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NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoc citreum* and *Weissella confusa*

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Abstract—Dextrans are the main exopolysaccharides produced by *Leuconostoc* species. Other dextran-producing lactic acid bacteria include *Streptococci*, *Lactobacilli*, and *Weissella* species. Commercial production and structural analysis has focused mainly on dextrans from *Leuconostoc* species, particularly on *Leuconostoc mesenteroides* strains. In this study, we used NMR spectroscopy techniques to analyze the structures of dextrans produced by *Leuconostoc citreum* E497 and *Weissella confusa* E392. The dextrans were compared to that of *L. mesenteroides* B512F produced under the same conditions. Generally, *W. confusa* E392 showed better growth and produced more EPS than did *L. citreum* E497 and *L. mesenteroides* B512F. Both *L. citreum* E497 and *W. confusa* E392 produced a class 1 dextran. Dextran from *L. citreum* E497 contained about 11% α-(1→2) and about 3.5% α-(1→3)-linked branches whereas dextran from *W. confusa* E392 was linear with only a few (2.7%) α-(1→3)-linked branches. Dextran from *W. confusa* E392 was found to be more linear than that of *L. mesenteroides* B512F, which, according to the present study, contained about 4.1% α-(1→3)-linked branches. Functionality, whether physiological or technological, depends on the structure of the polysaccharide. Dextran from *L. citreum* E497 may be useful as a source of prebiotic gluco-oligosaccharides with α-(1→2)-linked branches, whereas *W. confusa* E392 could be a suitable alternative to widely used *L. mesenteroides* B512F in the production of linear dextran.

Keywords: Exopolysaccharides; Dextrans; *Leuconostoc citreum*; *Leuconostoc mesenteroides*; *Weissella confusa*; H2BC

1. Introduction

Exopolysaccharide (EPS) production by lactic acid bacteria (LAB) has a great commercial potential since most LAB are generally regarded as safe. Current challenges in the utilization of EPSs from LAB include not only strain improvement and enhancement of EPS production, but also production of EPSs with specific structures and sizes that impart the desired functional properties.[1,2] Studies have shown that LAB produce both heteropolysaccharides and homopolysaccharides. The former are composed of different monosaccharides, such as D-glucose, D-galactose, and L-rhamnose, and may contain acetamido sugars, phosphates, or acetyl groups whereas the latter are composed of only one monosaccharide, such as D-glucose (α-glucans or β-glucans), D-fructose (fructans), or D-galactose (polygalactans).[1,3]

EPSs produced by *Leuconostoc* are primarily dextrans.[4,5] Other LAB that produce dextrans include various *Streptococcus* and *Lactobacillus* species.[6] Additionally, dextran production has typically served as a phenotypic test in the identification of bacteria classified in the genus *Weissella*.[7] Collins et al.[7] proposed the genus *Weissella* in 1993 during a study of *Leuconostoc*-like micro-organisms. The genus now includes eight species: *Weissella confusa*, *Weissella halotolerans*, *Weissella*

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kandleri, *Weissella minor*, *Weissella viridescens*, *Weissella paramesenteroides*, *Weissella thailandensis*, and *Weissella hellenica*. The first five species were formerly affiliated to the genus *Lactobacillus,* and the sixth to the genus *Leuconostoc*.[8]

Dextrans are α-glucans with a linear backbone made of α-(1→6)-linked D-glucopyranosyl units. They are divided into three classes according to their structural features. Class 1 dextrans have consecutive α-(1→6)-linked D-glucopyranosyl units with α-(1→2), α-(1→3), or α-(1→4)-linked branches. Class 2 dextrans (alternans) contain alternating α-(1→3) and α-(1→6) linkages with α-(1→3)-linked branches. Class 3 dextrans (mutans) have consecutive α-(1→3) linkages with α-(1→6)-linked branches.[9] Class 1 dextrans vary in their type and degree of branching, length of branch chains, spatial arrangement, and molecular weight.[9–11] According to Jeanes et al.,[4] based on periodate oxidation-reaction analysis, the amount of α-(1→6) linkages in a specific dextran can vary from 50% to 97% of the total number of glycosidic linkages. These structural variations depend on cultivation conditions and on the producing strains.[4,12,13]

Commercially produced dextran is used for various purposes in its native or chemically modified form. Native dextrans are used in clinical applications as blood-plasma substitutes, as standards for size-exclusion chromatography, and as ingredients in cosmetics, bakery, and frozen dairy products.[14] The production of functional polymers by chemical modification of dextrans has recently been reviewed.[14] Dextran derivatives include cross-linked dextrans used in the production of Sephadex columns and bioactive dextran derivatives such as dextran sulfates and phosphates.[14] Presently, interest in dextran-producing strains and their respective dextransucrases for the production of prebiotic gluco-oligosaccharides (GOS) is growing.[15,16]

Commercial production and structural analysis of dextrans has mainly focused on dextrans from *Leuconostoc* species, particularly strains of *Leuconostoc mesenteroides*.[4,5,9–11,14,17] In contrast, structural analysis of dextrans from *Leuconostoc citreum* and *Weissella* species has received little attention. *L. citreum* NRRL B-742 (formerly *L. mesenteroides* NRRL B-742)[13] has been reported to produce a dextran with both α-(1→3) and α-(1→4)-linked branches.[5] Recently, Olivares-lllana et al.[18] isolated *L. citreum* CW28 from *Pozol* (a fermented corn beverage), which produced an inulin-like polysaccharide.

