The role of dextran production in the metabolic context of *Leuconostoc* and *Weissella* Tunisian strains

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Abbreviations: cfu, colony forming unit; Dsr, dextransucrases; EPS, exppolysaccarides; HoPS, homopolysaccharudes; GC-MS, gas chromatography-mass spectrometry; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; MDH, maplate dehydrogenase; NCBI, National Center or Biotechnology Information; PEP, phosphoenolpyruvate; PYR, pyruvate; TEM, transmission electronic microscopy

Abstract

High molecular weight dextrans improve the rheological properties of fermented products, and have immunomodulatory and antiviral activity. We report on $5.84x10^7$-$2.61x10^8$ Da dextrans produced by *Leuconostoc lactis* AV1n*, Weissella cibaria* AV2ou, and *Weissella confusa* V30 and FS54 strains. Dextransucrases catalyze dextran synthesis by sucrose hydrolysis concomitant with generation of fructose. The four bacteria have ~160 kDa dextransucrases detected by zymograms. Each bacterium showed different interplay of dextran production and metabolic fluxes. All bacteria produced lactate, and, AV2ou apart, synthesized mannitol from fructose. Moreover, FS54 hydrolyzed dextran blue and the concentration of dextran produced by this bacterium decreased during the stationary phase. The binding of AV1n to Caco-2 cells and to polystyrene plates was higher under conditions for dextran synthesis. Thus, we provide here the first instance of a *Weissella* dextranase, associated to a dextransucrase ability, and of a positive influence of dextran on adhesion and aggregation properties of a bacterium.

Keywords: *Leuconostoc, Weissella,* dextran, exopolysaccharides, sucrose metabolism, bacterial adhesion

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria which belong to the phylum *Firmicutes*. Their main metabolic product is lactate and many of them are "generally recognized as safe" by the USA food and drug administration (Makarova et al., 2006). LAB are important to the food industry, because they produce during fermentations flavor enhancing compounds and prevent spoilage mainly by the production of lactate and bacteriocins (Kandler, 1983; Zarour et al., 2017a). LAB include, among others, the *Leuconostoc* and *Weissella* genera, heterofermentative bacteria closely related in their phenotypic characteristics (Fusco et al., 2015) and belonging to the same family of *Lactobacillaceae* (Zheng et al., 2020). Some *Leuconostoc* and *Weissella* strains produce exopolysaccharides (EPS), and specifically, dextran-type homopolysaccharides (HoPS). (Besrour-Aouam et al., 2019; Fusco et al., 2015). Dextran is a general term applied to polyglucans with a linear backbone containing at least 50% of D-glucopyranosyl residues attached by α-(1-6) linkages with varying percentages of α-(1-4), α-(1-3) or α-(1-2) branches (Bounaix et al., 2010). Dextransucrases (Dsr) synthesize dextran by hydrolysis of the glycosidic bond of sucrose and uses the released energy to transfer D-glucopyranosyl residues to the growing polymer, concomitantly releasing fructose (Werning et al., 2012). Diversity in the degree and type of branching, and chain length, confers to dextrans various properties which have applications in the cosmetic, pharmaceutical and food industries (Lakshmi Bhavani & Nisha, 2010; Pérez-Ramos, Nácher-Vázquez, Notararigo, López, & Mohedano, 2015).

Dextrans with a relatively low molecular weight (Mw) are used as a blood-plasma substitute in the pharmaceutical industry. Scientific evidences support that, in addition to food texture improvement, high Mw dextrans are postbiotics with antiviral and immunomodulatory activities (Nácher-Vázquez et al., 2015; Zarour et al., 2017b). Consequently, as there is currently a consumer demand for food that promotes well-being, high Mw dextran-producing LAB have interest for the bakery industry, since the *in situ* production of the HoPS could improve volume and shelf-life of the bread without other additives. Thus, the *in situ* production of dextran by *Weissella* strains in wheat sourdough allowed improvement of the bread volume and crumb softness (Katina et al., 2009), and also contributed to the nutritional enrichment and shelf life of gluten-free bread (Galle et al., 2012). Replacing hydrocolloid additives, the low-branched dextran producer *Leuconostoc* and *Weissella* strains conferred a thick, but not too elastic texture to pureed carrots (Juvonen et al., 2015). Furthermore, dextrans are used in ice cream preparation as cryoprotectants, in fermented dairy products to increase creaminess and viscosity and to reduce syneresis, and in low-fat cheese to enhance its texture by increasing moisture content (Kothari, Das, Patel, & Goyal, 2014).

Dextran-producing LAB have also industrial interest for the development of new functional fermented food and innovative nutritious and functional beverages (Zannini et al., 2013). These overall properties have promoted a scientific interest for the isolation and testing of new dextran-producing LAB from different habitats.

In addition, some *Weissella* and *Leuconostoc* strains produce mannitol (Galle, Schwab, Arendt, & Ganzle, 2010; Xu et al., 2017; Zarour, Prieto, Pérez-Ramos, Kihal, & López, 2018). This polyol has a higher sweetening power than sucrose, without tooth-decaying effects, and does not induce hyperglycemia because it is partially metabolized by humans (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). Consequently, the characterization of new mannitol-producing bacteria is of industrial interest. Thus, the aim of this study was to select strains, from new ecological niches, with beneficial functional properties, and thus potentially useful for the development of innovative Tunisian functional food. Therefore, we here report on the characterization of four Tunisian dextran-producing LAB. We have physicochemically characterized their dextrans and investigated their production and their influence on the bacterial metabolic fluxes as well as the adhesion and aggregation properties of the producer strains. We have detected a dextranase activity in *Weissella*

confusa FS54. Moreover, we have demonstrated that the *Lc. lactis* and the two *Weissella confusa* strains (V30 and FS54) produce mannitol.

2. Materials and methods

2.1. Bacterial strains and growth conditions. The four LAB strains used in this work were isolated in Tunisia: AV1n and AV2ou from avocado, and V30 and FS54 from leaf and rhizosphere of olive tree, respectively. Two of them were previously isolated and typed as *Leuconostoc lactis* AV1n (Besrour-Aouam et al., 2019) and *Weissella confusa* FS54 (Fhoula et al., 2013). In this work, the AV2ou and V30 strains were isolated and were typed by partial sequencing of its 16S rRNA coding gene, as *Weissella cibaria* (Genbank ID: MN907097), and *Weissella confusa* (Genbank ID: MT106834) strains, respectively. The ability of these LAB to produce dextran was investigated. The bacteria were grown at 30 °C in CDM medium, instead of complex media, which contain polysaccharides that could contaminate the EPS produced by the test organisms. Also, the influence of the use of sucrose instead of glucose during growth was assessed in real time using a Varioskan microplate reader (Figure S1).

The LAB were grown at 30 °C in the MRS broth supplemented with 2% of either glucose (MRSG) or sucrose (MRSS) or in a chemically defined medium (Sánchez et al., 2008) supplemented with 0.8% sucrose (CDMS) or 0.8% glucose (CDMG).

2.2. Production, purification, quantification and characterization of the EPS. For their production, LAB grown at 30 °C in MRSS to $OD_{600\ nm}$=1.0 were diluted 1:10 in CDMS medium and incubated at 30 °C until the beginning of the stationary phase. The EPS were isolated and purified from culture supernatants by ethanol precipitation and dialysis as previously described (Nácher-Vázquez et al., 2015). The EPS concentration was determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and EPS purity was tested fluorometrically using specific fluorescent staining kits for DNA, RNA and proteins as reported (Zarour et al., 2018). EPS characterization was performed by monosaccharide composition determination, Fourier transform infrared (FTIR) spectroscopy and methylation analysis as previously described (Notararigo et al., 2013). The range of Mw of purified dextrans was determined by size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) detector as previously reported (Zarour et al., 2018).

2.3. Detection of Dsr and dextranase activities. For Dsr testing, LAB cultures grown in MRSG or MRSS to OD_{600nm}=1.0. were diluted 1:100 in CDMG or CDMS and grown to $OD_{600\ nm}$=1.0. The culture supernatants were loaded onto a SDS-(3–8%)-polyacrylamide gradient gel, and subjected to electrophoresis at room temperature and constant 100 V. Then, the gel was washed three times with sodium acetate buffer (20 mM sodium acetate, pH 5.4, 0.05 g/L $CaCl_2$ and 0.1% Triton X-100) for 1 h to renature the proteins, and incubated overnight in the same buffer supplemented with 10% sucrose. Thereafter, dextransucrase activity was revealed by periodic acid-Schiff staining. The, proteins were stained with 0.25% coomassie brilliant blue. The Mw was estimated by reference to the Mark 12 Protein Unstained Standards.
For detection of dextranase activity, bacteria were grown in liquid MRSG to an OD_{600nm}=1.0. Then 5 µL of the culture containing $5x10^8$ cfu were spotted on MRSG-agar plates supplemented with 0.4% dextran blue and incubated for 20 days at 30 ºC.

2.4 Detection of EPS production at cellular level by transmission electron microscopy (TEM). Mid-exponential phase cultures, grown in either MRSG or MRSS at 30 ºC, were sedimented by centrifugation (11,200 x *g*, 3 min, 4 °C) with the aim of retaining the dextran attached to the bacteria. The pellet was resuspended in 0.1 M $AcNH_4$, pH 7. For the TEM analysis, the samples were prepared as previously described (Pérez-Ramos, Mohedano, Pardo, & López, 2018). The preparations were examined in a JEOL JEM-1230 microscope operating at an accelerating voltage of 100 kV.

2.5. LAB binding capability. To test for bacteria-enterocyte interactions, the Caco-2 enterocyte cell line was grown as a monolayer of differentiated and polarized cells as previously described (Nácher-Vázquez et al., 2017a) Bacterial cultures were grown as in section 2.4 and the adhesion assays were performed in Dulbecco's Modified Eagle medium (DMEM) containing 0.5% glucose (G) or in DMEM supplemented with 1% sucrose (S) to give a final concentration of $1.25x10^6$ colony forming units/mL and a ratio 1:10 bacteria:enterocytes as previously described (Zarour et al., 2018). However, as AV1n unable to produce high concentration of dextran at 37 °C (Besrour-Aouam et al., 2019), incubation was conducted at 30 °C for 2 h. Afterwards, the number of cell-associated bacteria was determined by plate counting, as previously described (Nácher-Vázquez et al., 2017a). Biofilm formation assays, on polystyrene plates were conducted in MRSS and MRSG as previously described (Stepanović et al., 2007) for 48 h at 30 °C.

2.6. Analysis of dextran synthesis and metabolic fluxes. LAB central metabolism was tested in the presence of sucrose as previously described (Zarour et al., 2018). Briefly, LAB grown in MRSS to OD_{600nm} = 1.0 were diluted 1:10 in CDMS (30 mM sucrose final concentration) and grown for 24 h at 30 ºC. Growth was monitored at $OD_{600nm,}$ and media acidification by pH measurement. Culture supernatants recovered by centrifugation (9300 x *g*, 10 min, 4 °C) were used to determine concentration of EPS by the phenol-sulphuric acid method, and sucrose and their metabolites by gas chromatography-mass spectrometry (GC-MS) using *myo*-inositol as internal standard.

