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# Biodiversity of Exopolysaccharides Produced from Sucrose by Sourdough Lactic Acid Bacteria

Marie-Sophie Bounaix,[†] Valérie Gabriel,[†] Sandrine Morel,[‡] Hervé Robert,[†] Philippe Rabier,[†] Magali Remaud-Siméon,[‡] Bruno Gabriel,[†] and Catherine Fontagné-Faucher*,[†]

[†]Laboratoire de Biologie appliquée à l'Agroalimentaire et à l'Environnement, Institut Universitaire de Technologie-Université Paul Sabatier, 24 rue d'Embaquès, F-32000 Auch, France, and [‡]Université de Toulouse, INSA, UPS, INP, LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France, CNRS, UMR5504, INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France

The distribution and diversity of natural exopolysaccharides (EPS) produced from sucrose by thirty heterofermentative lactic acid bacteria strains from French traditional sourdoughs was investigated. The EPS production was found to be related to glucansucrase and fructansucrase extracellular activities. Depending on the strain, soluble and/or cell-associated glycansucrases were secreted. Structural characterization of the polymers by $^{1}H$ and $^{13}C$ NMR spectroscopy analysis further demonstrated a high diversity of EPS structures. Notably, we detected strains that synthesize glucans showing amazing variations in the amount of α-(1→2), α-(1→3) and α-(1→6) linkages. The representation of *Leuconostoc* strains which produce putative alternan polymers and α-(1→2) branched polymers was particularly high. The existence of glucan- and fructansucrase encoding genes was also confirmed by PCR detection. Sourdough was thus demonstrated to be a very attractive biotope for the isolation of lactic acid bacteria producing novel polymers which could find interesting applications such as texturing agent or prebiotics.

**KEYWORDS: Glucans; homopolysaccharides; exopolysaccharides; sourdough; *Leuconostoc*; *Weissella*; lactic acid bacteria**

## INTRODUCTION

Sourdough fermentation plays an important role in baking technology by improving aroma, texture, shelf life and mineral bioavailability (*1*, *2*). Sourdough is composed of a fermented mixture of water and wheat and/or rye flour that contains a complex microbiota mainly composed of lactic acid bacteria (LAB) and yeasts (*3*). It is traditionally obtained from successive natural fermentation steps or can be produced using a starter culture containing one or more selected strains (*1*, *3*). Typical sourdough LAB mainly belong to hetero- and homofermentative strains of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* genera (*3*, *4*). Many inherent properties of sourdough (i.e., lactic fermentation, proteolytic activity, synthesis of volatile compounds and antimicrobial substances) rely on the metabolic activities of its resident LAB (*5*). Another interesting metabolic feature is the production of exopolysaccharides (EPS) as it has been recently reported (*6*−*10*). Several studies provided evidence that EPS produced by sourdough LAB have the potential to improve dough rheology and bread texture, and may be used to replace or reduce more expensive hydrocolloids (*8*, *9*, *11*).

Both homo- and heteroexopolysaccharides are produced by LAB. Heteropolysaccharides are composed of different monosaccharides (e.g., glucose, galactose, fructose and rhamnose). They are synthesized in small amounts ($mg \cdot L^{-1}$) from intracellular sugar nucleotide precursors by various different glycosyltransferases (*12*). In contrast, homopolysaccharides are either glucan or fructan polymers (*13*−*15*). They are synthesized in larger amounts ($g \cdot L^{-1}$) from sucrose by secreted or cell-anchored glucansucrases and fructansucrases that convert sucrose respectively into glucans or fructans, with the concomitant release of either fructose or glucose (*14*, *16*). The energy of the glycosidic bond of sucrose enables the efficient polymerization of the glucosyl or fructosyl residues (*14*, *16*, *17*). Glucansucrases from LAB are all classified in the glycoside-hydrolase family 70 (*18*). The glucans produced by these enzymes are divided in four categories: (i) dextran, characterized by a linear backbone of α-(1→6) linked glucosidic residues with α-(1→2), α-(1→3) or α-(1→4) linked branches; (ii) mutan, a water-insoluble linear polymer containing more than 50% α-(1→3) glucosidic linkages in the linear backbone; (iii) alternan which contains alternating α-(1→6) and α-(1→3) glucosidic linkages with some degree of α-(1→3) branching and (iv) reuteran which is a glucan mostly composed of α-(1→4) glucosidic bonds with some α-(1→6) branches (*14*, *15*). Two types of fructans are produced by fructansucrases from glycoside-hydrolase family 68: levan and inulin, which contain fructose units linked by *β*-(2→6) and *β*-(2→1) glycosidic bonds, respectively (*11*, *13*, *15*). EPS

*Corresponding author. Phone: +33 (0)562616302. Fax: +33 (0)562616301. E-mail: cathy.faucher@iut-tlse3.fr.


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properties vary widely with the monosaccharide composition, the type of glycosidic linkages, the degree of branching and the polymer size (*14*). Many glucansucrase and fructansucrase encoding genes have been cloned and sequenced. From sequence comparison, primers targeting the conserved catalytic domain have been designed to screen for genes encoding glucan- and fructansucrases in LAB (*9*, *19*).

Homopolysaccharide production by cereal LAB have been mainly reported for strains of the *Lactobacillus* genera and more specifically *Lb. sanfranciscensis* and *Lb. reuteri* (*7*, *9*, *20*, *21*). In addition, dextran production is a frequent phenotype in *Leuconostoc mesenteroides* strains (*14*). Sourdough lactobacilli (*Lb. sanfranciscensis, Lb. fermentum*, *Lb. pontis, Lb. panis, Lb. reuteri, Lb. acidophilus*) were shown to produce fructans (levan or inulin) as well as glucans (dextran, reuteran or mutan) as recently reviewed (*11*). Production of dextran by a *Ln. mesenteroides* strain isolated from panettone (*8*) and of glucan and/or fructan from sourdough *Weissella* species has also been reported (*6*, *9*).

