue, and how this could influence the viscosity of the EPS solution for technological applications.

ASSOCIATED CONTENT

Supporting Information.

Figure S1, Tables S1-S8 and CASPER report for the top-ranked *O*-deacetylated structure. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.

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ABBREVIATIONS

DEAE, Diethylaminoethanol; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EPS, exopolysaccharide; GRAS, generally-regarded-as-safe; LAB, lactic acid bacteria; L. Lactobacillus; MI, minimum relative intensity; MRS, Man Rogosa Sharpe; MW, molecular weight; NMR, nuclear magnetic resonance; PANSY, parallel acquisition NMR spectroscopy; PS, polysaccharide; SDM, semi-defined medium; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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Table 1. ^1H and ^{13}C NMR chemical shifts (ppm) at 70 °C of the resonances of the *O*-deacetylated EPS of *Lactobacillus plantarum* C88 and inter-residue correlations from ^1H , ^1H -NOESY and ^1H , ^{13}C -HMBC spectra.

Sugar residue		$^1\text{H}/^{13}\text{C}$						Correlation to atom (from anomeric atom)	
		1	2	3	4	5	6	NOE	HMBC
$\rightarrow 2$)- α -D-Glcp-(1 $\rightarrow$	A	5.495 [4.0]	3.730	3.886	3.544	4.094	~3.831	H3, C	C3, C
		(0.27)	(0.19)	(0.17)	(0.12)	(0.25)			
		97.51 {171}	77.67	72.44	70.32	72.30	61.37		H3, C
		(4.52)	(5.20)	(-1.34)	(-0.39)	(-0.07)	(-0.47)		
$\rightarrow 3,4,6$)- α -D-Galp-(1 $\rightarrow$	B	5.231 [n.r.] ^a	4.187	4.187	4.501	4.468	3.890, 4.109	H2, A H1, A	C2, A
		(0.01)	(0.41)	(0.38)	(0.55)	(0.44)			
		97.85 {171}	68.41	79.58	77.59	70.80	70.06		H2, A
		(4.67)	(-0.94)	(9.45)	(7.31)	(-0.50)	(8.02)		
$\rightarrow 3$)- β -D-Glcp-(1 $\rightarrow$	C	4.861 [7.7]	3.425	3.666	3.644	3.417	3.746, 3.905	H4, B	C4, B
		(0.22)	(0.18)	(0.17)	(0.22)	(-0.04)			
		103.30 {163}	73.26	83.56	71.01	76.22	61.80		H4, B
		(6.46)	(-1.94)	(6.80)	(0.30)	(-0.54)	(-0.04)		
β -D-Glcp-(1 $\rightarrow$	D	4.687 [7.5]	3.395	3.533	3.426	3.474	3.747, 3.934	H3, B	C3, B
		(0.05)	(0.15)	(0.03)	(0.01)	(0.01)			
		104.80 {160}	74.50	76.57	70.75	76.88	61.91		H3, B
		(7.96)	(-0.70)	(-0.19)	(0.04)	(0.12)	(0.07)		
β -D-Galp-(1 $\rightarrow$	E	4.445 [7.8]	3.547	3.653	3.942	3.684	~3.790		C6, B
		(-0.09)	(0.10)	(0.06)	(0.05)	(0.03)			
		104.13 {159}	71.77	73.65	69.58	75.97	61.80	H6a, B H6b, B	H6a, B H6b, B
		(6.76)	(-1.19)	(-0.13)	(-0.11)	(0.04)	(-0.04)		
$\rightarrow 3,4$)- α -D-Galp-(1 $\rightarrow$	B'	5.227	4.163	4.187	4.485	4.268	3.713, 3.804		
		(0.01)	(0.38)	(0.38)	(0.54)	(0.24)			
		96.32	68.48	79.58	77.19	71.21	61.41		
		(3.14)	(-0.87)	(9.45)	(6.91)	(-0.09)	(-0.63)		

³ $J_{\text{H1,H2}}$ values are given in Hertz in square brackets and ¹ $J_{\text{H1,C1}}$ values in braces. Chemical shift differences as compared to corresponding monosaccharides are given in parentheses.³² The spin system **B'** correspond to a minor component equivalent to 0.2 equivalents per repeating unit. Additional chemical shifts assignments from the minor component are: $\delta_{\text{H}}/\delta_{\text{C}}$ 5.499/97.32 (H1/C1 in residue **A'**) and 3.706/76.81 (H2/C2 in residue **A'**); δ_{H} 4.698 (H1 in residue **D'**) and 4.882 (H1 in residue **C'**).

^a n.r. = not resolved.

Table 2. ^1H and ^{13}C NMR chemical shifts (ppm) at 70 °C of the resonances of the native EPS of *Lactobacillus plantarum* C88.