In this study, we report on the structure of dextrans produced by *L. citreum* E497 and *W. confusa* E392. The dextrans were compared to that of *L. mesenteroides* B512F produced under the same conditions. Structural analysis was carried out using one- and two-dimensional (1D and 2D) nuclear magnetic resonance spectroscopy techniques.

2. Results

2.1. Isolation of exopolysaccharides

EPSs were extracted from the cell mass of *W. confusa* E392 and *L. citreum* E497 grown on De Man, Sharpe and Rogosa agar supplemented with 2% (w/v) sucrose (MRS-S). For comparison, EPSs from *L. mesenteroides* B512F were also produced and extracted under the same conditions. In general, *W. confusa* E392 showed better growth and produced more EPS than did *L. citreum* E497 and *L. mesenteroides* B512F. The cell mass from *L. mesenteroides* B512F was more liquid, less viscous and dried more easily on the media surface compared to that of *L. citreum* E497 and *W. confusa* E392. Protein assays carried out using the Bradford protein assay kit indicated negligible soluble protein content in the extracts. This merited exclusion of protein purification procedures. Monosaccharide composition analysis by gas chromatography (GC), after methanolysis and trimethylsilylation, showed that EPSs from *L. citreum* E497 and *W. confusa* E392 contained only glucose, thus indicating that EPSs from these strains are glucans. The recovery of glucose from the extracted EPS (dry matter) was 84.3 ± 4% and 83.6 ± 2%, respectively.

2.2. Structural analysis by NMR spectroscopy

2.2.1. Evaluation of the anomeric signals in the NMR spectra. The 1D ^{1}H spectra of EPS from *L. citreum* E497 and *W. confusa* E392 are shown in Figures 1 and 2, respectively. The ^{1}H spectrum of EPS from *L. mesenteroides* B512F (not shown) resembled that of EPS from *W. confusa* E392. The peak at 4.52 ppm in the spectra is from HDO. The ^{1}H spectra of EPS extracted from *L. citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F showed a typical dextran α-(1→6) chain-extending anomeric signal centered at 4.98 ppm.[10,19] The vicinal coupling constant of this main chain anomeric signal (δ_H 4.98 ppm, d, $J_{1,2}$ 3.2 Hz, H-1), more clearly seen in the 1D ^{1}H spectrum of EPS extracted from *W. confusa* E392 (Fig. 2), was consistent with α-linked glucose units. The spectra of the extracted EPSs also had anomeric signals due to branching found between 4.9 and 5.3 ppm in dextrans.[19] The ^{1}H spectrum of EPS from *L. citreum* E497 contained two additional intense anomeric signals at 5.11 and 5.18 ppm, and a low intensity anomeric signal at 5.32 ppm. In the spectrum of EPS from *W. confusa* E392 and *L. mesenteroides* B512F, only one additional low intensity anomeric signal was observed at 5.32 ppm. Altogether, these anomeric signals represent four spin systems (δ_H 4.98, 5.11, 5.18 and 5.32 ppm) which were labeled A–D according to the increasing chemical shift (Figs. 1 and 2). The relative intensities (%) of the peaks starting from the high-field anomeric proton were 74.8%, 11.0%, 10.7% and 3.5%

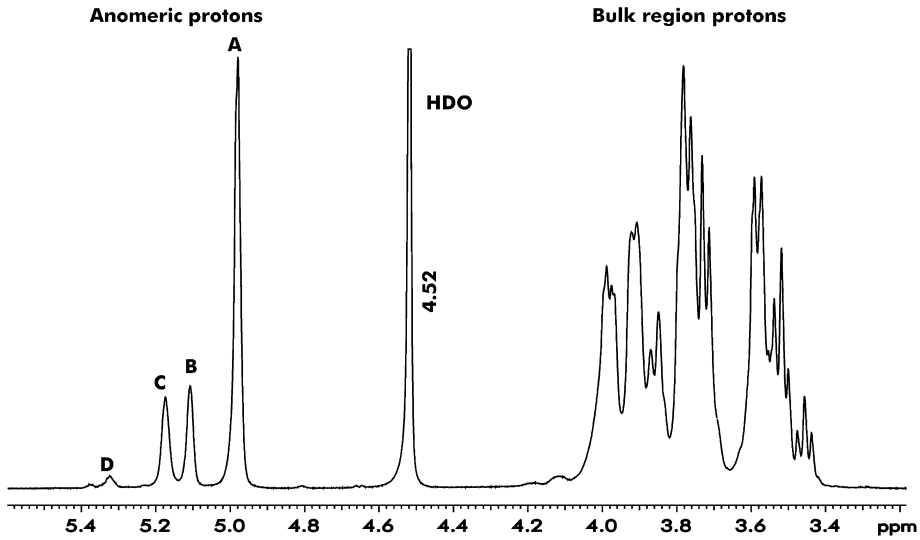

Figure 1. The 1D ^{1}H spectrum of EPS extracted from *L. citreum* E497 recorded at 500 MHz in D_2O at 50 °C. Anomeric protons are labeled A–D according to the increasing chemical shifts. The peaks are referenced to internal acetone ($^{1}H = 2.225$ ppm).