2.7. Statistical analysis. The data are expressed as the mean of three independent experiments (with at least two replicates per experiment) and the corresponding standard deviation. For analysis of LAB adhesion to enterocytes and LAB biofilm formation, a two factorial Randomized Complete Block Design (RCBD) was applied, with experiments performed on different days treated as random blocks. The effects of media, strains, and their interaction were analyzed with a two-way analysis of variance. A p value of ≤ 0.05 was considered significant. Mean pairwise comparisons were computed using Tukey's test ($p = 0.05$). All analyses were performed with the R software version 4.0.0 (RCoreTeam, 2020).

2.8. Informatics analysis of genomes, genes, and inferred protein sequences. To identify proteins with a particular function, and their coding genes, we searched the National Library of Medicine of the National Center or Biotechnology Information (NCBI) using the Entrez shear engine. The DNA sequences of the bacterial genomes and their inferred proteins were compared with those of other bacteria deposited at the NCBI database, using the Basic local alignment search tool. The DNA sequence of the draft genomes of *Lc. lactis* AV1n (Gene bank ID: WSZI00000000.1) and *W. confusa* FS54 (unpublished results) and their products were also analyzed with the informatics tools of the Rapid Annotation using Subsystem Technology (RAST) web page (https://rast.nmpdr.org/).

3. Results

3.1. Dextran production by the LAB and characterization of the polymers. The four Tunisian strains generated mucosal colonies when grown on MRSS medium containing 2% sucrose, the substrate for the dextran synthesis, but not on MRSG-agar, (Figure 1). The EPS production by the three *Weissella* in MRSS resulted in the generation of viscous and slightly diffuse colonies, whereas the AV1n strain produced more compact and lenticular colonies (Figure 1).

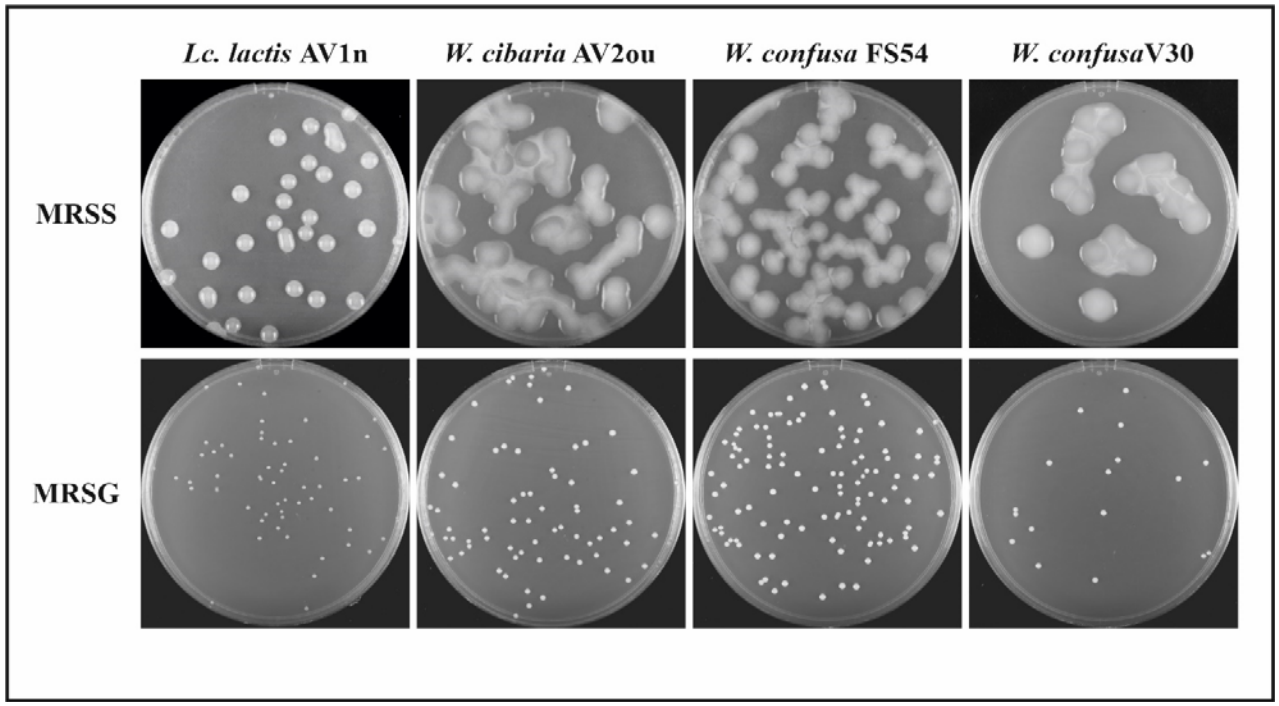

Figure 1. Detection of EPS production on solid media by the indicated LAB. Appearance of bacterial colonies after growth on MRSS-agar or MRSG-agar for 48 h at 30 °C.

During exponential growth, AV1n displayed a higher growth rate (μ) in CDMS than in CDMG (1.67±0.08 *versus* 1.00±0.10). The opposite behavior was observed for FS54 (μ=0.81±0.04 in CDMS *versus* μ=0.93±0.06 in CDMG) accompanied by a short exponential phase and apparently a lower final biomass (estimated by the OD of the cultures) in CDMS medium, indicating that this bacterium preferentially utilize glucose *versus* sucrose. For the other two *Weissella* strains the carbon source had little influence on the growth rate (μ=0.95-0.96 for AV2ou and μ=0.77-0.78 for V30), and only a slight decrease in the final biomass of AV2ou in CDMS *versus* CDMG. Moreover, the four LAB synthesized EPS in CDMS and not in CDMG (results not shown). Therefore, to produce EPS for purification we grew the strains in CDMS up to the end of the exponential phase (to avoid contamination of the EPS by bacterial debris due to cell lysis). High Mw polymers were recovered from the culture supernatants by precipitation with only 50% ethanol (final concentration) at 4 ºC to avoid co-precipitation of interfering compounds. For further purification, the precipitated EPS were dialyzed to remove low Mw contaminants. These treatments produced four EPS samples with a purity higher than 98%, free of RNA, and with low contamination of DNA (0.01-0.02%), and no detectable protein (except for the polymer from FS54 (Table 1).

Table 1. Detection of contaminants in the EPS preparations

Sample / Biomolecules / Bacteria	Supernatant[a]				After precipitation and dialysis[b]			
	EPS (%)	Protein (%)	DNA (%)	RNA (%)	EPS (%)	Protein (%)	DNA (%)	RNA (%)
Lc. lactis AV1n	98.21	1.73	0.06	ND	99.99	ND	0.01	ND
W. cibaria AV2ou	96.41	3.50	0.09	ND	99.99	ND	0.01	ND
W. confusa V30	96.13	3.76	0.11	ND	99.99	ND	0.01	ND
W. confusa FS54	92.53	7.23	0.20	0.04	98.49	1.49	0.02	ND

100% corresponds to the total concentration of detected biomolecules (EPS, proteins, DNA and RNA) in each sample.
[a]The concentration of proteins, DNA and RNA was measured directly in the supernatant. EPS concentration was determined after precipitation with ethanol.
[b]The concentration of the biomolecules were determined from preparations of the freeze-dried compound at a concentration of 1 mg/mL dissolved in water.
ND indicate levels below the limit of detection corresponding to 0.5 µg/mL of DNA, 20 ng/mL of RNA and 1 µg/mL of proteins.

The levels of the LAB-EPS in the culture supernatants ranged from 2.33 g/L for AV1n to 0.44 g/L for FS54, and their recovery after purification varied from 34% for AV2ou to 20% for FS54 (Table 2). Analysis by SEC-MALLS of the purified EPS revealed in each case only one polydisperse polymer, and as expected, all of them with high Mw being the highest average value for the product of AV1n (2.61×10^8 Da) and the lowest for that synthesized by FS54 (5.84×10^7 Da). In addition, the radius of gyration of the four EPS varied from 100-172 nm, these being the highest and the lowest values for the polymers of AV2ou and AV1n, respectively (Table 2).

Table 2. Analysis of the EPS production and their molecular weights

Bacteria	EPS in supernatant[a]	Purified EPS		
	Total amount (mg)	Total amount (mg)	Mw (Da)	Rx (nm)
***Lc. lactis* AV1n**	699	219	2.61×10^8	100.30
***W. cibaria* AV2ou**	300	103	1.24×10^8	172.35
***W. confusa* V30**	369	107	1.10×10^8	168.85
***W. confusa* FS54**	132	27	5.84×10^7	107.15

[a]Supernatants of 300 mL cultures.

The purified EPS samples were also subjected to physicochemical characterization. Analysis of the monomeric composition by GC-MS revealed that the EPS produced by the three *Weissella* strains were glucan, composed strictly of glucose moieties (data not shown). In addition, the FTIR spectra of the four HoPS preparations presented the same profile (Figure 2A) and they were typical of polysaccharides (Salazar, Ruas-Madiedo, Prieto, Calle, & de Los Reyes-Gavilan, 2012).

