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Evaluation of lactic acid bacteria strains isolated from fructose-rich environments for their mannitol-production and milk-gelation abilities

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ABSTRACT

Mannitol is a sugar alcohol, or polyol, widely used in the food industry because of its low-calorie properties. Industrial production of mannitol is difficult and expensive. However, certain bacterial species are known to produce mannitol naturally, including certain lactic acid bacteria and fructophilic lactic acid bacteria (LAB). In this study, bacterial strains isolated from fructose-rich sources, including flowers, leaves, and honey, were identified by 16S rRNA sequence analysis as *Leuconostoc*, *Fructobacillus*, *Lactococcus*, and *Lactobacillus* species and 4 non-LAB species. DNA profiles generated by pulsed-field gel electrophoresis discriminated 32 strains of *Leuconostoc mesenteroides* and 6 *Fructobacillus* strains. Out of 41 LAB strains isolated, 32 were shown to harbor the *mdh* gene, which encodes the mannitol dehydrogenase enzyme, and several showed remarkable fructose tolerance even at 50% fructose concentrations, indicating their fructophilic nature. Several of the strains isolated, including *Leuconostoc mesenteroides* strains DPC 7232 and DPC 7261, *Fructobacillus fructosus* DPC 7237, and *Fructobacillus fructosus* DPC 7238, produced higher mannitol concentrations than did the positive control strain *Limosilactobacillus reuteri* DSM 20016 during an enzymatic screening assay. Mannitol concentrations were also examined via HPLC in 1% fructose de Man, Rogosa, and Sharpe medium (FMRS) or 1% fructose milk (FM). Among the strains, *Fructobacillus fructosus* DPC 7238 displayed high fructose utilization (9.27 g/L), high mannitol yield (0.99 g of mannitol/g of fructose), and greatest volumetric productivities (0.46 g/L per h) in FMRS. However, *Leuconostoc mesenteroides* DPC 7261 demonstrated the highest fructose utilization (8.99 g/L), mannitol yield (0.72 g of mannitol/g of fructose), and volumetric productivities (0.04 g/L per h) in FM. Storage modulus G′ (>0.1 Pa) indicated a shorter gelation time for *Limosilactobacillus reuteri* DSM 20016 (8.73 h), followed by *F. fructosus* DPC 7238 (11.57 h) and *L. mesenteroides* DPC 7261 (14.52 h). Our results show that fructose-rich niches can be considered important sources of fructophilic LAB strains, with the potential to be used as starter cultures or adjunct cultures for the manufacture of mannitol-enriched fermented dairy products and beverages.

Key words: FLAB, *Leuconostoc*, *Fructobacillus*, mannitol, milk gelation

INTRODUCTION

Lactic acid bacteria (**LAB**) are ubiquitously present in a variety of environments, including fermented dairy products, meat products, cereals, plants, flowers, fruits, and vegetables (Mozzi et al., 2006; Dhakal et al., 2012; Ruiz Rodríguez et al., 2019). Often, LAB from diverse sources display diverse metabolic properties as a result of environmental adaptation (Tyler et al., 2016). In particular, fructose-rich niches are potential reservoirs for candidate LAB species with undiscovered functional traits (Di Cagno et al., 2013; Endo and Salminen, 2013). Species such as *Weissella confusa*, *Weissella cibaria*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis*, and *Enterococcus durans* are the most frequent species isolated from these sources (Askari et al., 2012; Ong et al., 2012; Leong et al., 2014). In the recent past, fructophilic lactic acid bacteria (**FLAB**) have been reported in fructose-rich fruits, flowers, and vegetables (Di Cagno et al., 2013; Endo and Salminen, 2013; Olofsson et al., 2014). Their fructophilic nature, or their ability to tolerate high concentrations of fructose, was thought to be unique to the *Fructobacillus* genus, a genus which arose from a reclassification of the *Leuconostoc* species. However, this property is also observed in some *Lactobacillus* species (Endo et al., 2018; Filannino et al.,

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2019; Ruiz Rodríguez et al., 2019), suggesting that other genera and species are also fructophilic in nature. To date, *Fructobacillus fructosus*, *Fructobacillus durionis*, *Fructobacillus ficulneus*, *Fructobacillus tropaeoli*, *Apilactobacillus kunkeei*, *Apilactobacillus apinorum*, *Fructilactobacillus florum*, and *Lactiplantibacillus plantarum* have been reported as FLAB species from fructose-rich sources, including fructose-consuming insects (Endo et al., 2010; Ruiz Rodríguez et al., 2017; Gustaw et al., 2018). Recently, FLAB have received some interest due to certain properties, including the production of polyols and acting as probiotic cultures for honeybees (McFrederick et al., 2012; Vásquez et al., 2012; Endo et al., 2018).

The FLAB differ from other LAB species because they prefer fructose over glucose as a growth substrate (Endo et al., 2009). Growth of FLAB on glucose is poor but can be enhanced in the presence of external electron acceptors such as pyruvate, oxygen, or fructose (Endo and Okada, 2008). Many heterofermentative LAB and FLAB (*Limosilactobacillus fermentum*, *Lactobacillus intermedius*, *Limosilactobacillus reuteri*, *F. tropaeoli*, and *F. fructosus*) synthesize mannitol when an alternative electron acceptor such as fructose is present in the medium (Ortiz et al., 2017). Mannitol is a sugar alcohol, or polyol, widely used in the food, pharmaceutical, and chemical industries. Because of its low calorie content, mannitol is increasingly attracting interest as a sugar substitute, or sweetener, for diabetics and others with sugar intolerance. One-step conversion of fructose to mannitol is catalyzed by the enzyme mannitol dehydrogenase (MDH; EC 1.1.1.67), requiring either NADH or NADPH as cofactors (Bhatt et al., 2012). Expression of the *mdh* gene is markedly induced by the presence of fructose in the early stages of microbial growth (Ortiz et al., 2017).