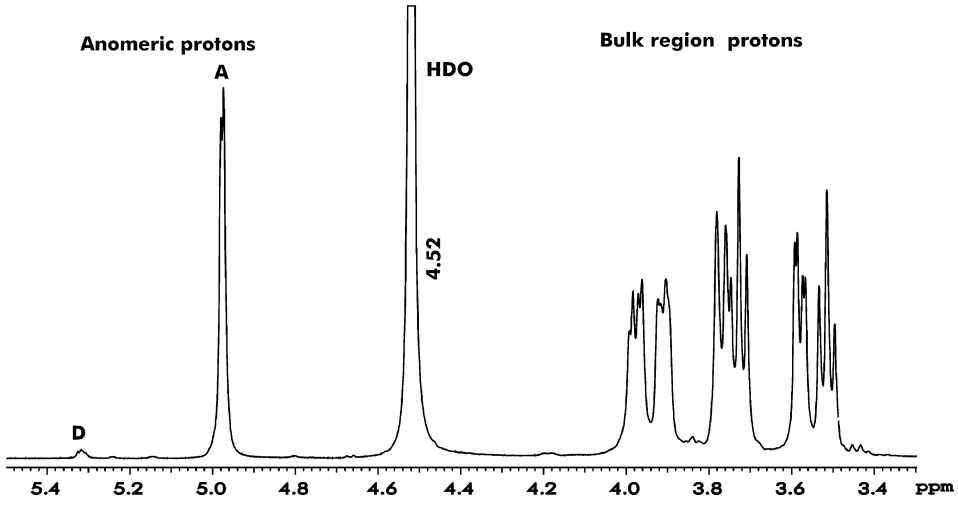

Figure 2. The 1D ^{1}H spectrum of EPS extracted from *W. confusa* E392 recorded at 500 MHz in D_2O at 50 °C. Anomeric protons are labeled A and D in accordance with the anomeric protons in EPS extracted from *L. citreum* E497 (Fig. 1). The peaks are referenced to internal acetone ($^{1}H = 2.225$ ppm).

for *L. citreum* E497; 97.3% and 2.7% for *W. confusa* E392; and 95.9% and 4.1% for *L. mesenteroides* B512F.

Carbon chemical shifts were determined using 2D heteronuclear single quantum correlation spectroscopy (HSQC). The HSQC spectrum of EPS from *L. citreum* E497 (Fig. 3) had anomeric ^{13}C chemical shifts at 99.3, 97.8, 97.0, and 100.9 ppm corresponding to ^{1}H anomeric signals at 4.98, 5.11, 5.18, and 5.32 ppm (spin systems A, B, C, and D), respectively. The additional signals in the anomeric region with ^{1}H chemical shifts at 4.58 and 5.38 ppm in the HSQC spectrum of EPS from *L. citreum* E497 (Fig. 3) shared the same ^{13}C chemical shift (99.3 ppm) as the main chain anomeric signal (δ_H 4.98 ppm). Furthermore, the signals were symmetrically separated from the signal at 4.98 ppm and were therefore considered as artifacts. These artifacts are most likely adiabatic phase cycling sidebands.[20] A similar phenomenon also occurred in the HSQC spectrum of EPS from *W. confusa* E392 (data not shown). The ^{1}H anomeric signals of EPS from *W. confusa* E392 had ^{13}C chemical shifts at 99.3 and 100.9 ppm, which are equivalent to spin systems A and D in EPS from *L. citreum* E497. These anomeric ^{13}C chemical shifts also confirm that the EPSs contain α-anomeric carbons rather than β-anomeric carbon atoms whose signals are found downfield from 102 ppm.[21]

2.2.2. Assignment of the chemical shifts for each spin system. In order to assign the chemical shifts for each spin system, additional 2D experiments were carried out

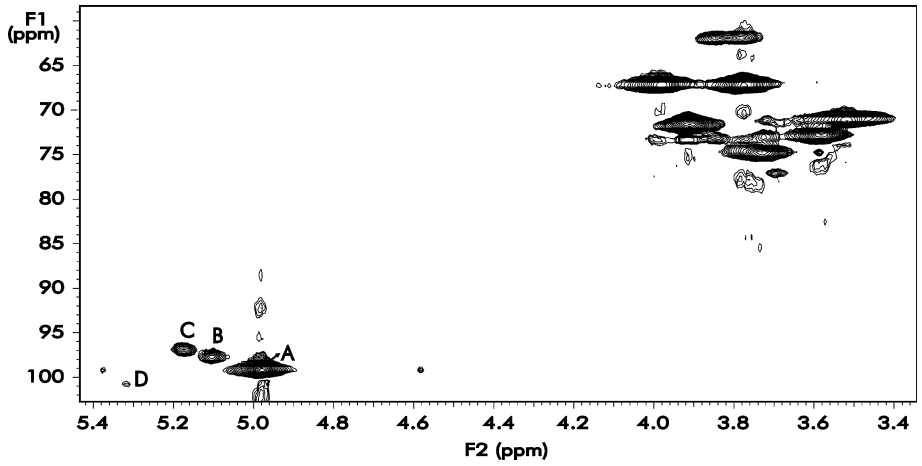

Figure 3. HSQC spectrum of EPS extracted from *L. citreum* E497 recorded at 500 MHz in D_2O at 50 °C. Anomeric correlation peaks are labeled A–D according to the increasing proton chemical shifts. Peaks are referenced to internal acetone ($^1H = 2.225$ ppm and $^{13}C = 31.55$ ppm).

on EPS extracted from *L. citreum* E497. These included double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear two-bond correlation spectroscopy (H2BC), and heteronuclear multibond connectivity (HMBC) experiments. From the HSQC spectrum of EPSs from *L. citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F it was possible to determine all the chemical shifts for spin system A. These chemical shifts were easily assigned with the DQF-COSY spectrum of EPS extracted from *L. citreum* E497, in contrast to spin systems B and C whose signals were obscured by signal overlap. To avoid ambiguity in assigning spin systems B and C, we used H2BC and HMBC spectra. Complete assignment of the spin system with ^1H anomeric signal at 5.32 ppm (spin system D) was difficult due to its low intensity in all samples. The complete assignment of ^1H and ^{13}C chemical shifts for spin systems A, B, and C is given in Tables 1 and 2, respectively.