The aim of this paper was to investigate EPS production by LAB strains previously isolated from traditional French sourdoughs (*22*). Thirty heterofermentative LAB strains were screened for their capacity to produce EPS from sucrose. Glycansucrase activities and corresponding produced glycans from *Leuconostoc* and *Weissella* strains were characterized. The presence of glucansucrase and/or fructansucrase encoding genes was also examined.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions.** Thirty strains of *Leuconostoc citreum* (11 strains), *Leuconostoc mesenteroides* (8 strains), *Weissella cibaria* (5 strains), *Weissella confusa* (1 strain), *Lactobacillus sanfranciscensis* (4 strains) and *Lactobacillus brevis* (1 strain) belonging to Culture Collection of the Laboratoire de Biologie appliquée à l'Agroalimentaire et l'Environnement-Université Paul Sabatier (UPS-LBAE, Auch, France) were used in this study. They were originally collected from nine traditional sourdoughs from the Midi-Pyrénées area (France) (*23*). The first letter used for strain nomenclature refers to the sourdough sample in which it was initially recovered. These strains were previously identified by molecular methods (*22*). Seven other LAB strains have been used as references for EPS production: *Ln. mesenteroides* ATCC $8293^T$ and NRRL B-512F, *Ln. citreum* NRRL B-742, NRRL B-1299 and NRRL B-1355, *W. cibaria* DSM $15878^T$ and *W. confusa* DSM $20196^T$. All strains were maintained frozen at −25 °C as 20% (v/v) glycerol stocks and routinely propagated in De Man, Rogosa and Sharpe (MRS) medium at 30 °C (Biokar, Beauvais, France). The carbohydrate fermentation pattern of the strains was investigated using API 50CH system (API system, BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The results were recorded after 24 and 48 h of incubation at 30 °C. Similarity between strains was evaluated by Pearson correlation, using StatBoxPro program, and strains were clustered considering a threshold of 90% homology.

**Preliminary Screening for EPS Producing Strains.** EPS synthesis was preliminarily determined in duplicate by growing cell colonies on agar plates containing modified MRS medium with 40 $g \cdot L^{-1}$ sucrose instead of 20 $g \cdot L^{-1}$ glucose (namely MRS-sucrose). Cell suspension ($OD_{600}$ = 0.3) was prepared in MRS broth by growing strains 24 h at 30 °C. Cells from 0.5 mL of culture were harvested by centrifugation (3260*g*, 5 min), washed with 1 mL of sterile water and resuspended in 0.2 mL of sterile water. Then, inoculation was performed by spotting 2 $\mu$L of bacterial suspension (about $5 \times 10^7$ bacteria, 10 spots/plate) on MRS and MRS-sucrose agar media. After incubation at 30 °C for 24 to 48 h, the strains which produced slimy colonies were recorded as capable of producing EPS and classified according the visual appearance (compact, creamy or liquid slime). The diameter of the zone of the bacterial growth was also measured.

**Glycansucrase Activity Measurements and Cellular Localization.** The strains that produced EPS on agar plates were evaluated for glycansucrase activity. Each strain was first cultivated in MRS broth at 25 °C for 20 h, and then a 100 mL culture was prepared (initial $OD_{600}$ = 0.3) in MRS−sucrose, with pH adjusted to 6.9 with 5 M sterile NaOH. The incubation was performed at 25 °C, 100 rpm until pH 5.0. Finally, pH was adjusted at 5.4 with 5 M sterile NaOH in order to optimize glycansucrase activity (*24*). The culture was centrifuged at 4 °C (12100*g*, 20 min) to separate culture supernatant containing soluble glycansucrase and pellet with cell-associated activity. Cells were washed twice with 20 mM sodium acetate buffer pH 5.4 and resuspended in the initial volume with the same buffer. Supernatant and pellet samples were immediately checked for glycansucrase activity and then kept at −25 °C until further use. Glycansucrase activity was also measured twice after the freezing step. Enzymatic activity was assayed by following of the reducing power released from sucrose by the 3,5-dinitrosalicylic acid method (DNS) (*25*). One glycansucrase unit is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of fructose and/or glucose per minute at 30 °C, in 20 mM sodium acetate buffer (pH 5.4) and in the presence of 292 mM sucrose.

**Polymer Production and Purification.** Culture supernatant or washed cell suspension having a glycansucrase activity of at least 0.7 $U \cdot mL^{-1}$ was used for EPS production in standard conditions. EPS synthesis was thus performed at 30 °C in a total volume of 20 mL with 20 mM sodium acetate buffer pH 5.4 containing 292 mM sucrose and 0.5 $U \cdot mL^{-1}$ enzyme fraction. The enzymatic reaction was conducted during 24 to 48 h until sucrose was depleted (except for the G15 cell-associated glycansucrase sample for which only 75% of the sucrose was consumed after 120 h). Otherwise when enzymatic activity was too low to perform such EPS production ($< 0.7$ $U \cdot mL^{-1}$), the polymers were recovered with an inoculum loop from EPS slime that surrounds colonies grown on MRS−sucrose agar plates at 30 °C for 48 h. The purification of the polymers was carried out as follows. The samples were heated at 95 °C for 15 min to inactivate enzymes potentially able to degrade the polymer and centrifuged at 12100*g* for 20 min at 4 °C. The supernatant was precipitated with 3 volumes of cold absolute ethanol (v/v) at 4 °C overnight. After precipitation, the mixture was centrifuged (conditions as above) and the pellet containing EPS was dissolved in Milli-Q water. Finally, EPS was precipitated twice with 60% (v/v) cold ethanol, centrifuged and dissolved in Milli-Q water. Thereafter, EPS was lyophilized for 72 h. According to the production method, purified polymers represented 600 to 750 mg (starting from 2 g of sucrose) or about 3 to 8% of the cellular mass collected.

**Determination of EPS Monosaccharide Composition.** Acid hydrolysis of purified glycans (about 3 mg) was performed in duplicate by addition of 750 $\mu$L of perchloric acid (1.75 M). Samples were incubated at 80 °C for 2 h in order to release fructose residues from fructans and avoid their degradation, and for 16 h to release glucose from glucan polymers. After incubation, 250 $\mu$L of KOH (5 M) was added in order to neutralize perchlorate. Precipitated potassium perchlorate was removed by centrifugation (6660*g*, 5 min) and the supernatant was diluted in $H_2SO_4$ 0.001 N and filtered through a 0.45 $\mu$m pore size filter. Monosaccharide composition was determined by high performance liquid chromatography (HPLC) using an ion-exclusion ORH-801 column (300 mm × 6.5 mm, Interaction Chromatography, France) and ICS refractive index detector (RI 8120) under the following conditions: mobile phase 0.001 N $H_2SO_4$, with a flow rate of 0.7 $mL \cdot min^{-1}$ and column temperature at 25 °C.