Sugar residue		$^1\text{H}/^{13}\text{C}$							Me	CO
		1	2	3	4	5	6			
$\rightarrow 2)$ - α -D-Glcp-(1 $\rightarrow$	A	5.652	3.765	3.930	3.525	4.085	3.808, 3.848			
		(0.42)	(0.23)	(0.21)	(0.11)	(0.25)				
		95.69	75.94	72.19	70.16	72.19	61.32			
		(2.70)	(3.47)	(-1.59)	(-0.55)	(-0.18)	(-0.52)			
$\rightarrow 3,4,6)$ - α -D-Galp2Ac-(1 $\rightarrow$	B	5.390	5.358	4.438	4.578	4.447	3.906, 4.117	2.211		
		(0.17)	(1.58)	(0.63)	(0.63)	(0.42)				
		94.39	70.42	76.93	77.14	70.92	70.05	21.58	173.95	
		(1.21)	(1.07)	(6.80)	(6.86)	(-0.38)	(8.01)			
$\rightarrow 3)$ - β -D-Glcp-(1 $\rightarrow$	C	4.868	3.418	3.734	3.463	3.416	3.729, 3.923			
		(0.23)	(0.17)	(0.23)	(0.04)	(-0.04)				
		103.01	73.23	80.79	72.10	76.45	61.64			
		(6.17)	(-1.97)	(4.03)	(1.39)	(-0.31)	(-0.20)			
β -D-Glcp-(1 $\rightarrow$	D	4.585	3.292	3.504	3.407	3.463	3.734, 3.926			
		(-0.05)	(0.04)	(0.00)	(-0.01)	(0.00)				
		105.02	73.93	76.45	70.69	76.78	61.81			
		(8.18)	(-1.27)	(-0.31)	(-0.02)	(0.02)	(-0.03)			
β -D-Galp-(1 $\rightarrow$	E	4.441	3.535	3.646	3.935	3.683	~3.777			
		(-0.09)	(0.09)	(0.06)	(0.04)	(0.03)				
		104.13	71.69	73.63	69.51	75.99	61.75			
		(6.76)	(-1.19)	(-0.13)	(-0.11)	(0.04)	(-0.04)			

Chemical shift differences as compared to corresponding monosaccharides are given in parentheses.³²

FIGURES AND LEGENDS

Figure 1. ^1H NMR spectra of the (+)-2-butyl glycosides of: **a)** the hydrolyzate of the EPS of *Lactobacillus plantarum* C88, **b)** D-glucose and **c)** D-galactose. The ratio of D-Glc and D-Gal in the ^1H NMR spectrum of panel **a** is 2:1, respectively. **d)** The anomeric region of the ^1H , ^{13}C -HSQC spectrum of the (+)-2-butyl glycosides of the hydrolyzate of the EPS of *Lactobacillus plantarum* C88 recorded at a ^1H frequency of 700 MHz. **e)** Overlay of the anomeric region of the ^1H , ^{13}C -HSQC spectra of the (+)-2-butyl glycosides of D-glucose and D-galactose (in grey and black color, respectively).

Figure 2. The ^1H and ^{13}C NMR spectra of the *O*-deacetylated EPS of *Lb. plantarum* C88 (a and b, respectively) and selected region of the ^1H , ^1H -TOCSY spectrum (c) showing correlations from anomeric protons and the H4 and H5 protons in residue **B**. The ^{13}C NMR spectrum with proton decoupling was acquired during the spin-lock of the ^1H , ^1H -TOCSY experiment ($\tau_m = 120$ ms) using a PANSY experiment. Selected regions of the multiplicity-edited ^1H , ^{13}C -HSQC NMR spectrum showing the region for the ring atoms and hydroxymethyl groups (in which the cross-peaks from the latter appear in red) (d) and the anomeric region (e). Resonances from anomeric and substitution positions are annotated, as well as all the resonances of residue **B**. Resonances from minor spin systems are indicated with primed characters.

Figure 3. CASPER output of the five top-ranked structures for the *O*-deacetylated EPS of *Lb. plantarum* C88, in CFG format (blue and yellow filled circles represent glucopyranose and galactopyranose residues, respectively). The relative deviations for structures 1-5 are 1.00, 1.15, 1.16, 1.16 and 1.19, respectively. For standard carbohydrate listing of the ten top-ranked structures see [Table S4 in the Supporting Information](#).

Figure 4. Selected regions of the $^1\text{H}, ^{13}\text{C}$ -H2BC and HMBC NMR spectra (*top* and *bottom*, respectively) spectrum showing correlations from anomeric protons and the H4 and H5 protons in residue **B**.

Figure 5. **a)** Diffusion-filtered ^1H NMR spectrum (the residual signal from the HDO peak is denoted by an asterisk) and **b)** ^1H -decoupled ^{13}C spectrum of the native EPS of *Lactobacillus plantarum* C88. The resonances from the *O*-acetyl group are indicated with black filled triangles. **c-d)** Selected regions of the multiplicity-edited $^1\text{H},^{13}\text{C}$ -HSQC spectrum of the native EPS of *Lactobacillus plantarum* C88 showing the region for the ring atoms and hydroxymethyl groups (in which the cross-peaks from the latter appear in red) (**c**) and the anomeric region (**d**). Resonances from anomeric and substitution positions are annotated, as well as all the resonances of residue **B**. Cross-peaks originating from a minor spin system are indicated with double primed capital letters.

Figure 6. Structure of the repeating unit of the EPS of *Lactobacillus plantarum* C88 in CFG-notation (*top*) and standard nomenclature (*bottom*).

Figure 7. Comparison between experimental and CASPER-predicted ^1H and ^{13}C chemical shifts (bottom and top, respectively) of the *O*-deacetylated and native repeating units (left and right, respectively) of the EPS of *Lactobacillus plantarum* C88. The ^1H and ^{13}C chemical shifts of the *O*-acetyl group are not shown in the panels. The largest chemical shift deviations are annotated.

Table of contents graphic