The H2BC experiment is helpful in bridging HSQC and HMBC experiments as it simplifies the identification of two-bond correlations.[22,23] Intra-residue correlation peaks from H-1→C-2, H-3→C-2, H-3→C-4, H-5→C-4, and H-5→C-6 in the H2BC spectrum of EPS from *L. citreum* E497 were especially useful in assigning spin system C (Fig. 4). Overlap between H-2 of spin system B and H-4 of spin system C (3.56 and 3.57 ppm, respectively) in the NMR spectra of EPS from *L. citreum* E497 made it difficult to determine their respective ^{13}C chemical shifts using the HSQC spectrum (Fig. 3). However, in the H2BC spectrum, a clear cross-peak between spin system B(H-1) and B(C-2) confirms the chemical shift for B(C-2) at 72.9 ppm. Similarly, the H-5 ^1H chemical shifts for spin systems A, B, and C are rela-

Table 1. Assignments of ^1H chemical shifts (ppm) for the spin systems A, B, and C in EPSs extracted from *L. citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F determined by 1D and 2D NMR experiments recorded at 500 MHz in D_2O at 50 °C

Strain	Spin System	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
L. citreum E497 *W. confusa* E392 *L. mesenteroides* B512F	A	4.98	3.58	3.73	3.52	3.91	3.77	3.98
L. citreum E497	B	5.11	3.56	3.78	3.45	3.91	3.79	3.84
	C	5.18	3.71	3.85	3.57	3.92	3.79	3.98

Table 2. Assignments of ^{13}C chemical shifts (ppm) for spin systems A, B, and C in EPSs extracted from *L. Citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F determined by 2D NMR experiments recorded at 500 MHz in D_2O at 50 °C

Strain	Spin system	C-1	C-2	C-3	C-4	C-5	C-6	C-6′
L. citreum E497 *W. confusa* E392 *L. mesenteroides* B512F	A	99.3	72.9	74.8	71.2	71.8	67.3	67.3
L. citreum E497	B	97.8	72.9	74.5	71.0	73.4	62.0	62.0
	C	97.0	77.1	73.2	71.0	71.8	67.2	67.2

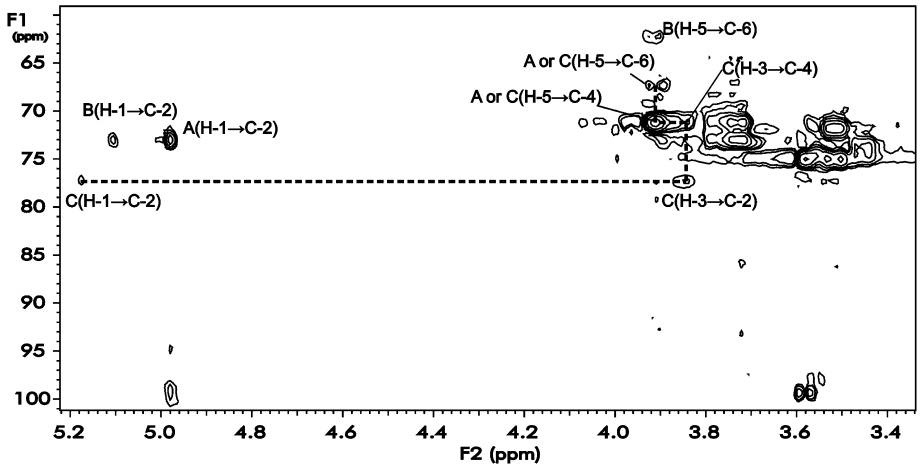

Figure 4. H2BC spectrum of EPS extracted from *L. citreum* E497 recorded at 500 MHz in D_2O at 50 °C. The figure shows significant cross-peaks for chemical shift assignment. A(H-1→C-2) represents the intra-residue correlation peak between H-1 and C-2 of spin system A. The dashed line indicates the correlations used in assigning spin system C.

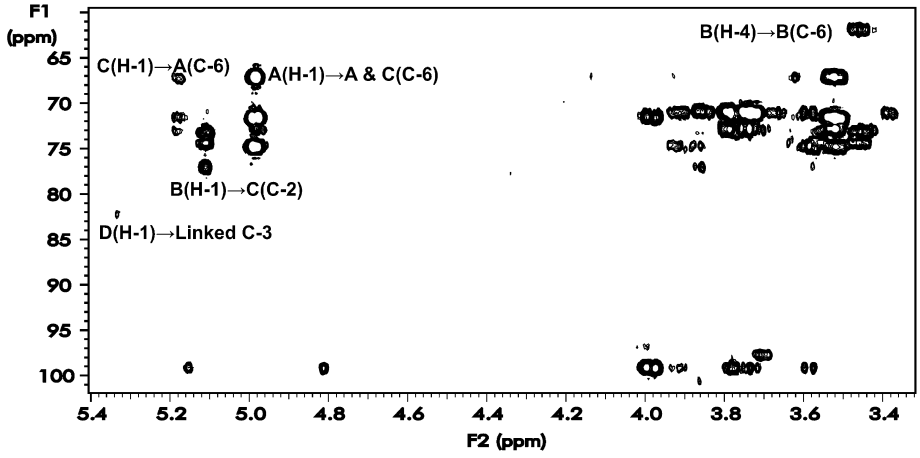

Figure 5. HMBC spectrum of EPS extracted from *L. citreum* E497 recorded at 500 MHz in D_2O at 50 °C. The figure shows anomeric ^{1}H connectivities to ^{13}C over the glycosidic bond. A(H-1)→A and C (C-6) represents an inter-residue correlation peak between H-1 and C-6 of consecutive A spin systems and a correlation peak between H-1 of spin systems A and C-6 of spin system C.

tively similar (3.91, 3.91, and 3.92 ppm, respectively), but the C-5 ^{13}C chemical shift for spin system B is farther downfield (73.4 ppm) than those of spin systems A and C (71.8 ppm). In this case, cross-peaks between H-1 and C-5 in the HMBC spectrum (Fig. 5) were appropriate for confirming which spin system had the downfield C-5 signal. The cross-peak between B(H-4) (3.45 ppm) and B(C-6) in the HMBC spectrum (Fig. 5) confirmed the assignment of the unbound C-6 signal (62.0 ppm) to spin system B.