**Endodextranase Hydrolysis of Purified Dextrans.** EPS were characterized by susceptibility to endodextranase hydrolysis. About 10 mg of polymers was dissolved in a 50 mM sodium acetate buffer pH 5.4 (final concentration 10 $g \cdot L^{-1}$). Dextran hydrolysis was performed by 10 $U \cdot mL^{-1}$ *Chaetomium gracile* endodextranase (Sankyo Co., Japan) during 24 h at 37 °C. One unit of dextranase corresponds to the enzyme amount that releases 1 $\mu$mol of isomaltose (measured as maltose) per minute in a 50 mM sodium acetate buffer (pH 5.4) at 37 °C, using dextran as substrate. Dextran 2,000 kDa (Sigma-Aldrich, St. Quentin Fallavier, France) was used as a positive control. Samples were centrifuged (6660*g*, 10 min) and supernatants were diluted in Milli-Q water and filtered through a 0.45 $\mu$m pore size filter before analysis. Digestion products were analyzed by high performance anion exchange chromatography and pulsed amperometric detection (HPAEC−PAD) using a 4 × 250 mm Dionex Carbopac PA100 column. A gradient of sodium acetate from 6 to 300 mM in 28 min in 150 mM NaOH was applied at 1 $mL \cdot min^{-1}$ flow rate. Detection was performed using a Dionex ED 40 module with a gold

working electrode and an Ag/AgCl pH reference (*16*). Glucose and isomaltose were used as calibration standards (Sigma, France).

**NMR Spectroscopy.** Samples (12 mg) were exchanged twice with 99.9 atom % $D_2O$, lyophilized and dissolved in 600 $\mu$L of $D_2O$. $^1H$ chemical shifts ($\delta$) are expressed in ppm by reference to internal acetone ($\delta$ = 2.225 ppm) and $^{13}C$ chemical shifts ($\delta$) are expressed by reference to the methyl-carbon of internal acetone ($\delta$ = 31.08 ppm). All NMR spectra were recorded on an Avance II (Bruker) 500 MHz spectrometer (500 MHz for $^1H$ NMR and 125 MHz for $^{13}C$ NMR) using a 5 mm *z*-gradient TBI probe. The data were acquired and processed using TopSpin 2.1 software. The temperature was 298 K. 1D $^1H$ NMR spectra were acquired by using a 30° pulse, 8000 Hz sweep width and 5 s total relaxation delay. A total of 16 scans were recorded. 1D $^{13}C$ NMR spectra were recorded using an inverse gated sequence taken from the Bruker pulse sequence library, and using a 90° pulse, 25,000 Hz sweep width, 2.5 s relaxation delay and 0.63 s acquisition time. A total of 30,000 scans were recorded. Commercial dextran, inulin and levan (Sigma-Aldrich, St. Quentin Fallavier, France) were used for chemical shift reference. The various signals were assigned as described by Seymour et al. (*26*, *27*), Uzochukwu et al. (*28*), and Maina et al. (*29*) for dextrans and Shimamura et al. (*30*) for levan. The percentages of α-(1→2) and α-(1→3) linkages in glucans were calculated from the relative intensities of the corresponding anomeric carbons or protons.

**Molecular Mass Determination.** Molecular weight of the purified glycans was determined by using high performance size exclusion chromatography (HPSEC). Analyses were performed with an ICS pump equipped with a 20 $\mu$L injection loop. Detection was performed with an ICS refractive index detector (RI 8120). A Shodex OH-Pack SB-805 column and guard column were used at 70 °C, using 0.45 mM of $NaNO_3$ as eluent at a flow rate of 0.3 mL.min$^{-1}$. Samples were dissolved in Milli-Q water (5 g·L$^{-1}$) and filtered through a 0.45 $\mu$m pore size filter before injection. A calibration curve was obtained by using commercial dextrans of 1360, 409.8, 147.6, 48.6, 11.6, and 5.2 kDa (Fluka, Sigma-Aldrich, St. Quentin Fallavier, France).

**PCR Amplification of Glycansucrase Encoding Genes.** *Leuconostoc* and *Weissella* total DNA was prepared from overnight cultures grown in MRS medium according to Robert et al. (*22*). Screening for EPS-synthesizing enzyme encoding genes was performed using two primers sets targeting glucansucrase and fructansucrase genes, respectively. Primer pair DegFor (5′-GAYAAYWSNAAYCCNRYNGTNC-3′) and DegRev (5′-ADRTCNCCRTARTANAVYKNG-3′) previously defined by Kralj et al. (*19*) amplifies a 660 bp DNA fragment from glucansucrase genes. Primer set FTF2-F (5′-GAYRTYTGGGAYWSNTGGC-3′) and FTF2-R (5′-GCWGANCCNGACCATTSTTG-3′) is predicted to give an amplicon of 220 bp from levansucrase genes. These degenerate primers have been constructed to target conserved amino acid sequences DVWDSWP and QEWSGSA present in the catalytic core of the LAB levansucrases, as determined by sequence alignment obtained from CAZy Web site (*18*). PCR reactions were performed, with a Gradient Master Thermocycler (Eppendorf), in a total volume of 20 $\mu$L containing 1 $\mu$L of template DNA (approximatively 5 to 10 ng) and using conditions previously described by Kralj et al. (*19*), for glucansucrase genes. The PCR conditions for amplification of the fructansucrase genes were as follows: 1 $\mu$M of each primer, 0.2 mM dNTP, 1× reaction buffer, 3 mM $MgCl_2$ and 0.5U RedGoldstar Taq polymerase (Eurogentec). The PCR reaction involved an initial denaturation step (94 °C, 5 min), followed by denaturation (94 °C, 45 s), annealing (55 °C, 30 s) and elongation (72 °C, 30 s), for a total of 30 cycles, and a final elongation step (72 °C, 10 min). PCR products were visualized through gel electrophoresis on 1.7% (w/v) agarose gel in 0.5× TBE buffer, following by ethidium bromide staining and detection by UV illumination. A Smart Ladder (Eurogentec, Angers, France) was used to estimate the size of the bands.