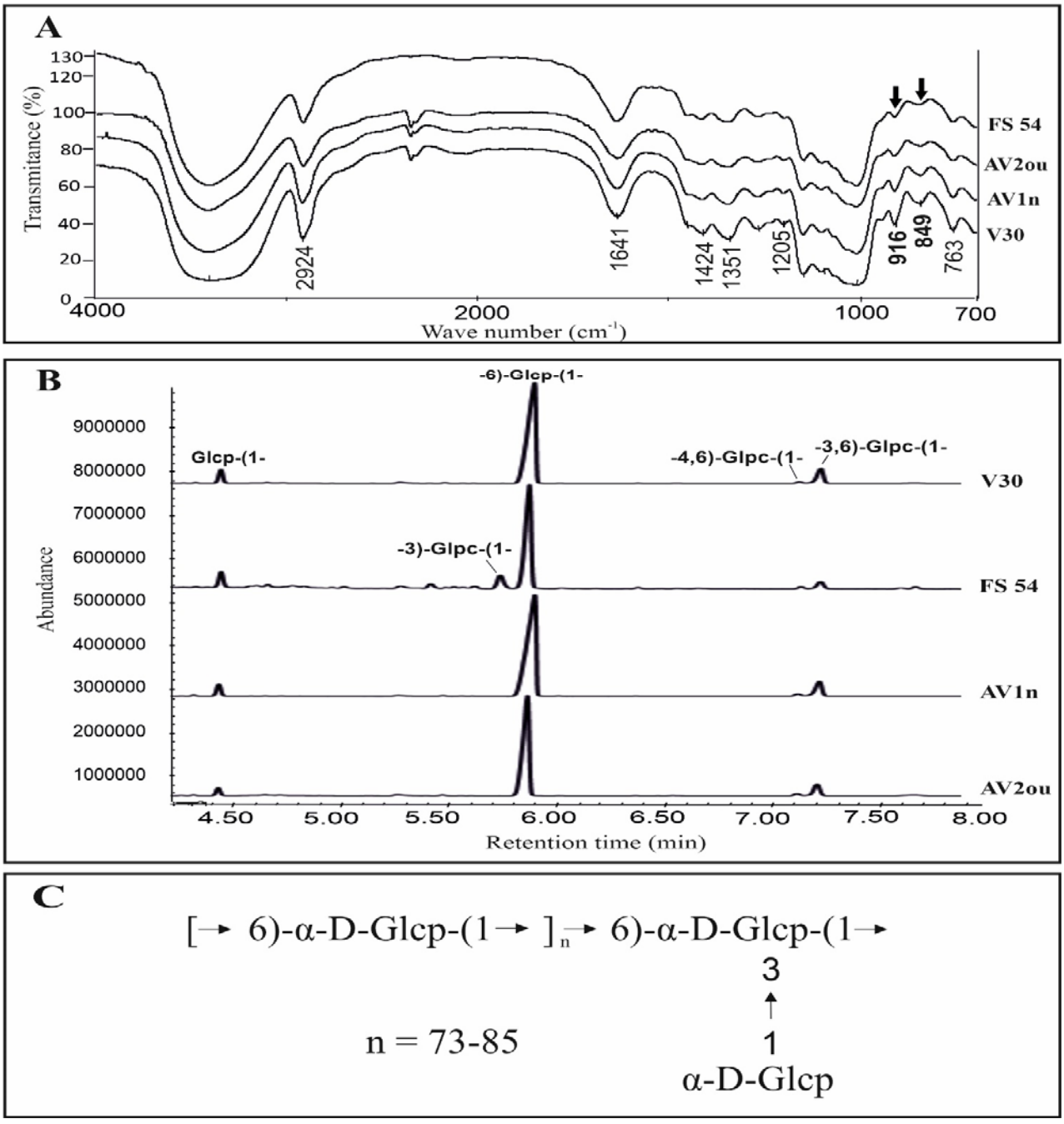

Figure 2. Physicochemical characterization of the dextran produced by the indicated LAB. (A) infrared spectrum of the EPS. (B) Types of the EPS bonds deduced by the methylation analysis. (C) Structure of the dextrans.

The detection in the four EPS preparations of absorption bands around 850 $cm^{-1}$and 920 cm^{-1} corresponding to α-anomers (Notararigo et al., 2013) and the absence of bands characteristic of β-linkages, showed that the polymers were α-glucans. This analysis also confirmed the absence of non-glucidic components (phosphate, sulphates or proteins). The methylation analysis (Figure 2B) showed that the four EPS had a main chain of glucopyranose units with α-(1-6) linkages (73.0%-85.0%), partially branched at the *O*-3 position (6.0%-9.6%) and *O*-4 position (0.5%-1.7%) by a single α-glucopyranose unit. Therefore, the overall data defined the polymers as dextran-type HoPS with the structure depicted in Figure 2C. The methylation analysis also revealed that FS54 EPS contains a small concentration of glucopyranose units with α-(1-3)-linkages, which may correspond to short side chains in the dextran structure (Figure 2B). In fact, previous works have demonstrated that approximately 40% of side chains of commercial dextrans are composed of single α-D-glucopyranosyl units, 45% contain two units and only 15% of them have more than 2 units (BeMiller, 2003).

3.2. Dextransucrase activity pattern of the dextran-producing LAB. The above and previous results (Besrour-Aouam et al., 2019) demonstrated that indeed the four LAB studied produced dextrans. These polymers are synthesized by the Dsr, which are extracellular enzymes. Moreover, the *dsrLL* gene of AV1n has been previously sequenced and its homologous and heterologous overexpression confirmed that the DsrLL enzyme synthesizes *in vivo* dextran (Besrour-Aouam et al., 2019). Therefore, in order to detect the enzymes produced by the four LAB, their culture supernatants were fractionated by SDS-PAGE, and upon addition of sucrose *in situ* synthesis of the glucans was performed at a pH 5.4 and the activity bands were detected with Schiff's reagent (Figure 3A).

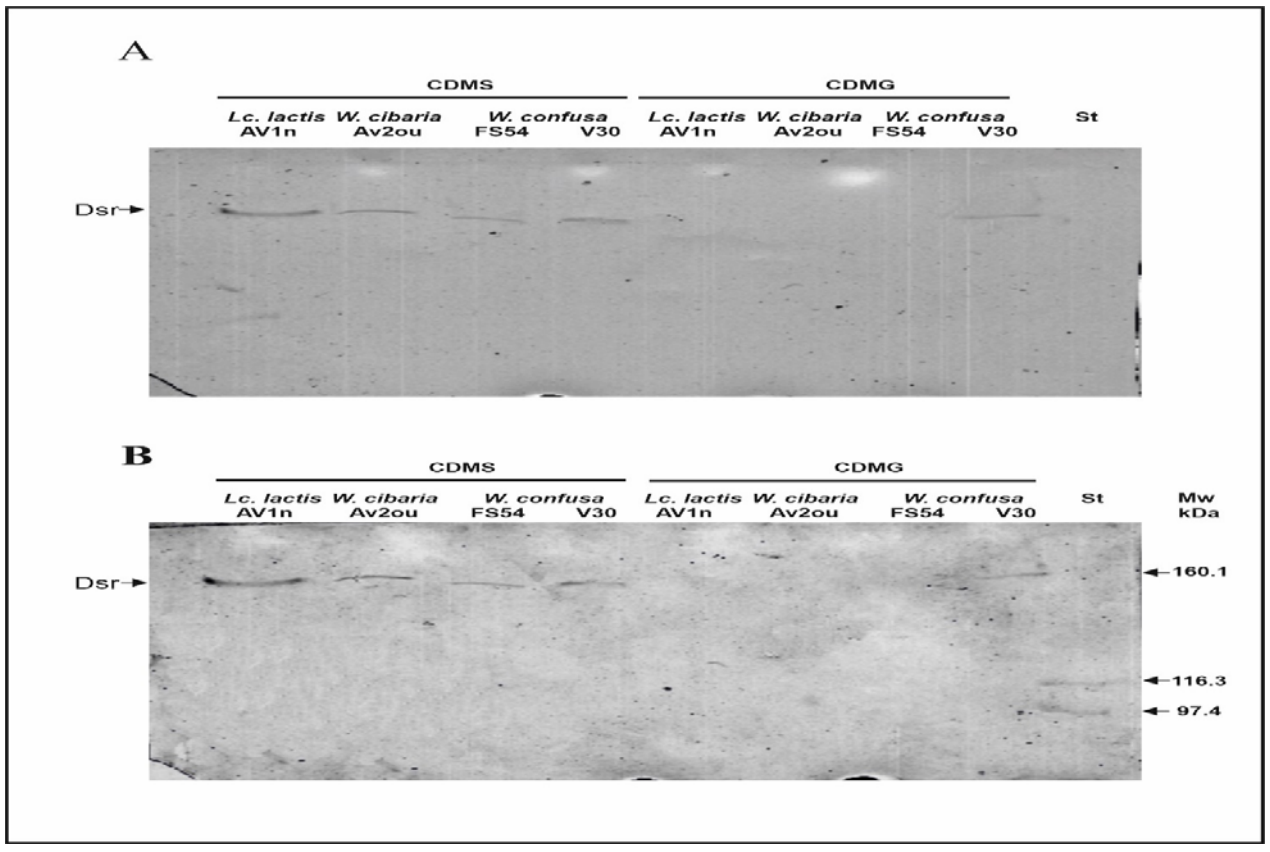

Figure 3. *In situ* detection of dextransucrase activity in cell free supernatants of LAB cultures. The indicated bacteria were grown in the presence of sucrose (CDMS) or glucose (CDMG), the culture supernatants were subjected to SDS-PAGE and after protein renaturalization, they were analyzed *in situ* for Dsr activity (A), and then stained with Coomassie brilliant blue (B). St, protein Mw standard (Mark 12).

The gel was also stained with coomassie brilliant blue for protein visualization (Figure 3B). The zymogram revealed that when grown in CDMS the supernatants of all LAB cultures contain one active Dsr detected as a band at the expected position for the extracellular AV1n DsrLL of 160.1 kDa (Figure 3A). This band was not observed in the supernatants of AV1n grown in CDMG (Figure 3A), nor was the presence of a non-active protein (Figure 3B). Thus, these results support the previously demonstrated sucrose mediated induction of *dsrLL* transcription in this LAB (Besrour-Aouam et al., 2019). With regard to the *Weissella* strains, when grown in CDMG, only in the supernatant of the V30 culture was a band detected, revealed by both the activity analysis (Figure 3A) and by staining (Figure 3B), indicating that Dsr V30 is synthesized in the presence of either sucrose or glucose.

3.3. Analysis of LAB capability to adhere to Caco-2 cells and to form biofilm. The presence of EPS can affect the *in vitro* binding of their producing LAB to biotic and abiotic surfaces (Nwodo, Green, & Okoh, 2012; Zarour et al., 2017b), although in some cases there is no correlation of the bacterial behavior in these two types of interactions (Rohani, Papizadeh, & Pourshafie, 2018). Therefore, the influence of dextran synthesis on the ability of LAB to bind to human enterocytes (Figure 4A) as well as to form biofilm on polystyrene plates (Figure 4B) was investigated. The LAB adhesion assays to Caco-2 cells were performed in the absence (G) or the presence of sucrose (S) (Figure 4A). Prior to exposure to Caco-2 cells, analysis of the bacterial cells by TEM showed that, as expected, dextran was only present in the LAB cultures grown in the presence of sucrose **(**inset in Figure 4A). High levels of binding to enterocytes were observed for AV1n in both conditions tested and also for V30-G (Figure 4A). Furthermore, the experiments revealed three types of bacterial behavior when sucrose was included in the assay. AV1n was the only bacterium able to adhere to the enterocytes at a higher level in the presence of sucrose (48.8±3.1% AV1n-S *versus* 27.8±2.7% AV1n-G). An opposite pattern was observed for V30 with binding ability significantly higher in the absence of dextran production (38.1±4.2% V30-G *versus* 14.3±2.2% V30-S). Moreover, adhesion of AV2ou and FS54 were only slightly, and not statistically significantly (p value > 0.05), affected by the presence (11.4±0.7% and 10.6±1.2%), or absence (9.6±2.2% and 15.0±3.1%) of sucrose in the medium.
To assay binding to an abiotic surface, LAB were grown in either MRSG or MRSS and the assay was performed in the same corresponding medium. The four LAB formed biofilms efficiently, apart from AV1n in MRSG medium, where very low levels of biofilm were detected (Figure 4B). This last result indicated the importance of the presence of dextran for biofilm formation by AV1n. Moreover, among the four LAB, two behaviors were observed (Figure 4B). In the presence of the Dsr substrate (MRSS), not only AV1n, but also AV2ou formed biofilm more efficiently, this effect being more pronounced for the former bacterium (3.4±0.4 in MRSS *versus* 0.8±0.2 in MRSG) than for the latter (3.5±0.2 in MRSS *versus* 2.4±0.3 in MRSG). In contrast, apparently synthesis of the dextrans interfered with the biofilm formation capability of V30 (3.6±0.3 in MRSG *versus* 2.7±0.3 in MRSS) and FS54 (3.1±0.3 in MRSG *versus* 2.7±0.2 in MRSS). In agreement with these results observation of the cell pellet of AV1n sedimented after 24 h of growth in MRSS revealed a visible layer of EPS, which seems to cover the cells of this bacterium but not of that of FS54 (Figure S2) and the other LAB (results not shown).