2.2.3. Connectivity over the glycosidic linkages. The glycosidic linkages between the spin systems in the EPSs analyzed were determined by the comparison of literature and chemical shift data determined here. Furthermore, the connections were confirmed with an HMBC experiment on EPSs from *L. citreum* E497. Dextrans typically have the ^{13}C anomeric signals (C-1) downfield at ~90 ppm, C-2, C-3, C-4 and C-5 appear in the 70–75 ppm region, and C-6 is normally upfield at ~60 ppm. Upon glycosylation, the two carbons involved are downfield displaced by ~10 ppm.[17] As shown in Tables 1 and 2, EPSs from *L. citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F have the A spin system, which corresponds to α-(1→6)-linked D-glucopyranosyl units in the dextran backbone.[14] Seymour et al.[17] have shown that the dextran backbone contains a bound C-6 with a chemical shift at 66.6 ppm (67.2 ppm in this work), which is significantly farther downfield than a free C-6 at 61.4 ppm (62.0 ppm in this work). Similarly, residue C not only has a bound C-6 chemical shift at 67.2 ppm, but also H-2 and C-2 signals (3.71 and 77.1 ppm, respectively) significantly farther downfield than that of spin systems A (3.58 and 72.9 ppm) and B (3.56 and 72.9 ppm). Spin system C was therefore identified as the branch points in the main chain (2,6-di-O-

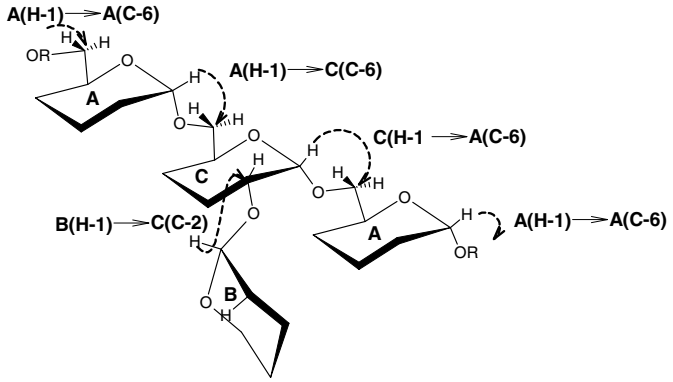

Figure 6. A schematic representation of EPS from *L. citreum* E497. A, B, and C correspond to the main spin systems found in the EPS. A(H-1)→C (C-6) represents an inter-residue correlation peak between H-1 of spin system A and C-6 of spin system C.

substituted α-D-glucopyranosyl). The difference between H-2 and C-2 chemical shifts of spin system C and those of spin systems A and B was clearly visible in the DQF-COSY (not shown) and H2BC spectra (Fig. 4), respectively. Other than the anomeric signal, spin system B had no significantly shifted ^{1}H and ^{13}C signals to indicate glycosylation.

The HMBC spectrum of EPS from *L. citreum* E497 is presented in Figure 5. Cross-peaks for the three main anomeric signals were observed from spin system A H-1 (4.98 ppm) to spin systems A and C C-6 (67.2 ppm), from spin system B H-1 (5.11 ppm) to spin system C C-2 (77.1 ppm), and from spin system C H-1 (5.18 ppm) to spin system A C-6 (67.2 ppm). A schematic representation of these connectivities is shown in Figure 6. The correlation peak between C H-1 (5.18 ppm) and A C-6 (67.2 ppm) was only observed when the average $^{n}J_{CH}$ (n >1) coupling constant (default 8–9 Hz) used in the HMBC experiment was lowered to 4 or 6 Hz.

Though the spin system with ^{1}H and ^{13}C anomeric signals at 5.32 and 100.9 ppm could not be assigned, analysis of various dextrans has shown that it occurs in α-(1→3) branched dextrans.[10,19,24] Additionally, Seymour et al.[25] showed that bound C-3 of the 3,6-di-O-substituted α-D-glucopyranosyl residue in dextrans is usually shifted downfield to 82.9 ppm. Due to the low intensity of spin system D, such a signal in the 82–83 ppm region could not be clearly established in the HSQC spectrum of EPS extracted from *L. citreum* E497. However, by increasing the number of transients to 64 during the acquisition of the HSQC spectrum of EPS extracted from *W. confusa* E392 (data not shown), a correlation peak at δ_H 3.86 and δ_C 82.3 ppm was observed. Furthermore, the occurrence of a weak cross-peak signal from 5.32 ppm to a bound C-3 at 82.3 ppm in the HMBC spectrum of EPS from *L. citreum* E497 confirmed the presence of α-(1→3)-linked branches (Fig. 5). The difference in the chemical shift (0.6 ppm) of the bound C-3 reported here and that reported by Seymour et al.[21] is due to the difference in temperatures at which the spectra were obtained. A relatively similar difference also occurred in the chemical shift of a bound C-2 reported here (77.1 ppm) and that reported by Seymour et al. (77.8 ppm).[21]