## RESULTS

**Screening for EPS Production.** Thirty heterofermentative sourdough LAB strains were screened for EPS production from sucrose by growing strains on MRS solid medium containing sucrose (4%, w/v) at 30 °C for 48 h. In these conditions, 23 strains displayed a mucoid phenotype revealing the presence of EPS and 7 strains did not produce any EPS (**Table 1**). As shown in **Figure 1**, three types of mucoid phenotypes were distinguished among EPS-producing strains: liquid, creamy and compact. Among the EPS$^+$ strains, 8 strains showed a liquid mucoid phenotype, 11 strains showed a creamy morphology and only 4 strains revealed a compact phenotype. The largest spots ( > 20 mm) were

**Table 1.** Results of Screening for EPS Production by Sourdough Strains[a]

| species | strain | API 50CH profiles | EPS phenotype |
|---|---|---|---|
| *Ln. citreum* | A7 | Lc2 | liquid (15) |
| | B1 | Lc4 | negative |
| | B2 | Lc2 | compact (12) |
| | B3 | Lc3 | negative |
| | B7 | Lc2 | liquid (16) |
| | B13 | Lc1 | compact (8) |
| | C10 | Lc2 | liquid (30) |
| | C11 | Lc2 | compact (10) |
| | C12 | Lc5 | creamy (16) |
| | E16 | Lc2 | liquid (25) |
| | H6 | Lc4 | creamy (14) |
| *Ln. mesenteroides* | A9 | Lm3 | compact (7) |
| | E18 | Lm4 | negative |
| | G15[b] | Lm3 | liquid (22) |
| | G28[b] | Lm3 | liquid (16) |
| | H9 | Lm1 | negative |
| | K24[b] | Lm3 | liquid (15) |
| | K29[b] | Lm3 | liquid (16) |
| | K30 | Lm2 | creamy (9) |
| *W. cibaria* | C36-1 | Wc2 | creamy (17) |
| | D38 | Wc2 | creamy (19) |
| | D39 | Wc1 | creamy (19) |
| | H25 | Wc2 | creamy (17) |
| | K39 | Wc2 | creamy (19) |
| *W. confusa* | C39-2 | Wc2 | creamy (12) |
| *Lb. sanfranciscensis* | A16 | Lsf1 | negative |
| | B27 | Lsf2 | negative |
| | O12 | Lsf3 | creamy (9) |
| | O31 | Lsf1 | creamy (12) |
| *Lb. brevis* | A15 | nd | negative |

[a] The first letter in strain denomination refers to the sourdough sample in which it was initially recovered (A, B, C, D, E, G, H, K, O). Carbohydrate fermentation patterns (API 50CH profiles) were defined within species, considering a threshold of 90% homology. EPS phenotype (negative, compact, creamy, liquid) was recorded from culture on MRS agar plates containing sucrose after 48 h at 30°C. Values in parentheses are the diameter (mm) of the zone of the bacterial growth. [b] Strains G15, G28, and K24, K29 were classified as distinct strains based on difference in morphology of the colonies on standard MRS agar medium (without sucrose).

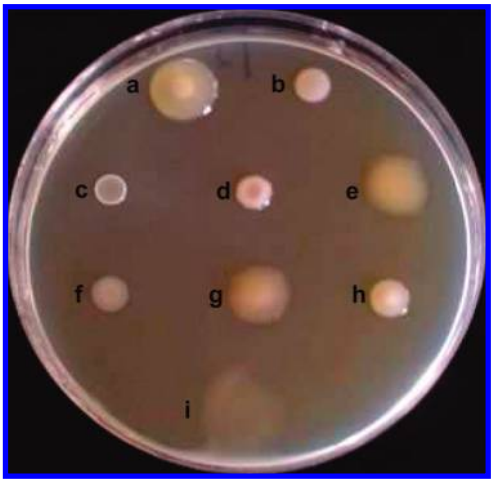


**Figure 1.** Morphology of bacterial spots from D38 (**a**), C39-2 (**b**), B3 (**c**), B2 (**d**), B7 (**e**), NRRL B-512F (**f**), G28 (**g**), K30 (**h**) and G15 (**i**) strains grown on MRS agar plates containing sucrose (40 g·L$^{-1}$) at 30 °C after 48 h. EPS$^-$ phenotype (**c**) and EPS$^+$ phenotype described as compact (**d**), creamy (**a**, **b**, **f**, **h**), liquid (**e**, **g**, **i**).

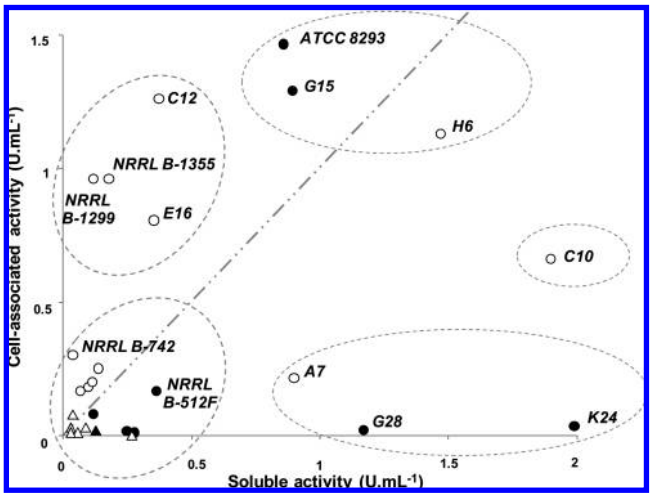


**Figure 2.** Distribution of glycansucrase activity from soluble and cell-associated fractions: *Leuconostoc citreum* (○), *Leuconostoc mesenteroides* (●), *Weissella cibaria* (△) and *Weissella confusa* (▲). Clusters (- - -) and median (— · · —). Each value is the average figure obtained by two glycansucrase activity measurements with a relative error lower than 10%.

observed for the liquid mucoid phenotype. $EPS^+$ strains were recovered from different species: *Ln. citreum* (9 strains), *Ln. mesenteroides* (6 strains), *W. cibaria* (5 strains), *W. confusa* (1 strain) and *Lb. sanfranciscensis* (2 strains). Carbohydrate fermentation profile on API 50CH led us to distinguish several biochemical patterns for each species (**Table 1**). All the strains were able to ferment glucose, fructose, maltose and sucrose. The main carbohydrates allowing group differentiation were arabinose, ribose, xylose, galactose, mannose, cellobiose, melibiose, trehalose and raffinose (data not shown). The strains were isolated from nine different sourdoughs and EPS-producing strains were found in each sourdough (**Table 1**). Sourdoughs contain between one and five different EPS-producing strains. Following this preliminary screening, we further characterized the type of glycansucrase activity responsible for the synthesis of EPS from sucrose.