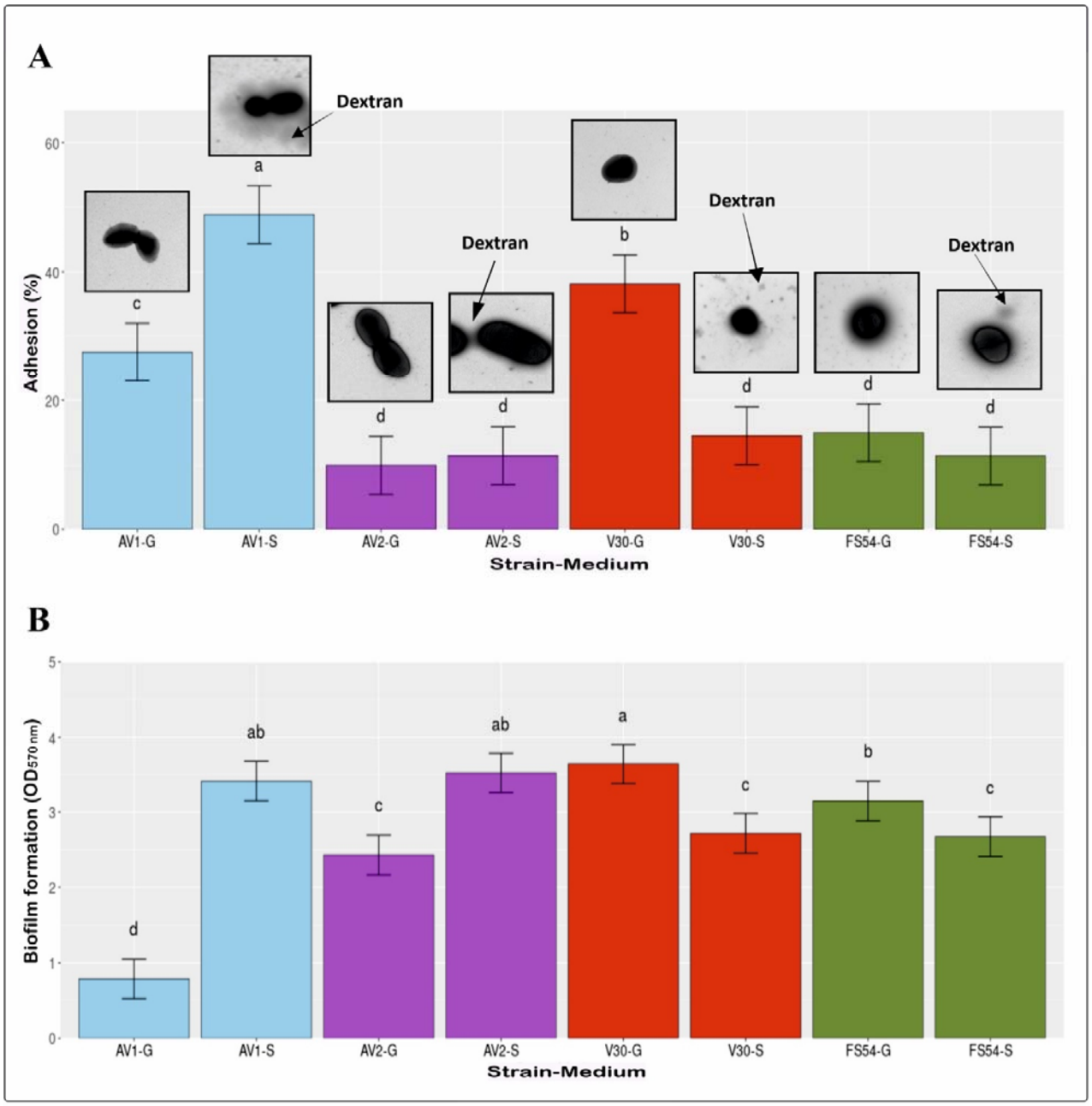

Figure 4. Analysis of LAB interaction with biotic and abiotic surfaces. (A) LAB binding to Caco-2 human cell line. The assays were performed in DMEM (G) and DMEM supplemented with 1% sucrose (S) during 2 h. Adhesion levels of the indicated LAB strains are expressed as the percentage of cfu. 100% corresponds to the number of bacteria added to the Caco-2 cells. The insets show pictures of the LAB obtained by TEM. The arrows mark the dextran. (B). LAB binding to polystyrene plates. Biofilm formation was performed in MRSG (G) or MRSS (S) for 48 h at 30 ºC. The results represent the total biomass that constitute the biofilm, determined by the $OD_{570\ nm}$ using the crystal violet staining technique. Experiments were performed in triplicate and with three replicates per each assay. The ANOVA statistical analysis of the results is depicted. A p value ≤ 0.05 was considered significant. Tukey's test was employed (α=0.05) to test the statistically significant differences between samples. Means with the same letter were not significantly different.

3.4. Central metabolism and EPS production. Dsr enzymes convert the sucrose substrate into high Mw polymers, with the concomitant release of fructose (Bounaix et al., 2010) . This synthesis, taking place extracellularly, allows an easy monitoring of the levels of sucrose and metabolic products by GC-MS analysis of the LAB culture supernatants. Thus, we have investigated the interrelation of the metabolic fluxes in conditions of dextran production during the planktonic growth of the four LAB in CDMS medium as well as the evolution of the medium pH (Figure 5 and Table S1). All LAB produced dextran only during the exponential phase of growth and secreted lactate during both the exponential and the stationary phases (Figure 5). After growth for 24 h, the highest lactate concentration was observed for AV2ou and FS54 (50.92±0.4 Mm and 44.94±0.1 mM) and the lowest for AV1n and V30 (~28 mM) (Table S1). These results correlated with the final pH of the cultures 4.8 and 4.9 for the former two and 5.3 for the latter LAB (Figure 5).

For all LAB, the maximum concentration of the EPS in the culture supernatants were concomitant with the highest extracellular levels of fructose (Table S1). Fructose accumulation in culture supernatants has been previously described for *Leuconostoc* and other dextran-producing LAB during their consumption of sucrose (Dols, Chraibi, Remaud-Simeon, Lindley, & Monsan, 1997; Nácher-Vázquez et al., 2017a; Santos, Teixeira, & Rodrigues, 2000; Zarour et al., 2018). Here, the four LAB generated transient extracellular accumulation of fructose followed by further decrease (Figure 5), presumably due to its internalization. Afterwards, fructose can be the substrate for mannitol synthesis in a reaction catalyzed by a mannitol dehydrogenase (Wisselink et al., 2002) Thus, the metabolic analysis revealed that beside AV2ou, the other three LAB produced mannitol (Figure 5 and Table S1).

The maximum concentrations of the EPS expressed as molecules of glucose ranged from 12.53±0.73 mM for AV1n to 4.62±0.18 mM for FS54, and intermediate values were observed for AV2ou and V30 strains (9.21±0.15 mM and 10.78±0.06 mM) (Table S1). These levels of the HoPS corresponded to 2.25-0.85 g/L and were similar to that synthesized by the dextran-producing *Lc. mesenteroides* strains (1-3 g/L) (Notararigo et al., 2013; Zarour et al., 2017b) and *Lactobacillus sakei* MN1 (2 g/L) (Nácher-Vázquez et al., 2015) under the same growth conditions.

The efficiency of the Dsr is influenced by various factors, including the pH of the culture medium (Ruhmkorf et al., 2013). The optimal pH value for Dsr activity is around pH 5.4 (Bounaix et al., 2010), and in fact the activity of the Dsr of the four LAB was detected at pH 5.5 (Figure 3A). In addition, the biomass has to be taken in consideration for efficiency of the EPS production, and estimation of the biomass by the OD of the cultures revealed a specific dextran production for OD_{600nm}=1 of 5.0 mM, 5.4 mM, 4.8 mM and 3.3 mM for the AV1n, AV2ou, V30 and FS54 strains, respectively. At the late stationary phase (after 24 h of growth), most of the EPS (87%-92%) synthesized by the LAB during the exponential phase of growth was recovered from the cultures supernatants, except for that of FS54, with a recovery of 48% of the maximum level (Table S1). This result indicated the existence of a dextranase activity in this LAB, and this hypothesis was supported by the ability of the FS54 strain and not the others to generate halos on MRSG-agar plates supplemented with 0.4% of dextran blue (inset in Figure 5).

Each bacterium showed a different pattern of coupling of EPS production and metabolic fluxes (Figure 5). Thus, production of EPS by AV2ou, V30 and FS54 strains apparently stopped, when only 75%, 66.8% and 57.1% of the sucrose added to the medium (30 mM) had been consumed. However, lactate was still increasing and mannitol was still produced by the *W. confusa* strains. By contrast, AV1n consumed more than 95% of the sucrose after 3 h of growth, when the maximum value of the EPS production took place. Under the same growth conditions, *Lc. mesenteroides* RTF10 and *Lb. sakei* MN1 consumed approximately 90% of the sucrose added to the medium during the first 7 h of growth, and when the culture entered the stationary phase (Nácher-Vázquez et al., 2017a) and *Lc.*

mesenteroides CM9 and *Lc. mesenteroides* CM30 showed the same pattern of sucrose consumption (Zarour et al., 2018).