3. Discussion

In most cases, structural analysis of EPS involves tedious isolation procedures, especially when EPSs are extracted from complex liquid media.[26,27] In addition to protein and lipids, complex liquid media can contain a variety of polysaccharides, such as mannans from yeast extract, which may complicate EPS purification. De Vuyst and Degeest[1] speculated that different research groups report different structures of EPSs due to poor isolation and purification procedures or to the production of more than one type of EPS by the same strain. The latter variation may depend on different factors such as cultivation conditions and medium composition. In studies by Jeanes et al.,[4] dextrans were isolated from cultures grown in a liquid medium containing sucrose, tryptone, yeast extracts, and a phosphate source at 25 °C for five days. In the present investigation, dextrans were produced on nutritionally rich solid media containing 2% sucrose (MRS-S agar) in a carbon dioxide atmosphere at 30 °C for five days. These conditions may not represent the optimum for dextran production by these strains and the structure of dextrans determined may change under more optimal conditions. As Kim et al.[12] showed, incubating dextransucrase isolated from *L. mesenteroides* B512FMCM (a mutant of *L. mesenteroides* B512F) at a different sucrose concentration or temperature significantly affected the degree of branching in the resultant dextrans. Nevertheless, growth of the strain on solid media in the present study led to a simple dextran isolation procedure. Proteins, polysaccharides, and other contaminating molecules from the media were sufficiently excluded by keeping the media surface intact during cell mass removal.

Previously, structural analysis of dextrans had extensively been carried out with chemical methods such as periodate oxidation and methylation analysis,[4,5] enzyme-aided structure analysis,[28] 1D ^{1}H and ^{13}C NMR spectroscopy,[10,17,19] and also by 2D NMR spectroscopy.[29] In this work, structure analysis was accomplished with 1D and 2D NMR spectroscopy techniques.

Based on NMR spectroscopy data, EPSs produced by *L. citreum* E497 and *W. confusa* E392 are branched dextrans that typically have a backbone made of consecutive α-(1→6)-linked D-glucopyranosyl units. *L. citreum* E497 produces an α-(1→2)-branched dextran with few α-(1→3) branches. The degree of branching determined from the relative intensities of the ^{1}H anomeric signals was about 10.7% for 2,6-di-O-substituted glucopyranosyl units, 11% for α-(1→2)-linked branches and about 3.5% for α-(1→3)-linked branches. Structurally related α-(1→2)-branched dextrans have been reported in various *L. mesenteroides* strains such as *L. mesenteroides* NRRL B-1298, NRRL B-1299, NRRL B-1396, and NRRL B-1399.[5,30] Duenas-Chasco et al.[29] also reported a highly α-(1→2)-branched dextran from *Lactobacillus* spp. G-77. The structure of dextran from *L. citreum* E497 closely resembles that of *L. mesenteroides* NRRL B-1396. By methylation analysis, Seymour et al. showed that dextran from *L. mesenteroides* NRRL B-1396 contains about 77.8% α-(1→6) linkages, 9.1% 2,6-di-O-substituted glucopyranosyl units, 3.3% 3,6-di-O-substituted glucopyranosyl units, and 9.8% terminal D-glucose residues.[21] Methylation analysis by Slodki et al. showed a relatively similar composition, but indicated the presence of 2% 4,6-di-O-substituted glucopyranosyl units in dextrans from this strain.[5]

Dextran from *W. confusa* E392 is linear with few (2.7%) α-(1→3)-linked branches. This dextran represents a more linear dextran than that of *L. mesenteroides* B512F, which in this work was found to contain about 4.1% α-(1→3) linkages based on the relative intensities of the anomeric signals. Dextrans from *L. mesenteroides* B512F are often reported to contain a slightly higher number (5%) of α-(1→3) linkages by methylation analysis.[14] However, we have analyzed a commercial dextran produced by *L. mesenteroides* B512F (Ge Healthcare Biosciences, Uppsala, Sweden) and found that it contains 4.1% α-(1→3) branches based on the relative intensities of the anomeric signals. According to the manufacturer, this dextran contains 5% α-(1→3) branches. The accuracy of NMR spectra integration depends on the experimental conditions particularly ensuring that all nuclei have established equilibrium magnetization (relaxed) before subsequent pulsing. Additionally, post-acquisition processing such as phasing and baseline correction should be done if reproducible results are to be obtained. Notably, NMR integration techniques have been shown to vary the degree of branching in clinical dextrans from 4.8% to 5.5%.[31] In this study, the relative intensities of anomeric signals are compared for data acquired and processed in the same way. The slight difference in branching degree in dextrans produced by *L. mesenteroides* B512F reported here may therefore result from differences in the analytical methodology (the relative intensities of anomeric signals and methylation analysis) rather than a structural variation due to cultivation conditions.

The fine structure of dextrans still requires more investigation. Whether the branches are part of a comb-like structure or a ramified structure still needs to be investigated.[31] For example, α-(1→3)-linked branches in *L. mesenteroides* NRRL B512F can be single D-glucose units or may be extended by two or more α-(1→6)-linked D-glucose residues.[9] The α-(1→3) branch linkages in dextrans from *L. mesenteroides* NRRL B-1299 may occur as terminal linkages on non-reducing chain ends or may be located at intersections between continuous α-(1→6) linkages.[32] Additionally, studies on dextran from *L. mesenteroides* NRRL B-1299 by Bourne et al.[33] revealed the presence of isomaltosyl homologues connected via α-(1→2) linkages while Slodki et al.[5] found 2-*O*-mono-glucopyranosyl units.