**Glycansucrase Activity.** Extracellular glycansucrase activity recovered either in the cell culture supernatant or associated with the cell pellets from *Leuconostoc* and *Weissella* strains was then determined (**Figure 2**) and compared to that obtained from the reference *Ln. mesenteroides* or *citreum* strains NRRL B-512F, B-742, B-1299, B-1355 and ATCC 8293 producing glycansucrases which have been well characterized (**Figure 2**). Five clusters were defined according to the localization and level of activity. (i) The first cluster includes three *Leuconostoc* strains (A7, G28 and K24) which produce glycansucrases only in the supernatant and for which no cell-associated forms were detected. The level of activity produced ranged between 0.8 and 2 $U \cdot mL^{-1}$. (ii) The second one associates two *Ln. citreum* sourdough strains (E16, C12) and two reference-strains (NRRL B-1299 and B-1355) which only produce cell-associated glycansucrase activities with a production of about 1 $U \cdot mL^{-1}$. (iii) *Ln. mesenteroides* G15 and H6 and reference strain ATCC 8293 formed a third cluster. These latter strains produced both soluble and cell-associated glycansucrases with a production level around 1 $U \cdot mL^{-1}$. (iv) Strain C10 (*Ln. citreum*) stood apart with an activity of 2 $U \cdot mL^{-1}$ in the supernatant and about 0.7 $U \cdot mL^{-1}$ in the cell fraction. (v) Finally, the largest group includes 17 strains from *Leuconostoc* and *Weissella* species of which four are the reference strains NRRL B-742 (*Ln. citreum*), NRRL B-512F (*Ln. mesenteroides*), DSM 15878 (*W. cibaria*) and DSM 20196 (*W. confusa*). A low level of glycansucrase activity ( $< 0.3$ $U \cdot mL^{-1}$) was found both in the culture supernatant and the cell fraction.

**Exopolysaccharide Characterization.** The EPS produced from sucrose by the various strains were purified and submitted to acid hydrolysis followed by HPLC analysis of the monosaccharide. Depending on the level of glycansucrase activity, the production was carried out either in solid medium by recovering the slime or in liquid medium using soluble or cell-associated glycansucrase preparation. Acid hydrolysis of *Lb. sanfranciscensis* EPS released only fructose moieties showing that these strains produce only fructans. In contrast, all the *Leuconostoc* and *Weissella* strains produced glucans. For some strains (i.e., G15 and ATCC 8293), we observed that both glucose and fructose moieties were produced by acid hydrolysis indicating the presence of both glucans and fructans.

The EPS were further analyzed by $^1H$ and $^{13}C$ NMR spectroscopy, and the results are reported in **Tables 2** and **3**. As shown in **Figures 3** and **4**, four different patterns were obtained. Regarding group I (representative strain K24), the chemical shifts obtained by $^1H$ and $^{13}C$ (**Table 2**) are similar to that displayed by *Ln. mesenteroides* NRRL B-512F linear dextran with more than 95% α-(1→6) linked D-Glc*p* and less than 5% of α-(1→3) D-Glc*p* linked residues (**Figure 3**). The EPS classified in group II (representative strain G15) showed two anomeric protons at 4.98 and 5.33 ppm which were also assigned to α-(1→6) D-Glc*p* and α-(1→3) D-Glc*p* residues. However, the signals in the bulk region of the $^1H$ NMR spectrum (from 3.2 to 4.3 ppm) were very different from those obtained for group I glucan. The presence of the signals at 105.04, 81.13, 77.14, 76.04, 64.22, and 60.74 ppm in $^{13}C$ NMR spectroscopy strongly suggests that the sample is a mixture (**Figure 4**). The comparison of the chemical shifts with those displayed by commercial levan and linear dextran (**Figure 4** and **Table 2**) confirmed the presence of both levan and dextran. The $^1H$ and $^{13}C$ NMR spectra of the polymers from group III (representative strain A7) displayed all the chemical shift characteristics of α-(1→2) branched dextran. The $^1H$ NMR spectrum showed four anomeric protons at 4.98, 5.11, 5.19, and 5.32 ppm which were assigned to the anomeric proton of the α-(1→6) D-Glc*p*, the α-(1→2) D-Glc*p*, the 2,6-di-O-substituted α-D-Glc*p* and the α-(1→3) D-Glc*p* units, respectively (**Figure 3**). The $^{13}C$ NMR spectrum was totally in agreement with $^1H$ NMR. The anomeric signals specific for the α-(1→2) glucosidic linkages were clearly identified at 97.07 ppm and 96.24 ppm (**Figure 4**). The presence of the α-(1→2) linkage was further confirmed by the signal at 76.29 ppm which corresponds to C-2 involved in α-(1→2) linkages (*28*). The $^1H$ NMR and $^{13}C$ NMR spectra of the group IV (representative strain B2) are characteristic of the glucans containing α-(1→6) linkages together with a high content of α-(1→3) linkages (*28*). Indeed, the signal at 100.23 ppm corresponds to the anomeric carbon involved in the α-(1→3) linkages. We also observed, at 98.72 and 98.44 ppm, the anomeric signals corresponding to the α-(1→6) linked Glcp units and that of the 3,6-di-O-substituted α-D-Glc*p*. The presence of the α-(1→3) linkages is further confirmed by the signal at 81.14 ppm which corresponds to C-3 involved in α-(1→3) linkages (**Figure 4**).

Finally for glucans, we identified 18 dextrans with a percentage of α-(1→6) linkages varying from 92.2 to 99.1% and containing a low amount of α-(1→3) linkages (groups I and II, **Table 3**). In addition, 8 dextrans were found to express various percentages of α-(1→2) linkages (3.6 to 27.4%) along with α-(1→6) linkages varying from 71.6 to 92.2% and α-(1→3) linkages ranging from 0.4 to 4.2% (group III, **Table 3**). Finally, 4 *Ln. citreum* strains produced glucans with 23 to 40% α-(1→3) linkages (group IV, **Table 3**).