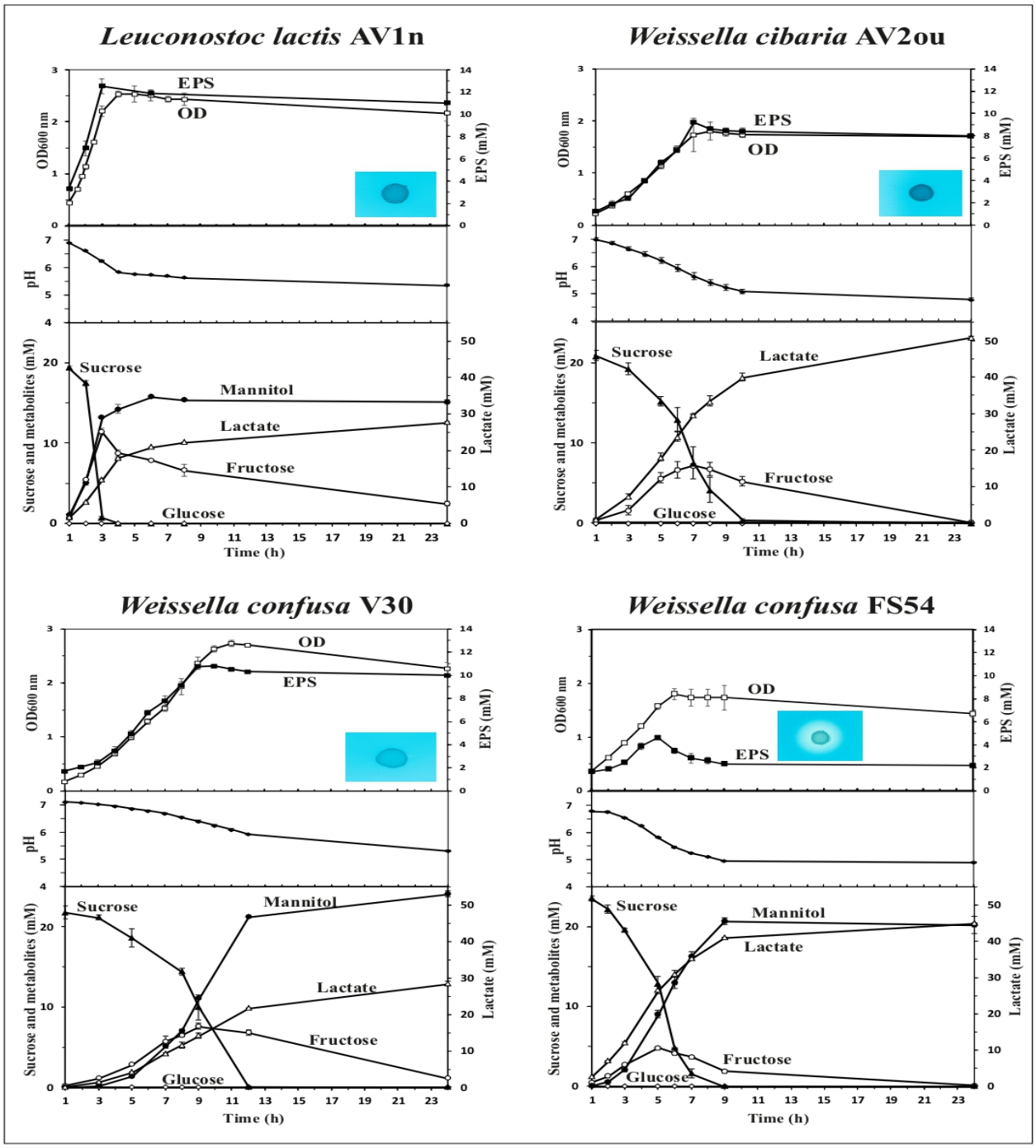

Figure 5. Analysis of EPS production and central metabolism of the LAB and detection of dextranase activity. The indicated bacteria were grown at 30 °C in CDMS medium. The growth and pH evolution were measured and sucrose and its metabolites were analyzed in the culture supernatants. The insets depict the detection of dextranase activity in MRS-dextran blue-agar medium. Symbols: □, $OD_{600\ nm}$; ■, dextran; ♦, pH; ▲, sucrose; ○, fructose; ◊ ,glucose; ●,mannitol and Δ, lactate.

Finally, after 24 h of growth the AV1n, V30 and FS54 strains were able to accumulate in their culture supernatants high concentration of the polyol (15.17 ± 0.26 mM, 24.08±0.37 mM and 20.27 ±1.08 mM, respectively).

4. Discussion

Leuconostoc and specially *Weissella* genera are of scientific interest for both their metabolic properties and their potential probiotic applications. In this work we investigated the metabolism involved in the production of dextran by three *Weissella* and one *Lc. lactis* strains isolated in Tunisia, as well as the characteristics and the physiological role of these HoPS . Previous studies of 123 EPS producing *W. confusa* and *W. cibaria* strains demonstrated that all produced dextran but only 18 of them produced fructan, supporting the view that α-glucan production is characteristic of this genus. Moreover, the dextran contained approximately 97% α-(1-6) glycosidic linkages in the main backbone and around 3% α-(1-3) linked branches (Malang, Maina, Schwab, Tenkanen, & Lacroix, 2015). which was also the case for the polymers analyzed in this study. Furthermore, *Lc. lactis* AV1n synthesizes the same type of dextran with 9% α-(1-3) branch linkages and 1.2% α-(1-4) linkages (Besrour-Aouam et al., 2019). This type of polymer is also synthesized by *Lb. sakei* MN1 and several *Lc. mesenteroides* strains (Nácher-Vázquez et al., 2015; Zarour et al., 2017b). However in LAB belonging to the *Leuconostoc* and *Lactobacillus* genera, branching at the *O*-4 position in combination with substitution at *O*-3 position is unusual, and it has been reported in only a few cases (Fraga Vidal, Moulis, Escalier, Remaud-Simeon, & Monsan, 2011; Llamas-Arriba et al., 2019).

High Mw dextrans with few branch linkages have good potential as rheology modifiers and can be used as food additives (Maina, Virkki, Pynnonen, Maaheimo, & Tenkanen, 2011; Zarour et al., 2017b). In this context, the LAB investigated in this work produced dextrans of high Mw ranging from $5.84x10^7$ Da to $2.61x10^8$ Da. These values are similar to those of the dextrans produced by several *Leuconostoc* and *Lactobacillus* strains, which ranged from $1.74x10^8$ Da to $4.41x10^8$ Da (Llamas-Arriba et al., 2019; Zarour et al., 2017b). However, they were higher than the $2x10^6$ Da and $1x10^7$ Da dextrans of *W. cibaria* CMGDEW3 (Ahmed, Siddiqui, Arman, & Ahmed, 2012) and *W. confusa* R003 (Netsopa, Niamsanit, Sakloetsakun, & Milintawisamai, 2018).

Moreover, the analysis, in an activity gel assay, of the enzymes present in LAB culture supernatants, allowed us to demonstrate that the four strains produce a dextran-synthesizing enzyme (Dsr) of ≃160 kDa. The regulatory controls of Dsr in LAB are diverse. Expression of the *dsr* gene in *Lc. mesenteroides* is induced in the presence of sucrose (Bounaix et al., 2010; Quirasco, Lopez-Munguia, Remaud-Simeon, Monsan, & Farres, 1999) in contrast to *Streptococcus mutans* and *Lactobacillus reuteri* which synthesize Dsr constitutively (Arskold et al., 2007; Monsan et al., 2001; Schwab & Ganzle, 2006) or *Lb. sakei* MN1, strain in which inhibition of *dsr* expression was observed in the presence of sucrose at 30 °C (Besrour-Aouam et al., 2019; Nácher-Vázquez et al., 2017b). Only a few reports provide data on the regulation of the expression of Dsr in *Weissella* spp. A recent study has demonstrated that cold temperature acts as an inducer of *dsrM* expression of *W. cibaria* 10M (Hu & Ganzle, 2018). Others authors have shown constitutive expression of Dsr in the presence of glucose and sucrose in *Weissella* strains (Bounaix et al., 2010). In this work, the zymogram test of LAB supernatants, also provided information on the influence of the carbon source present in the growth medium on Dsr enzyme synthesis. Dsr was detected in cultures grown in the presence of sucrose or glucose only in the case of the V30 strain. As far as we know this is the first instance of a sucrose-inducible expression of a Dsr in *Weissella.* It has been claimed that regulation of expression of fructansucrase is species and strain dependent (Malang et al., 2015), so this could also be the case for Dsr. Further studies will be needed to unravel the regulatory mechanisms involved in the expression of Dsr in *Weissella spp*.

When the four LAB were grown in CDM medium containing 30 mM sucrose, the AV1n strain showed the highest dextran synthesis (12.5 mM) with the highest Mw ($2.6x10^8$ Da), and among the *Weissella* strains, V30 had the highest production of the HoPS (10.8 mM) with a Mw of $1.1x10^8$ Da. Also, FS54 showed the lowest level of production (4.6 mM) of a polymer with the lowest Mw ($5.8x10^7$ Da). This last result could correlate with the fact that a dextranase activity was detected, and that the recovery of the EPS from the FS54 cultures supernatants decreases with the time of incubation. As far as we know, this is the first instance of a dextranase activity in a *Weissella* strain. Moreover, apart from *Streptococcus* strains, no dextranase activity has been described in any LAB (Zannini, Waters, Coffey, & Arendt, 2016).

Many factors, such as polymer size, and the specific chemical structure of the EPS molecule seem to affect adhesiveness of the producing-bacteria. We have previously demonstrated that the *O*-2 substituted (1-3)-β-D-glucan HoPS synthesized by *Pediococcus parvulus* strains is involved in the bacterial binding to colon epithelial cells (de Palencia et al., 2009; Garai-Ibabe et al., 2010). Also, several studies carried out on *Bifidobacterium* and *Lactobacilli* heteropolysaccharides describe them as positive mediators of interaction (Alp, Aslim, Suludere, & Akca, 2010; Sims et al., 2011). However, other studies have shown that elimination of high Mw EPS synthesis by knock-out or spontaneous single mutation of *eps* coding genes resulted in an increased adherence of lactobacilli strains (Horn et al., 2013; Lebeer et al., 2009). Previous works with dextran-producing LAB showed that polymer production resulted in either decreased bacterial adhesion, or did not affected it (Nácher-Vázquez et al., 2017a; Zarour et al., 2017b). In this work, we have identified these two types of behaviors, one in each of the two *W. confusa* strains. In addition, the results obtained here indicate that the dextran synthesized by AV1n potentiates almost two-fold the adhesion of this strain to Caco-2 cells. This result correlated with the TEM analysis, which revealed significant amounts of EPS attached to the bacterial cells even after sample manipulations prior to the adhesion assay. Also, the stickiness of the EPS may explain the generation of compact colonies during sessile growth of AV1n. Moreover, the positive effect of the dextran on the binding of AV1n to a biotic surface correlated with the 4.3-fold higher efficiency of the bacteria to form biofilm in the presence of sucrose than in the presence of glucose. As far as we know, this is the first instance of a positive influence of a dextran on bacterial binding to biotic and abiotic surfaces. Furthermore, in the case of AV2ou, a more moderate beneficial influence of the HoPS in biofilm formation was observed, accompanied by a tendency, not statistically significant, for binding to Caco-2 cells. In general, dextrans have a high solubility, and it is feasible that the low concentration of EPS attached to the bacteria, detected by TEM prior to the experiments, together with incubation in the presence of sucrose for 48 h for the biofilm formation *versus* 2 h for the adhesion assay, could be responsible for the detected differences. On the other hand, a correlation between the two processes was also observed for V30 with a negative effect of the dextran. A similar correlation had also been previously observed for *Lb. sakei* MN1, where high biofilm formation coincided with stronger adhesion to epithelial cells upon growth in MRSG (Nácher-Vázquez et al., 2017a). To explain the bacterial behaviour, it is feasible that, as we had proposed for *Lb. sakei* MN1 (Nácher-Vázquez et al., 2017a), the dextran synthesized during the assays in the presence of sucrose impairs the interaction of adhesins, or other bacterial components, with the Caco-2 cells and also diminishes the bacteria-abiotic surfaces interactions.

Sucrose is the most abundant disaccharide in the environment because of its origin in higher plant tissues and in this work we have analyzed the performance of the *Leuconostoc* and *Weissella* strains using this disaccharide as carbon source in the CDM medium. The DNA sequences of the genomes of various *Leuconostoc* and *Weissella* strains have been determined as well as the draft version of genomes from *Lc. lactis* AV1n (genome ID: WSZI00000000.1) and *W. confusa* FS54 (unpublished results) have been established. This information, together with the current knowledge of obligate

heterofermentative LAB and the analysis of metabolites in the LAB culture supernatants performed in this work have allowed us to propose the following metabolic pathways for sucrose utilization in the strains tested (Figures 6 and 7).