NMR spectroscopy analysis in this work did not establish the presence of branch chain-extending linkages or α-(1→2) or α-(1→3) interchain linkages. As Seymour et al.[21] noted, the anomeric signal at 98.2 ppm (97.8 ppm in this work) is clearly for an α-(1→2) branch-terminating α-D-glucopyranosyl unit. This glucose residue contains an unbound C-2 (72.9 ppm), similar to that found in unbranched chain-extending α-glucopyranosyl units. Additionally, the HMBC spectrum confirmed that this α-glucopyranosyl unit possesses an unbound C-6 (62.0 ppm). The presence of isomaltosyl homologues connected via α-(1→2) linkages or α-(1→2) branch extensions would certainly result in characteristic anomeric signals in NMR spectra. This has clearly been illustrated for dextrans with α-(1→3) branch linkages with extensions via α-(1→6) or α-(1→3)-linked D-glucopyranosyl units.[10] In dextran from *L. mesenteroides* B512F, Cheetham et al. [10] illustrated that α-(1→3)-linked branches with α-(1→6)-linked D-glucose extensions resulted in an apparent triplet (two overlapping doublets) centered at 5.28 ppm (5.32 ppm in this study). Though not well resolved, the broad signal at 5.32 ppm assigned to α-(1→3)-linked D-glucose in the 1D spectra of dextrans extracted from *L. citreum* E497, *W. confusa* E392, and *L. mesenteroides* B512F appears to be a triplet. Therefore, like dextran from *L. mesenteroides* B512F, dextrans from *L. citreum* E497, and *W. confusa* E392 may contain single unit α-(1→3)-linked D-glucose branches or may contain branches extended by α-(1→6)-linked D-glucose units. In most cases, chain intersections or branch chain-extending linkages have been established mainly by the analysis of structural segments (oligosaccharides)

produced from the native dextran.[6,10] Such oligosaccharides are preferable in NMR spectroscopy analysis due to improved resolution, particularly when solutions of the parent molecule are viscous. In the present study, we analyzed only the native dextrans.

Gluco-oligosaccharides with α-(1→6) and α-(1→2) linkages may possess prebiotic properties.[34,35] Moreover, Olano-Martin et al.[36] demonstrated with in vitro studies that dextrans and oligodextrans were good substrates for butyric acid production by intestinal microflora. Short-chain fatty acids such as butyric acid are potential compounds for preventing bowel cancer.[36] Therefore, using α-(1→2)-branched dextran from *L. citreum* E497 in food formulation, in their native form or as oligosaccharides produced by partial hydrolysis, may confer prebiotic properties to the product. In addition, the dextransucrase from *L. citreum* E497 may serve as an alternative to that of *L. mesenteroides* NRRL B-1299 in the production of prebiotic oligosaccharides. Interestingly, we have found α-(1→2)-branched dextrans not only from *L. citreum* E497, but also from two other *L. citreum* strains (E1082 and E389) isolated from barley grain and oats, respectively. The production of α-(1→2)-branched dextrans may be a feature of *L. citreum* strains isolated from cereals.

As a highly linear polysaccharide, dextran from *W. confusa* E392 may be a suitable alternative to dextrans from *L. mesenteroides* B512F. Linear dextrans are highly soluble and impart high viscosity depending on their molecular weight. An increase in α-(1→3) branch linkages tends to limit the solubility of dextrans.[9,11] Dextran from *W. confusa* E392 may be suitable for various applications such as food additives and in the production of size-exclusion chromatography standards. Additionally, chemical modification can render the dextran useful in various ways. To our knowledge, this is the first structural description of dextrans from *Weissella* species.

4. Experimental

4.1. Materials

The growth media used was De Man, Sharpe, and Rogosa agar (Oxoid, Bsingstoke, UK) supplemented with 2% (w/v) sucrose (MRS-S agar). Extraction reagents included water purified with a Milli-Q-Plus system (Millipore Corporation, Billerica, Bedford, MA, USA), EtOH from Altia Oy (Rajamäki, Finland), NaCl and KCl from Sigma–Aldrich Chemie (Riedel-de Haen, Seelze, Germany), $Na_2HPO_4{\cdot}2H_2O$ from J.T. Baker (Deventer, The Netherlands), and KH_2PO_4 from Merck (Darmstadt, Germany). Monosaccharide analysis reagents included D-glucose and bis(trimethylsilyl)trifluoroacetamide from Merck, HCl from Sigma–Aldrich Chemie, chlorotrimethylsilane from Fluka Chemie (Buchs, Germany), pyridine from Sigma–Aldrich Chemie (Steinheim, Germany), and heptane from Rathburn Chemicals Ltd (Walkerburn, Scotland). The protein analysis kit (Quick Start™ Bradford Protein Assay) was from Bio-Rad Laboratories, Inc. (Hercules, USA), and the NMR solvent D_2O (99.8%) was from Merck. The bacteria were from the culture collection of the Technical Research Centre of Finland (VTT) and included *L. citreum* VTT E-93497 isolated from a malting process, *W. confusa* VTT E-90392 (=DSM 20194, NCDO 1975) isolated from soured carrot mash, and *L. mesenteroides* NRRL B512F.