Endodextranase hydrolysis of the purified EPS was also performed. Various levels of resistance to dextranase have been found, which are in agreement with the NMR characterization

**Table 2.** $^{13}C$ NMR Chemical Shifts of Purified EPS, Compared with Linear Dextran from NRRL B-512F, Levan from Fluka, α-(1→2) Linked Dextran (*27*) and Alternan (*26*)

| α-(1→6) linear dextran from B-512F | levan | α-(1→6) and α-(1→2) linked dextran from B-1299 | α-(1→6)/α-(1→3) alternating linkage from B-1355 (alternan) | group I | group II | group III | group IV |
|---|---|---|---|---|---|---|---|
| | 105.06 | | | | 105.04 | | |
| | | | 100.55 | | | | 100.23 |
| 98.53 | | 98.71 | 99.02 | 98.54 | 98.54 | 98.55 | 98.72 |
| | | | 98.93 | | | | 98.44 |
| | | 97.24 | | | | 97.07 | |
| | | 96.39 | | | | 96.24 | |
| | | | 81.60 | | | | 81.14 |
| | 81.13 | | | | 81.12 | | |
| | 77.12 | | | | 77.14 | | |
| | | 76.47 | | | | 76.29 | |
| | 76.04 | | | | 76.04 | | |
| 74.23 | | 74.30 | 74.36 | 74.24 | 74.23 | 74.24 | 74.00 |
| | | 73.86 | | | | 73.71 | |
| | | 72.77 | | | | 72.73 | |
| | | | | | | 72.49 | |
| | | | | | | | 72.30 |
| 72.23 | | 72.34 | 72.62 | 72.23 | 72.24 | 72.21 | 72.20 |
| | | 71.17 | | | | 71.16 | |
| 71.01 | | | 71.16 | 71.02 | 71.02 | 71.01 | 71.00 |
| | | | | | | 70.92 | |
| | | | | | | | 70.85 |
| | | | | | | | 70.54 |
| 70.35 | | 70.41 | 70.62 | 70.36 | 70.36 | 70.35 | 70.16 |
| | | | | | | 70.20 | |
| | | | | | | | 69.97 |
| | | | | | | | 69.75 |
| | | | | | | 66.57 | |
| 66.35 | | 66.61 | 66.13 | 66.38 | 66.37 | 66.33 | 65.52 |
| | 64.20 | | | | 64.22 | | |
| | | 61.38 | | | | 61.21 | |
| | | | 61.35 | | | | 60.94 |
| | 60.74 | | | | 60.74 | | |

(**Table 3**). The values ranged from 4.3 to 37.4% for classical dextrans, from 37.9 to 82% for dextrans containing α-(1→2) linkages and from 64.0 to 97.8% for glucans with high percentage of α-(1→3) linkages. In addition, different dextranase resistance ratios were obtained for similar percentage of α-(1→6) linkages, indicating differences in polymer structure.

HPSEC analysis of the polymers revealed that they were totally excluded (data not shown) showing that the EPS have a size greater than $10^6$ Da.

**PCR Screening of Glucansucrase and Fructansucrase Encoding Genes.** The 23 $EPS^+$ and 7 $EPS^-$ sourdough strains, and several reference strains, have been screened for glucansucrase and fructansucrase encoding genes. The screening for potential fructansucrase genes revealed only 6 positive strains: *Lb. sanfranciscensis* O12, O31, *Ln. mesenteroides* G15, A9, NRRL B-512F and ATCC 8293. For both the *Lb. sanfranciscensis* O12, O31 and *Ln. mesenteroides* G15, ATCC 8293, the results were in correlation with the production of fructan suspected from EPS acid hydrolysis. Production of fructan was not detected for *Ln. mesenteroides* A9 even though PCR amplification was positive. The screening for putative glucansucrase genes revealed a positive signal for all the $EPS^+$ *Ln. mesenteroides* and *Ln. citreum* strains. Conversely, we did not obtain any amplification with DNA from the *Weissella* strains although EPS isolation revealed that all these strains produced a dextran. In addition, three *Leuconostoc* EPS-negative strains (B1, B3 and E18) gave a positive amplification with the primers targeting glucansucrase encoding genes.

## DISCUSSION

The results presented here clearly demonstrate a high biodiversity of EPS produced by sourdough LAB strains with sucrose substrate. Differences in cellular localization and activity level of the produced enzymes and in polymer structures were also highlighted.

The screening for EPS production performed on sucrose-containing agar plates revealed that 23 out of 30 heterofermentative sourdough LAB strains produced homopolysaccharides from sucrose. Nine *Ln. citreum*, five *Ln. mesenteroides*, five *W. cibaria* and one *W. confusa* strains produced only glucan, two *Lb. sanfranciscensis* strains produced fructan and one strain (*Ln. mesenteroides* G15) synthesized both glucan and fructan. Differences among strains in mucoid phenotype suggested the production of different types of EPS that was further confirmed by NMR results and dextranase resistance rate. Remarkably, EPS-producing strains have been found in every sourdough sample previously analyzed (*22*, *23*). This result confirms the assumption from Tieking et al. (*9*) that any given sourdough is likely to contain EPS-producing strains. For instance, sourdough C exhibited diversity both on EPS-producing LAB species (*Ln. citreum, W. cibaria* and *W. confusa*) and on polymer structure (dextran with α-(1→2) linkages, glucan with about 39% α-(1→3) linkages and dextran with low α-(1→3) ramifications, 2.4 to 3.0%).

Glycansucrase activity was quite variable among strains. The results pointed out that enzyme activity from *Ln. mesenteroides* strains is essentially soluble and that from *Ln. citreum* is