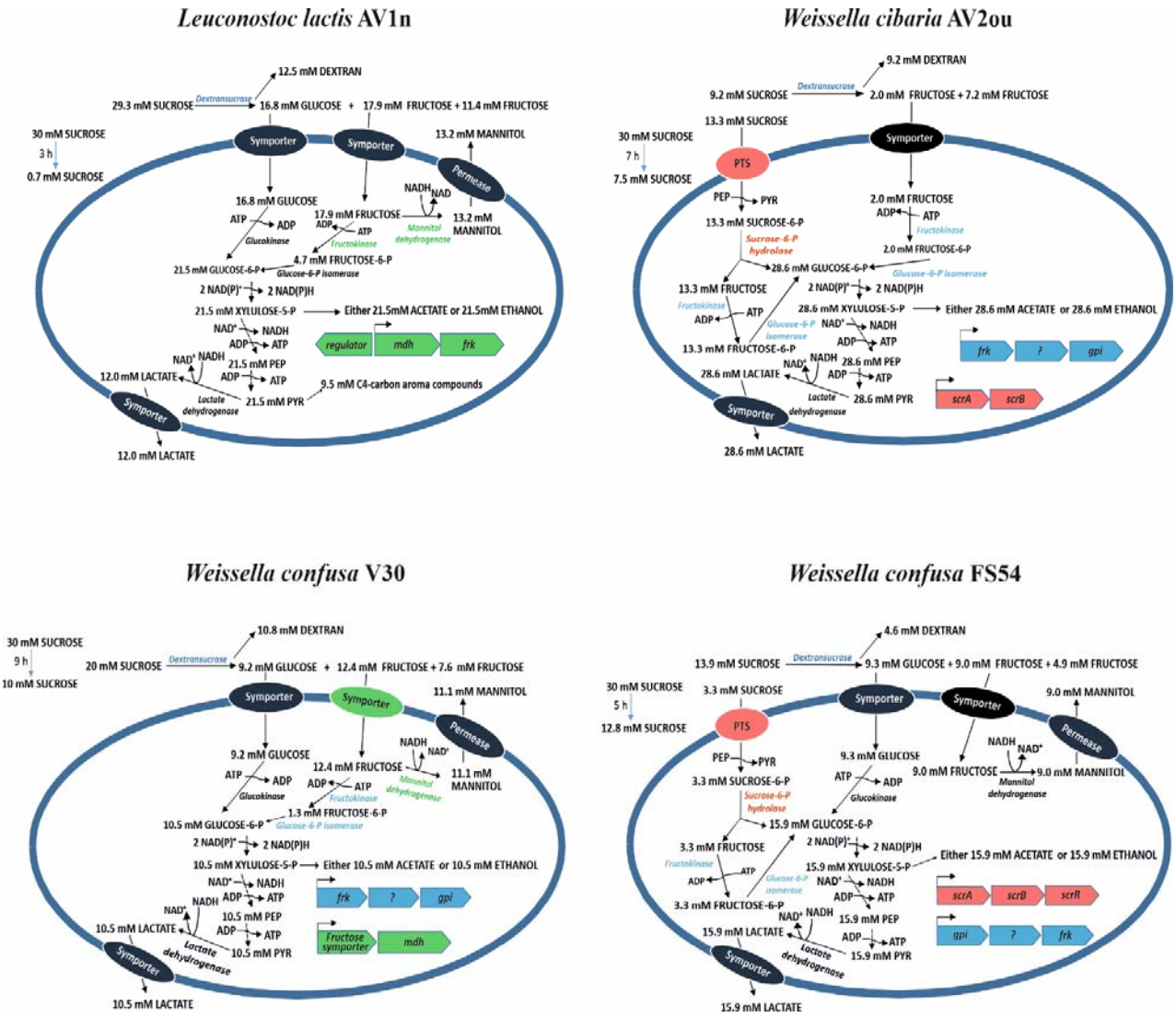

Figure 6. Predicted LAB pathways for sugar transport and catabolism with concomitant dextran production. The predicted metabolic situation when the maximum production of dextran was detected. Only some pertinent transport systems, reactions and enzymes involved in the pathways as well as some of their coding genes are indicated. The dotted lines indicate that several steps take place for the synthesis of the indicated compound. The concentration of the indicated compounds obtained experimentally (**Figure 5**) and detailed in **Table S1**, as well as those expected to be generated by the metabolic pathways are indicated.

During the exponential phase of growth, consumption of sucrose by AV1n was accompanied by production of EPS, fructose, mannitol and lactate. At the end of this phase (after 3 h of growth, Figure 6), out of the 30 mM sucrose present in the CDMS medium only 0.7 mM sucrose remained extracellularly. Since we have previously shown that expression of *dsrLL* is induced in the presence of sucrose (Besrour-Aouam et al., 2019) and the DsrLL is present in the CDMS cultures (Figure 3), it is feasible to assume that the enzyme hydrolyzes the missing 29.3 mM sucrose yielding 29.3 mM fructose plus glucose: 12.5 mM incorporated in the dextran as well as 16.8 mM as free sugar. No accumulation of glucose in the cultures supernatants was observed for either this LAB or the three *Weissella* strains analyzed, indicating a fast internalization into the bacterial cell. Thus, the free glucose could be transported to the cytoplasm by a glucose-proton symporter and the coding gene is present in the sequenced genome of the *Lc. Lactis* strains including Wikim 40 used as prototype (genome ID: CP016598.1, position 574140-575105) and AV1n (contig WSZI01000008.1, position 46045-47010). Afterwards, the monosaccharide will be metabolized, as in the other obligate heterofermentative LAB, by the phosphoketolase pathway (PKP) (Figure 6). The PKP is a low ATP-yielding process (only one molecule of ATP per molecule of glucose) and yields from glucose equimolecular concentration of lactate plus either acetate or ethanol. These last two compounds could not be detected by GC-MS under the conditions used in this work; therefore, the generation of these compounds will be not discussed. With regard to the fate of the fructose, at the end of the exponential phase, out of the 29.3 mM generated by the hydrolysis catalysed by the *Lc. lactis* Dsr, only 11.4 mM of this hexose remained extracellularly. Thus, apparently 17.9 mM fructose was internalized, presumably by a fructose proton symporter, such as is present in *Leuconostoc* (Koduru et al., 2017) and then metabolized to yield mannitol by the action of the mannitol dehydrogenase (MDH) or to enter in the PKP pathway after conversion to fructose-6-P by fructokinase (Frk). In the *Lc. lactis* genomes, including Wikim 40 (position 501552-505480) and AV1n (RAST ID: contig WSZI01000010.1, position 1-3766). *mdh* and *frk* genes are linked with the same polarity and preceded by a divergent gene encoding a putative transcriptional regulator belonging to the LacI family. Therefore, it seems that expression of the MDH and Frk is co-regulated and probably expressed, when fructose is internalized. After 3 h of growth, 13.2 mM mannitol was detected together with 12.0 mM lactate in the culture supernatants. Thus, the lactate concentration could be synthesized by the lactate dehydrogenase (LDH), utilizing 12.0 mM pyruvate (PYR) as substrate, out of the 21.5 mM of pyruvate generated by the metabolism of the internalized glucose (16.8 mM) and part of the internalized fructose (4.7 mM). Moreover, the C4-carbon biosynthetic pathway from PYR is induced in the presence of citrate in *Leuconostoc* (García-Quintáns, Blancato, Repizo, Magni, & P., 2008; Zarour et al., 2017a). Therefore, it seems that 9.5 mM pyruvate was devoted to the synthesis of these compounds that could not be detected by GC-MS under the conditions used in this work. During the stationary phase (Figure 7), AV1n consumed the remained 0.7 mM sucrose, and after 21 h in this growth phase, no more EPS was produced and even a slight decrease of the dextran levels (12.5 mM *versus* 11.0 mM) was observed, indicating that Dsr was not functional during this period and that sucrose followed a different fate.

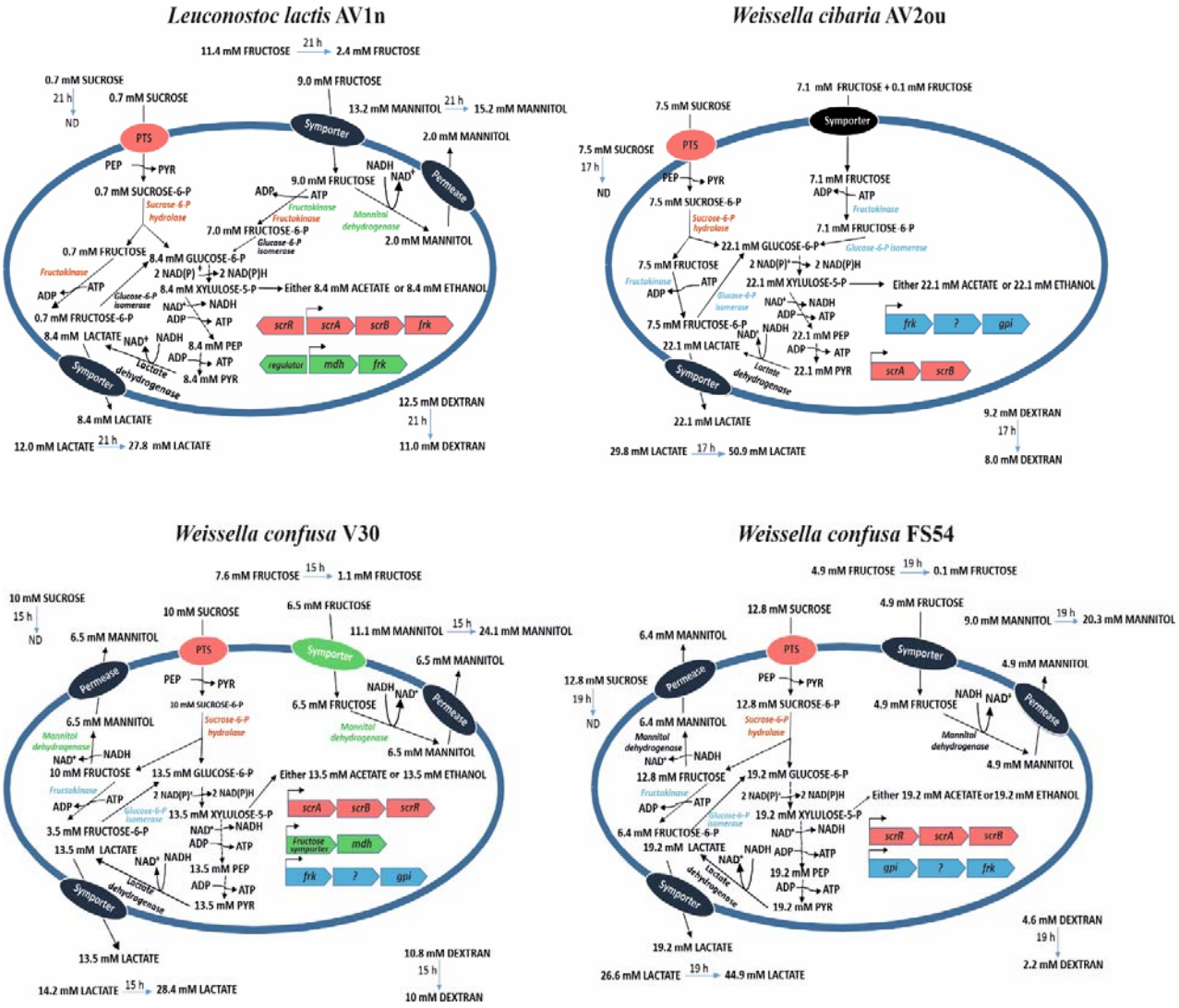

Figure 7. Predicted LAB pathways for sugar transport and catabolism concomitant with dextran production. Expected metabolic situation after 24 h of incubation. Only some pertinent transport systems, reactions, enzymes involved in the pathways as well as some of their coding genes are indicated. The dotted lines indicate that several steps take place for the synthesis of the indicated compound. The concentration of the indicated compounds obtained experimentally (**Figure 5**) and detailed in **Table S1**, as well as those expected to be generated by the metabolic pathways, are indicated.