4.2. Methods

4.2.1. Extraction of exopolysaccharides. The bacteria were grown on MRS-S agar in a carbon dioxide atmosphere created with airtight jars and Anaerocult A strips (Merck) at 30 °C for five days. The cell mass was carefully removed from the plates to ensure that the agar surface was unbroken. The cells were suspended in 200–450 mL of sodium phosphate buffer saline (PBS, 8.0 g NaCl, 0.2 g KCl, 1.4 g $Na_2HPO_4{\cdot}2H_2O$, and 0.2 g KH_2PO_4 in 1 L Milli-Q water, pH 7.4) in centrifuge bottles. The suspensions were shaken for 10 min and allowed to stand for 30 min. Subsequently, the cells were separated by centrifugation at 10,000 rpm for 40 min using a Sorvall RC-5C centrifuge (Du Pont Company, Delaware, USA). The supernatants were collected by decanting and centrifuged again under the same conditions. The cells were resuspended in PBS for a second extraction. Supernatants were stored at 4 °C.

EPSs were recovered from the supernatant by EtOH precipitation. Three volumes of EtOH were added to each supernatant, and the EPSs were allowed to precipitate for 48 h.[37] EPSs were recovered from the supernatant by centrifuging or decanting. For further analysis, a portion of the precipitate was placed in Eppendorf tubes and dried using Speed-Vac Plus equipped with a Universal Vacuum System Plus with Vapornet UVS400 (Savant Instruments, Inc., Holbrook, NY, USA) or by lyophilization using Edward Super Modulyo 12k, (Manor Royal, West Sussex, UK).

4.2.2. Protein assay. The amount of soluble protein in the extracted EPS was determined in order to assess the necessity of a protein removal procedure. The analysis was performed with a Bradford assay kit.[38] The color formed was measured with a Perkin Elmer Lambda 25 (Perkin Elmer, Shelton, USA) UV–vis spectrometer.

4.2.3. Analysis of monosaccharide composition. The monosaccharide composition of the extracted EPS was determined by GC after methanolysis and trimethylsily-

lation according to Sundberg et al.[39] For quantification, a calibration curve was prepared from D-glucose at five concentration levels. Samples for calibration curves were prepared by diluting the methanolyzed standards. All analyses were performed in triplicate. The samples were filtered with 0.45 μm membrane filters (Acrodisc 13, Pall Corporation, Ann Arbor, Michican, USA) and placed in GC vials. The following GC conditions were used for the analysis of the trimethylsilylated methyl glycosides: oven temperature 150 °C (5 min), 2 °C/min to 260 °C, injector temperature 225 °C, FID detector temperature 280 °C, injection vol 1 μL, split ratio 1:30, and flow rate 1 mL/min with helium as the carrier gas. The GC instrument used included a Hewlett Packard HP5890 Series II GC system with a flame ionization detector (FID) and a Hewlett Packard 7673 series injector and auto-sampler (Hewlett Packard, Palo Alto, USA). The column used was an HP-5 (30 m × 0.32 mm × 0.25 μm) from Agilent Technologies (Foster City, USA). The amounts of the monosaccharides were calculated as anhydro-sugar using a correction factor of 0.90 for D-glucose.

4.2.4. NMR spectroscopy analysis. NMR data were recorded on a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H and using 5 mm triple-resonance pulsed-field gradient (PFG) probes. NMR samples (10–25 mg/mL) were exchanged three times with D_2O, filtered, and then placed in NMR tubes (Wilmad NMR tubes, 5 mm, ultra-imperial grade, from Aldrich chemical company, Milwaukee, WI, USA). All the measurements were performed at 50 °C, and the chemical shifts were referenced to acetone ($^{1}H = 2.225$ ppm and $^{13}C = 31.55$ ppm).

The 1D ^{1}H spectra were acquired with a spectral width of 8000 Hz, pulse width of 9 μs, 4 s acquisition time, and 64 scans. For the DQF-COSY experiments, a spectral width of 3000 Hz was employed in both dimensions and a matrix of 2048 × 256 complex points (8 scans per t_1 value) was collected. The data were zero-filled to 4096 × 512, and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations. In TOCSY experiment, a spectral width of 3000 Hz was employed in both dimensions and a matrix of 1024 × 256 complex points (8 scans per t_1 value) was collected. A spin-lock time of 100 ms using the z-filtered TOCSY (Zdipsitocsy) pulse sequence was used.[40] The data were zero-filled to 2048 × 512, and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations.

For HSQC,[41] a spectral width of 25,000 Hz in F_1 and 5200 Hz in F_2 was used. A data matrix of 2048 × 256 complex points (16 scans per t_1 value) was collected and the average ^{1}H– ^{13}C coupling constant was estimated to be 140 Hz. The WURST 140 pulse sequence was used for ^{13}C decoupling during data acquisition. The data were zero-filled to 4096 × 512, and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations. For the H2BC experiment,[23] a spectral width of 20,000 Hz in F_1 and 8000 Hz in F_2 was used. A data matrix of 2048 × 144 complex points was obtained with $T = 21.8$ ms, a relaxation delay of 1.5 s, and 128 scans per t_1 value. The third-order low-pass filter was set for $125 < {}^{1}J_{CH} < 165$ Hz. WURST adiabatic decoupling was used during data acquisition. The data were zero-filled to 4096 × 1024, and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations. For the gHMBC experiment, the gradient selected absolute value HMBC pulse sequence was used.[42] A spectral width of 20 000 Hz in F_1 and 5200 Hz in F_2 was used. A data matrix of 2048 × 256 complex points (256 scans per t_1 value) was collected. The average $^{1}J_{CH}$ was set to 140 Hz and the average $^{n}J_{CH}$ ($n > 1$) was set to 4–6 Hz. The data were zero-filled to 4096 × 1024 and a sine-bell weighting function was employed in both dimensions prior to Fourier transformations.

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