**Table 3.** Glucan Classification[a] and Resistance to Dextranase Digestion

| | | | | percentage of branching type of glucan (%) | | | |
|---|---|---|---|---|---|---|---|
| group | strain | species | EPS system production | α-(1→6) | α-(1→2) | α-(1→3) | dextranase resistance rate[b] |
| I | NRRL B-512F | *Ln. mesenteroides* | SM | 96.3 | | 3.7 | 15.1 |
| | DSM 15878 | *W. cibaria* | SM | 97.0 | | 3.0 | 14.8 |
| | DSM 20196 | *W. confusa* | SM | 97.1 | | 2.9 | 14.9 |
| | A9 | *Ln. mesenteroides* | SM | 94.3 | | 5.7 | 28.8 |
| | C36−1 | *W. cibaria* | SM | 97.0 | | 3.0 | 14.8 |
| | C39−2 | *W. confusa* | SM | 97.6 | | 2.4 | 10.3 |
| | D38 | *W. cibaria* | SM | 96.8 | | 3.2 | 13.5 |
| | D39 | *W. cibaria* | SM | 96.7 | | 3.3 | 16.3 |
| | G15 | *Ln. mesenteroides* | S | 96.1 | | 3.9 | 7.7 |
| | G28 | *Ln. mesenteroides* | S | 95.5 | | 4.5 | 9.4 |
| | H6 | *Ln. citreum* | S | 94.2 | | 5.8 | 25.6 |
| | | | C | 92.2 | | 7.8 | 37.4 |
| | H25 | *W. cibaria* | SM | 96.9 | | 3.1 | 13.9 |
| | K24 | *Ln. mesenteroides* | S | 95.8 | | 4.2 | 12.8 |
| | K39 | *W. cibaria* | SM | 97.2 | | 2.8 | 17.7 |
| II | ATCC 8293 | *Ln. mesenteroides* | S[c] | 96.4 | | 3.6 | 21.5 |
| | | | C[c] | 94.0 | | 6.0 | 8.4 |
| | G15 | *Ln. mesenteroides* | C[c] | 99.1 | | 0.9 | 4.3 |
| III | A7 | *Ln. citreum* | S | 71.6 | 27.4 | 1.0 | 82.0 |
| | B7 | *Ln. citreum* | SM | 72.8 | 25.9 | 1.3 | 80.5 |
| | C10 | *Ln. citreum* | SM | 77.4 | 20.8 | 1.8 | 70.5 |
| | | | S | 85.7 | 13.5 | 0.8 | 51.4 |
| | | | C | 72.3 | 27.3 | 0.4 | 74.3 |
| | C12 | *Ln. citreum* | C | 72.5 | 26.6 | 0.9 | 71.5 |
| | K29 | *Ln. mesenteroides* | SM | 92.2 | 3.6 | 4.2 | 37.9 |
| | K30 | *Ln. mesenteroides* | SM | 87.4 | 10.6 | 2.0 | 58.9 |
| IV | B2 | *Ln. citreum* | SM | 60.5 | | 39.5 | 97.3 |
| | B13 | *Ln. citreum* | SM | 60.9 | | 39.1 | 97.8 |
| | C11 | *Ln. citreum* | SM | 60.3 | | 39.7 | 97.3 |
| | E16 | *Ln. citreum* | C | 76.7 | | 23.3 | 64.0 |

[a] The classification was based on the type and percentage of glucosidic linkages determined by NMR spectroscopy. EPS were recovered from the slime scraped off on solid media (SM) or were produced by soluble (S) or cell-associated (C) glucansucrases. [b] Susceptibility to hydrolysis by endodextranase (*31*). [c] A mixture of dextran and levan was produced for ATCC 8293 S (25% levan), ATCC 8293 C (31% levan) and G15 C (72% levan).

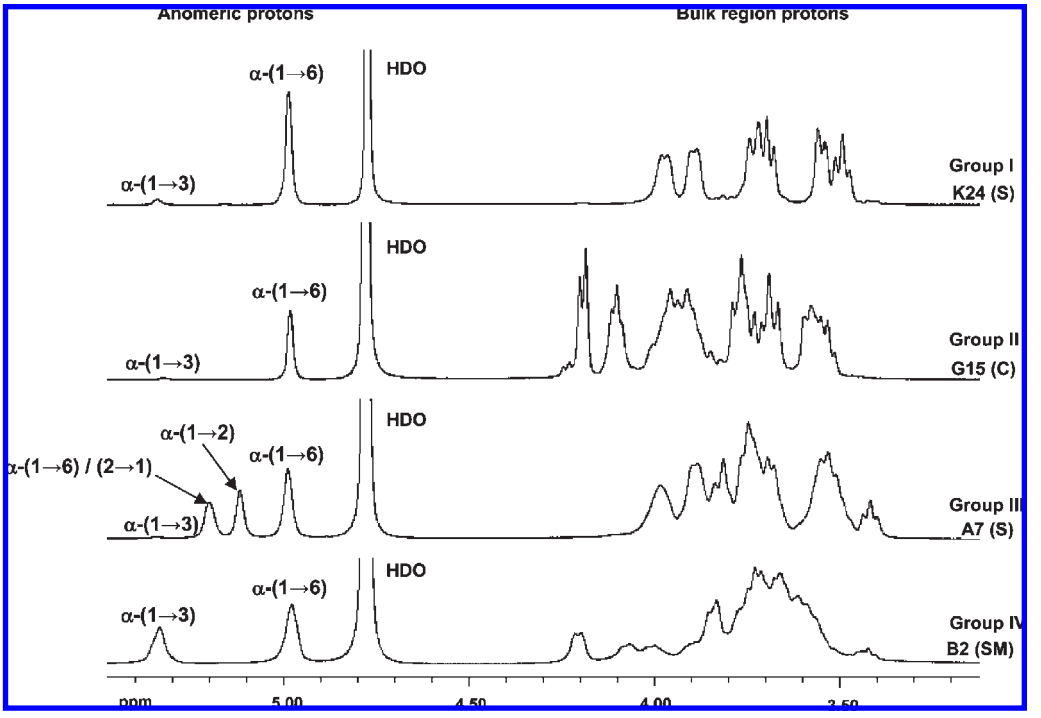


**Figure 3.** $^1$H NMR spectra of EPS extracted from cell-associated glycansucrase (C) for G15 (group II, mixture of levan and dextran), soluble enzyme (S) for A7 (group III) and K24 (group I) and slime from solid media (SM) for B2 (group IV), recorded in $D_2O$ at 298K. The peaks are referenced to internal acetone ($^1$H = 2.225 ppm).