Sucrose, like other sugars, could be internalyzed through two different systems prior to its catabolism: a phosphoenolpyruvate (PEP)-dependant phosphotransferase system (PTS), which results in accumulation of sucrose-6-phosphate (P) into the cell or a non-PTS sucrose permease allowing intracellular sucrose accumulation without chemical modification (Reid & Abratt, 2005). After, sucrose transport by any of the mechanism, *Leuconostoc* can support the synthesis of lactate throught the glycolytic PKP pathway by reactions catalyzed by various enzymes,(Koduru et al., 2017). We can not discriminate which pathway is used by AV1n and in principle more energy is required for the PTS phosphorylation step, due to a requirement of conversion of PEP in PYR, instead of conversion of the internalized sucrose by the sucrose phosphorylase enzyme, that only requires the usage of inorganic phosphate. However, theoretical and experimental results with *Lc. mesenteroides* support that in heterofermentative LAB, the PTS are working provided that PEP is already present in the bacterial cells (Koduru et al., 2017). This situation can take place at the beginning of the stationary phase in AV1n, when PEP could be already generated from the PKP glycolysis during the exponential phase of growth (Figure 6). In addition, sucrose operons involved in the PTS for sucrose transport and catabolism have been detected in various bacteria including LAB (Naumoff & Livshits, 2001). Thus, 0.7 mM sucrose could be internalized by a PTS (encoded by *scrA*), resulting in an accumulation of sucrose-6-phosphate, that upon conversion into glucose-6-P and fructose by hydrolysis mediated by the sucrose-6-P hydrolase (encoded by *scrB*) could be metabolized to two molecules of lactate (1.4 mM final concentration), if the fructose generated by its hydrolysis is only directed to the PKP pathway by action of the Frk (Figure 7). Supporting this hypothesis, a cluster of *scrAB-frk* genes preceded by a *scrR* encoding a transcriptional regulator, that could activate expression of the cluster in the absence of extracellular glucose generation, was detected in the genomes of Wikim 40 (position 1415788-1421138) and AV1n (contig WSZI01000014.1, position 162561-167840). In addition, during the stationary phase out of the 11.4 mM fructose present in the supernatant, only 9.0 mM was slowly transported to the cytoplasm and generated only 2 mM mannitol (Figure 7). If we take into consideration that two Frk will be present in the bacteria, encoded by genes located in either the *scr* cluster or in conjunction with the *mdh* gene, it would be expected that conversion of the internalized 9.0 mM fructose into fructose-6-P (7 mM) instead of mannitol (2 mM) would be favored. These hypothetical pathways would yield 8.4 mM lactate, that with the previously accumulated molecules of this compound sums up to a total of 20.4 mM lactate from sucrose. Therefore, the 27.8 mM lactate detected in the culture supernatant after 24 h growth should be generated, in addition to the proposed pathways, from different substrates present in the growth medium as, for example, citrate and certain amino acids (i.e. serine, alanine and asparagine), whose metabolism can also contribute to the lactate pool. (Fernandez & Zuniga, 2006; García-Quintáns et al., 2008).

In the case of AV2ou, the maximum dextran production (9.2 mM) was reached after 7 h of growth, at the beginning of the stationary phase (Figure 6), when 7.5 mM sucrose still remained in the supernatant and beside of the EPS, 7.2 mM fructose and 29.8 mM lactate were also detected (Figure 6 and Table S1). This balance indicates that during the first 7 h of growth, 9.2 mM sucrose was hydrolyzed by the Dsr and that 13.3 mM sucrose was internalized by a PTS transport, to finally generate a theoretical 28.6 mM lactate, very close to the detected 29.8 mM (Figure 6). Supporting this hypothesis, a putative cluster *scrA* and *scrB* lacking a flanking *scrR* was detected in the chromosome of the *W. cibaria* CH2 (genome ID: CP012873.1 position 1593503-1596675) and SRCM103448 (genome ID: CP035267.1 position 1339344-1342516) strains. Thus, the operon could be expressed constitutively even when the Dsr is active. In addition, mannitol was not detected in the culture supernatants. Several studies have reported the ability of some *Weissella* and *Leuconostoc* to produce high level of mannitol, nevertheless others claimed the absence of synthesis by strains of these genera (Galle et al., 2010; Xu et al., 2017; Zarour et al., 2018). In *Leuconostoc pseudomesenteroides*, the *mdh* gene is preceded by a co-

transcribed gene, whose product has been demonstrated to be a fructose permease by heterologous expression in *E. coli* (Heuser, Marin, Kaup, Bringer, & Sahm, 2009). This operon is carried by plasmids of *W. cibaria*, such as pMKC02 (Genbank ID: CP012875.1) and unnamed2 plasmid (Genbank ID: CP035269.1) of CH2 and SRCM103448 strains, respectively. Therefore, it is feasible that AV2ou lacks the operon-carrier plasmid and consequently could not synthesize MDH and its product mannitol. In the chromosome of SRCM103448 (genome ID: CP035267.1 position 649686-653298) a *frk* gene is clustered, together with a gene of unknown function, with the *gpi* gene, which encodes the glucose 6-P isomerase enzyme responsible for the conversion of the product of the Frk in glucose-6-P, which could be further converted into lactate by the PKP (Figure 6).Thus, it seems that the 2 mM fructose transported by an unidentified fructose permease will be converted to 2 mM glucose-6-P to further provide PEP for the PTS for sucrose transport and to fill the pool of lactate. After 7 h of growth, AV2ou seems to stop the usage or synthesis of Dsr, when entering into the stationary phase, even when 7.5 mM sucrose was still present in the supernatant. Although, *dsr* gene expression has been defined as constitutive in *Weissella* strains (Bounaix et al., 2010), the present results suggested that a possible repression could occur in the transition from exponential to stationary phase. By contrast it seems that the bacterium continues using the PTS to transport the remaining 7.5 mM sucrose together with the transport and catabolism of fructose to generate 22.1 mM lactate that together with the previously produced 29.8 mM will yield, after 17 h of growth, a theoretical 51.9 mM, very close to the detected 50.9 mM (Figure 7).

For the V30 and FS54 strains, although both accumulated fructose, mannitol and lactate in the supernatant, it appears that there are some differences in their pathways due to gene association and expression. Thus, the maximum EPS levels were reached at the late exponential phase for both strains, but were 2.3-fold higher for V30 than for FS54 (10.8 mM *versus* 4.6 mM, Figure 6). This could be due, at least partially to the fact that FS54 showed a dextranase activity, which could partially hydrolyze the dextran even during the exponential phase, presumably to use later the products of this catalysis as a carbon source. However, the use of dextran as a carbon source reserve for the bacteria is not clearly demonstrated, but it has been reported that *Streptococcus mutans* and *Streptococcus sobrinus* produce dextran-hydrolyzing enzymes (Zannini et al., 2016). In addition, this usage of dextran during exponential growth could be partially responsible for the 1.9-fold higher yield of lactate by FS54 than by V30 (26.6 mM *versus* 14.2 mM in Figure 6). However, inspection of the *W. confusa* strain genomes including the draft genome of FS54 indicates two types of behavior for fructose utilization. *W. confusa* VTTE-163496 seems to be a useful prototype to explain the results obtained for V30 (Figure 6). It carries, like *Lc. pseudomensenteroides*, the fructose permease coding gene clustered with the *mdh* gene (genome ID: contig PVSI01000045.1, position 5172-6920), without a regulator coding gene next to the putative operon. Also, a cluster including *frk* and *gpi*, like that detected in *W. cibaria* strains, is present in the VTTE-163496 genome (contig: PVSI01000035.1, position-19704-23281). Therefore, it seems that during the exponential phase of growth the V30 Dsr is responsible for the hydrolysis of 10.8 mM sucrose. Furthermore, the generated 9.2 mM glucose would be expected to be internalized through a proton symporter and further converted to lactate. Moreover, synchronization of only fructose permease and MDH expression, uncoupled of the Frk synthesis, could be responsible for conversion of 12.4 mM fructose into 11.1 mM mannitol and only 1.3 mM glucose-6-P substrate for lactate synthesis. Also, in the VTTE-163496 genome (contig: PVSI01000015.1, position-69943-74188), there is a *scr* operon, that in addition to *scrA* and *scrB* carries, downstream, a s*crR* gene. Expression of this operon seems to be responsible for transport and initial metabolism of the remaining 10 mM sucrose by its transcriptional activation in the stationary phase, yielding 10 mM fructose that could be converted to 6.5 mM mannitol and 3.5 mM glucose-6-P, increasing the total pool of this last compound to generate PYR, that upon action of the LDH will generate 13.5 mM lactate. This concentration together

with that produced during the exponential phase will reach a theoretical concentration of 27.7 mM lactate close to the real value observed of 28.4 mM. Finally, 6.5 mM fructose out of the 7.6 mM present in the supernatant will be internalized by the symporter and converted in 6.5 mM mannitol, the pool of this polyol therefore reaching the 24.1 mM detected (Figure 7).

Analysis of the draft genome of FS54 indicates that it follows the pathways depicted in Figure 6. The high levels of lactate, even during the exponential phase, indicated that in this state the operon encoding the PTS for sucrose transport and the sucrose 6-P hydrolase are already activated (Figure 6), although a *scrR* gene is an operon component. In this case, the *scrR* is located upstream of *scrA* and *scrB.* Therefore, if ScrR acts as activator of the operon transcription, the location of *scrR* could result in a higher translation of the regulator from the mRNA, provoking an earlier activation of the PTS for sucrose transport than in V30. Also, it seems that all sucrose transported by the PTS is finally converted to 6.6 mM lactate, following the same fate as the 9.3 mM glucose internalized through the symporter. Moreover, the 9.0 mM mannitol will be synthetized from all of the internalized 9.0 mM fructose through an unknown mechanism, whose coding genes seem to have no homology with those characterized in other LAB.

In the stationary phase of growth, FS54 behaved similarly to V30 (Figure 7), apart from the 2.4-fold reduction of dextran concentration (from 4.6 mM to 2.2 mM) presumably by hydrolysis. The PTS for sucrose transport and further metabolism seems to provide the synthesis of 19.2 mM lactate, conversion that will increase the lactate pool to 45.7 mM, close to the final detected value 44.9 mM. Also, from the sucrose internalization by the PTS and further metabolism, 6.4 mM mannitol will be generated from fructose catalysis, and constitute the pool of this polyol with the 4.9 mM generated by direct transport of 4.9 mM fructose and action of the MDH.

In summary, the results presented here indicate that, independently of the genus or species, each LAB analyzed displayed a different genetic and metabolic strategy when the carbon source is sucrose, and that production of dextran may take place concomitantly, or not, with mannitol synthesis. Further molecular and biochemical studies will be required to support this hypothesis. Moreover, the results presented here revealed that the four LAB have potential for being used in the production of functional food.

5. Conclusions

This is the first report that analyzes the metabolism of sucrose consumption, related to dextran synthesis, by *Weissella* and *Leuconostoc lactis* strains in CDM medium. The four LAB tested produce the same HoPS, however, *Weissella* strains showed a different sucrose metabolism compared to *Lc. lactis* AV1n. As far as we know, such sucrose consumption has never been described before in the *Weissella* genus. Also, the behavior of *Lc. lactis* AV1n is the first instance of a positive influence of dextran in the adhesion and aggregation properties of a LAB. Finally, we believe that this is the first instance of a dextranase activity encoded by a *Weissella confusa* strain.

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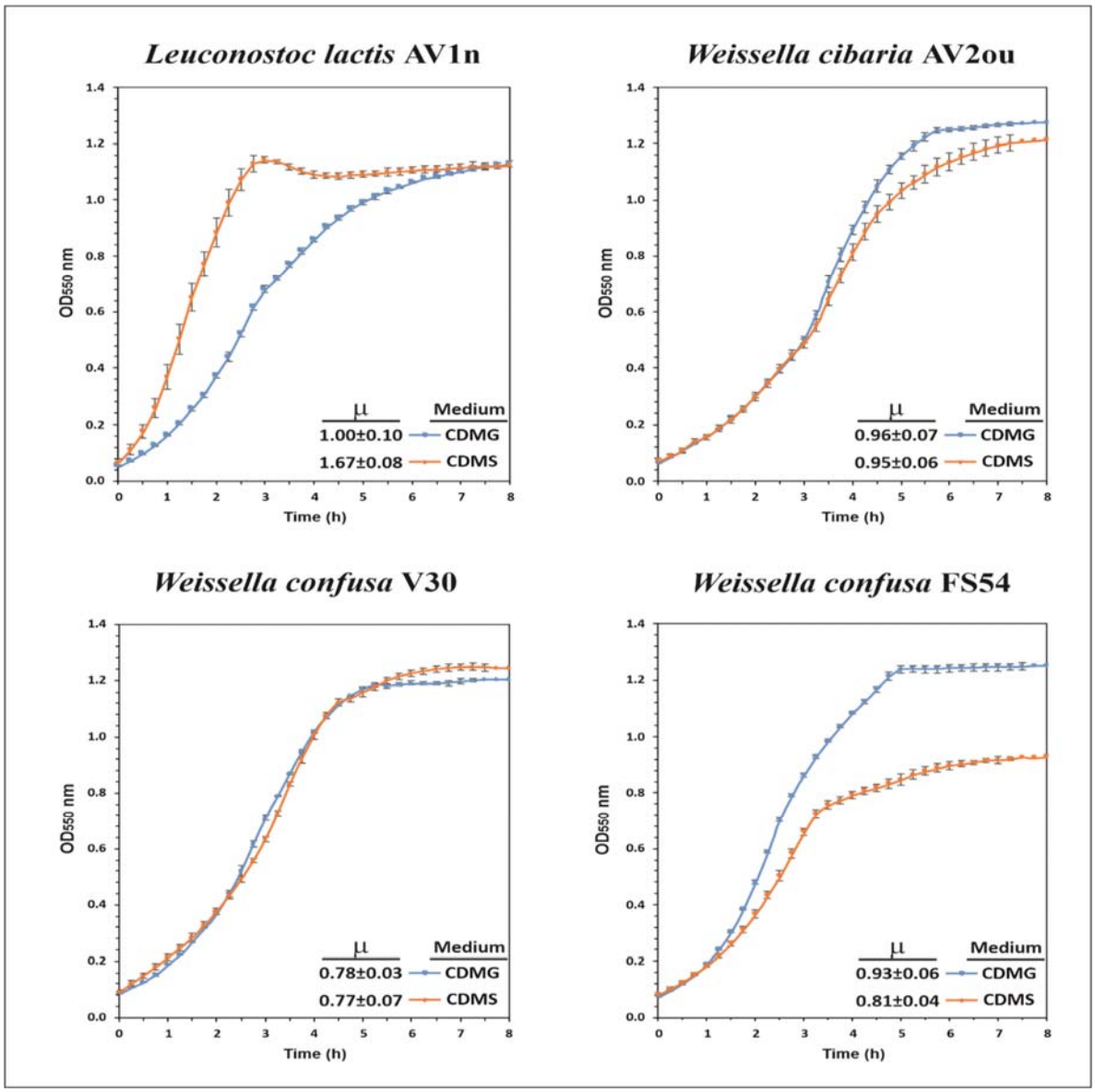
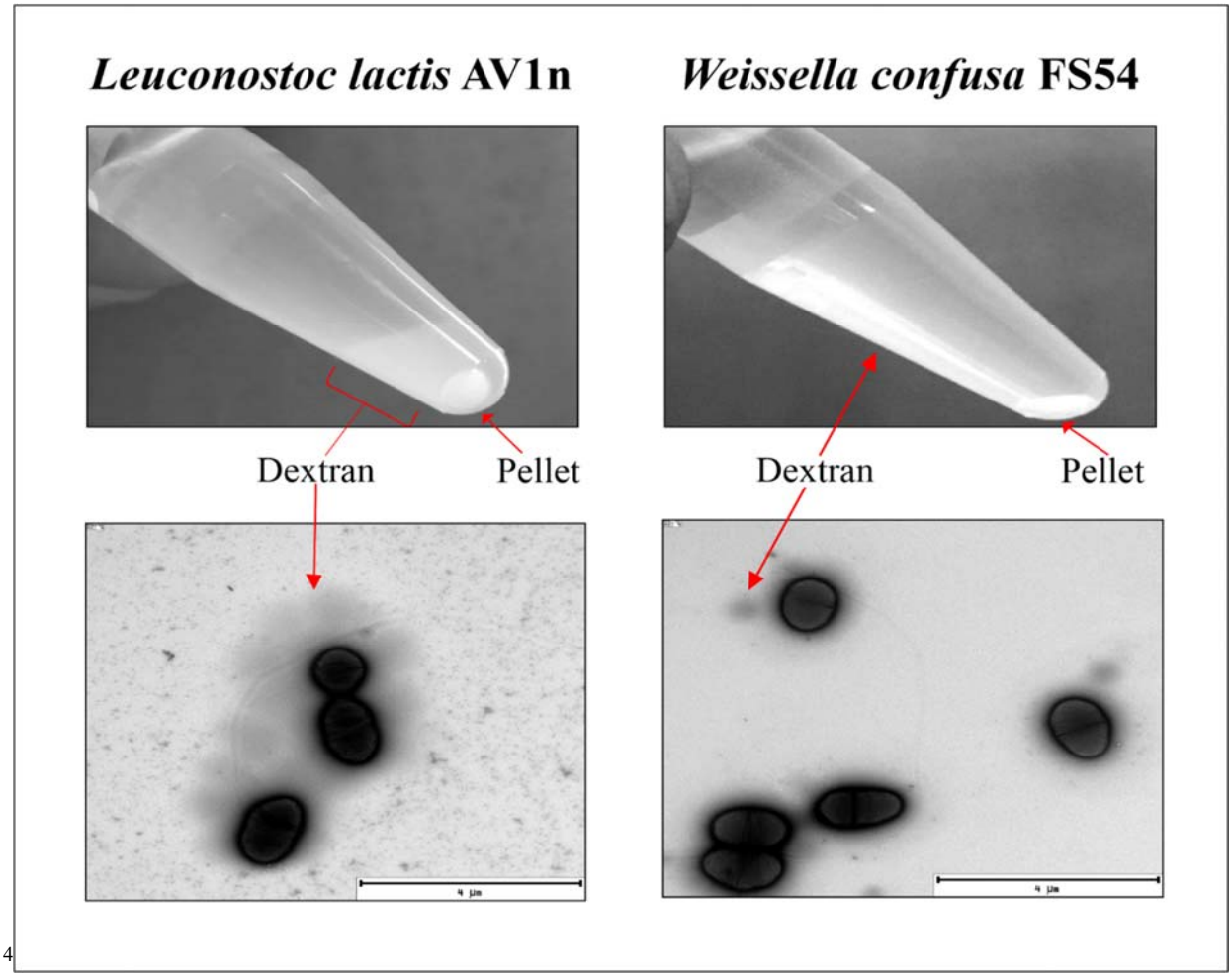


Figure S1. Influence of carbon source on the growth of the tunisian LAB. Bacterial growth in the presence of sucrose or glucose was spectrophotometrically monitored as follows. Exponential cultures grown in MRS containing either 2% glucose or 2% sucrose were diluted, respectively, in fresh CDM defined medium supplemented with either 0.8% glucose or 0.8% sucrose to OD_{550} nm = 0.07. Then, 200 µl of each culture were dispensed in triplicate in sterile 96-well optical white w/lid cell cultureU96 (Thermo Fisher Scientific) and the bacterial growth at 30 °C was monitored in real time by measuring the OD_{550} at 15 min intervals in a Varioscan Flask System (Thermo Fisher Scientific). The growth rate (µ) of the LAB during the exponential growth was calculated with the following formula:

$$\mu = \frac{\ln\left(\frac{ODt}{ODo}\right)}{(t - to)}$$



851 4

852 **Figure S2. Macroscopic and microscopic detection of dextran present in LAB cultures.** The indicated
853 strains were grown in MRS medium containing 2% sucrose. The upper panels show the sedimented bacteria
854 after 24 h of growth. The lower panels show analysis by transmission electronic microscopy of LAB
855 exponential cultures prepared as described in Materials and Methods section 2.7.
856

Table S1. A**nalysis of metabolic fluxes of LAB grown in CDMS at the end of exponential phase and at late stationary phase**

Bacteria	Concentration of compounds at the maximum production of EPS during the exponential phase[a] (mM)						Concentration of compounds at the late stationary phase[b] (mM)					
	Sucrose	EPS	Fructose	Glucose	Lactate	Mannitol	Sucrose	EPS	Fructose	Glucose	Lactate	Mannitol
Lc. lactis AV1n	0.71±0.20	12.53±0.72	11.41±0.42	ND	12.00±0.20	13.18±0.09	ND	11.02±0.18	2.43±0.3	ND	27.77±0.29	15.17±0.26
W. cibaria AV2ou	7.49±2.00	9.21±0.150	7.20±0.15	ND	29.84±0.11	ND	ND	7.99±0.06	0.10±0.02	ND	50.92±0.36	ND
W. confusa V30	9.96±1.57	10.78±0.06	7.60±0.37	ND	14.21±0.66	11.09±0.12	ND	9.98±0.13	1.14±0.07	ND	28.44±0.67	24.08±0.37
W. confusa FS54	12.85±0.97	4.62±0.18	4.86±0.92	ND	26.57±0.95	9.05±0.45	ND	2.20±0.10	0.13±0.01	ND	44.94±0.11	20.27±1.08

[a]Concentration of the substrate and the metabolites were determined after 3 h,5 h, 7 h and 9 h of growth for AV1n, FS54, AV2ou and V30 strains, respectively.
[b]Concentration of the substrate and the metabolites were determined after 24 h of growth.
ND, undetectable amount of the compound.