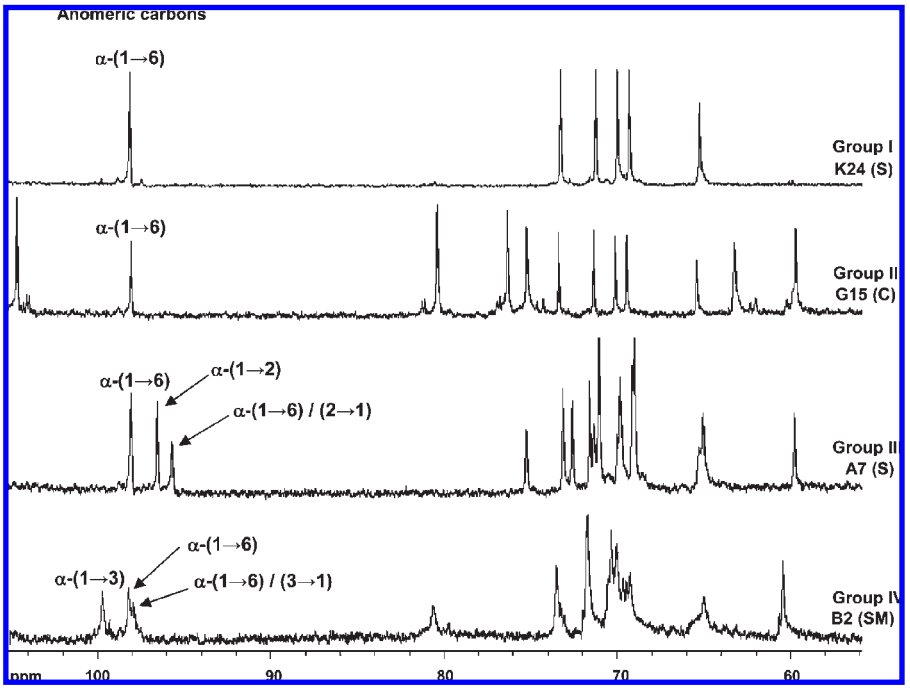


**Figure 4.** $^{13}C$ NMR spectra of EPS extracted from cell-associated glycan-sucrase (C) for G15 (group II, mixture of levan and dextran), soluble enzyme (S) for A7 (group III) and K24 (group I) and slime from solid media (SM) for B2 (group IV) recorded at 125 MHz at 298K in $D_2O$. The peaks are referenced to internal acetone (31.03 ppm).

preferentially cell-associated. Regarding *Weissella* strains, the glycansucrase activity was very low ( $< 0.3$ U·mL$^{-1}$). Cultivation conditions used in the present study could be not optimal for growth and/or EPS production of those strains. The presence of relevant glucan- and fructansucrase encoding genes was confirmed by PCR screening with primers targeting conserved domains of these enzymes. A good correlation was observed between the results of PCR amplification and those obtained from acid hydrolysis and NMR experiments. Remarkably, no PCR product was detected for the dextran-forming *Weissella* strains, as previously reported for two others glucan-forming *Weissella* strains (*6*, *10*).

Pure glucans and fructans were easily recovered by ethanol precipitation of EPS directly scraped from sucrose-containing agar cultures or produced by the soluble or cell-associated glycansucrases harvested from bacterial culture. $^{13}C$ NMR informed on the proportion of dextran and levan, and the susceptibility to endodextranase hydrolysis also provided relevant indications about the structure of the polymers (*31*). All sourdough LAB strains from *Leuconostoc* and *Weissella* genera produced only glucans except *Ln. mesenteroides* G15 strain, which synthesized both dextran and levan, approximately in a 1:3 ratio. For this latter, levansucrase activity was found as cell-associated. Production of both glucan and fructan was also found for *Ln. mesenteroides* strains NRRL B-512F and ATCC 8293. This confirmed Olvera et al. (*32*) molecular and enzymatic investigations on the presumptive structure of the polymers synthesizes by *Ln. mesenteroides* ATCC 8293$^T$. It well produces *in situ* both usual dextran and levan by soluble and cell-associated fractions, with a respective proportion of 3:1 and 2:1. Strains of *Lb. sanfranciscensis* synthesized only fructan, which is in agreement with previous works from Korakli et al. (*7*) and Tieking et al. (*9*), which reported levan production. Most glucan-producing *Leuconostoc* and *Weissella* strains produced classical dextrans with about 95% α-(1→6) linkages and low amounts of α-(1→3) glycosidic bonds. The structural description of dextrans from *Weissella* has been published recently as a linear polymer containing only α-(1→6) linkages (*33*) or with few (2.7%) α-(1→3)-linked branches (*29*). In the present study, the EPS produced by the *Weissella* strains were also dextrans, with α-(1→3) linkages varying from 2.4 to 3.3%. Nevertheless, production of fructan and/or glucan has also been described for sourdough *Weissella* strains (*6*).

Furthermore, we report here for the first time the production of α-(1→2) branched dextran by sourdough LAB strains. These polymers were synthesized by several strains belonging to *Ln. citreum* (A7, B7, C10 and C12) and *Ln. mesenteroides* (K29, K30) species. Production of such a type of dextran was reported for only few *Leuconostoc* strains (*26*−*29*), being first described by Seymour et al. (*26*) notably for *Ln. mesenteroides* NRRL B-1299 (which belong to *Ln. citreum* species, unpublished results). Maina et al. (*29*) recently described production of α-(1→2) branching dextrans by three strains isolated from cereal products. The results obtained in the present study strongly support the assumption that their production may be a feature of *Ln. citreum* strains isolated from cereals (*29*). In addition, four sourdough strains produced dextran with more α-(1→2) linkages (about 27%) than previously described by Uzochukwu et al. (*28*) and Maina et al. (*29*). Interestingly, strain *Ln. citreum* A7 had a soluble glycansucrase activity, in contrast to that of NRRL B-1299 which is cell-associated.

Another group of sourdough strains produces glucans with interesting structural characteristics. Actually, *Ln. citreum* B2, B13 and C11 synthesized glucans with a high percentage of α-(1→3) linkages, reaching 40%. These polymers had a very similar $^{13}C$ NMR spectrum with alternan from *Ln. citreum* strain NRRL B-1355, a polymer of glucopyranosyl residues alternately linked by α-(1→3) and α-(1→6) glycosidic bonds in the main chain (*26*, *34*). In addition, we observed that these polymers were more soluble in water than the usual dextrans. NMR results, the solubility feature and the high dextranase resistance rate (more than 97%) suggest that these polymers are alternans. However, whether the α-(1→3) branches are actually part of an alternan structure or a comblike structure still needs to be investigated.

Few strains are known to produce alternan, with the only description of *Ln. mesenteroides*/*citreum* NRRL B-1355, B-1501 and B-1498 and only one gene encoding alternansucrase has been so far sequence analyzed (*35*). Strain *Ln. citreum* E16 seems to produce quite a different polymer; with only 23.3% α-(1→3) linkages and a dextranase resistance rate of 64%. Further studies would reveal whether this strain synthesizes a mixture of alternan and dextran or a highly α-(1→3) branched dextran.

In conclusion, it was shown that production of homopolysaccharides is a widespread metabolic activity in heterofermentative sourdough LAB. Some strains described in the present study are likely to produce polymers, and derived oligosaccharides, with potential prebiotic properties (α-(1→2) linked dextran or putative alternan). Further studies will provide additional information on the natural biodiversity of *Leuconostoc* and *Weissella* sourdough glycansucrases. Overall, sourdough was demonstrated to be an attractive biotope for isolation of LAB producing polymers, and corresponding synthesizing-enzymes, with diverse industrial applications.

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