To date, FLAB remain unevaluated in dairy applications. Therefore, in this study, we aimed to isolate and characterize LAB, including FLAB, from fructose-rich niches and to evaluate these strains in terms of their in situ mannitol production and gelation behavior in milk.

MATERIALS AND METHODS

Sample Collection for Isolation of LAB/FLAB

In total, 19 samples from fructose-rich sources were collected from 3 different locations in Ireland. Eleven samples, comprising fresh-cut flowers, leaves, and honey, were obtained from a farm in County Tipperary. Four flower samples were obtained from a garden center in Kilworth, County Cork. Fruit (grapes and bananas) and vegetable (cauliflower and spinach) samples were purchased from a local market, in Fermoy, County Cork. All samples were collected aseptically and stored at 4°C until further analysis.

Isolation of FLAB Using Fructose-Containing Media

Five grams of each sample was added to 10 mL of maximum recovery diluent (MRD; 8.5 g/L of sodium chloride and 1.0 g/L of peptone) and homogenized. Appropriate serial dilutions of each sample suspension were plated onto 2 different agars supplemented with 10 g/L of fructose: fructose yeast peptone (**FYP**) agar, as described by Endo et al. (2009), and fructose de Man, Rogosa, and Sharpe agar (**MRS**; with fructose, this will be termed **FMRS**; Becton, Dickinson and Company, Wokingham, UK). The FYP and FMRS agar plates were incubated aerobically at 30°C for 24 to 48 h. Small to medium-sized colonies were selected and inoculated into each of FYP and FMRS broth containing 10 g/L of fructose, followed by incubation aerobically at 30°C for 24 h. Mixed cultures were purified by streak plate using FYP or FMRS agar. *Limosilactobacillus reuteri* DSM 20016 and *Lactococcus lactis* DPC 6665 were used as MDH-positive and -negative cultures, respectively, for the various assays performed in the study. These strains were cultured in FMRS at 37°C for DSM 20016 and M17 (Oxoid, Basingstoke, UK), supplemented with 0.5% (wt/vol) lactose at 30°C for DPC 6665.

Genotypic Characterization

Genomic DNA was extracted from the isolates using the Ultra Clean Microbial DNA Isolation Kit (Mo-Bio Laboratories, Cambridge, UK) after overnight growth of cultures at 30°C in FMRS. Species identification was determined using the method described by Alander et al. (1999). The 16S rRNA amplicons (≈1,500 bp) were then purified by ISOLATE II PCR and Gel Kit (BIO-52060; Bioline, Dublin, Ireland), according to the manufacturer's instructions. The concentration and purity of the isolated amplicons were measured using the Nanodrop-Spectrophotometer (NanoDrop 1000 Spectrophotometer; Thermo Scientific, Dublin, Ireland). Nucleotide sequences of purified PCR products were determined by Eurofins Genomics (European Custom Sequencing Centre, Germany). Sequence similarity analysis was conducted using the Basic Local Alignment Search Tool (BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene sequences were compared with those available at the National Collection for Biotechnological Information GenBank database (accession nos. NR_113960.1, NR_104925.1, NR_074957.1, NR_113579.1, MK986693.1,

NR_042285.1, NR_104573.1, NR_113901.1, NR_115605.1, NR_025341.1, NR_118557.1, NR_157602.1, and NR_113957.1). For species assignation, isolates that showed maximum similarity percentage (≥87.50%) with the reference strain in the database were considered.

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

The FLAB isolates were grown in FMRS broth at 30°C overnight. The cell pellet was obtained by centrifugation of 1.5 mL of culture at 5,000 × *g* for 10 min and washed twice with buffer (1 *M* NaCl, 10 m*M* Tris-HCl, pH 7.6). The pellet was suspended in 200 μL of the same buffer and vortexed for 5 s. To the cell suspension, an equal volume of 2% (wt/vol) low melting point agarose (Sigma-Aldrich Co., St. Louis, MO) in 0.125 *M* EDTA, pH 7.0, was added and dispensed into a plug mold. DNA plugs were prepared as per the method described by Pogačić et al. (2014) and according to the PulseNet protocol (Hunter et al., 2005). The strain *Salmonella enterica* ssp. *enterica* serovar Braenderup (ATCC BAA664) was used as reference strain. All the plugs were stored in TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) at 4°C until digestion.

For DNA digestion, plugs were equilibrated for at least 30 min in 1× CutSmart Buffer (New England Biolabs Inc., Ipswich, MA) at 4°C and transferred to fresh digestion buffer containing 20 U of restriction enzyme (*Sma*I or *Sfi*I for DNA from FLAB isolates, and *Xba*I for DNA from reference strain). Plugs with restriction enzyme added were incubated at the optimum temperature for activity of each enzyme (*Sma*I, 25°C for 5h; *Sfi*I, 50°C for 5 h; and *Xba*I, 37°C for 3 h). After digestion, the plugs were cut into slices, placed on the tip of the gel comb, and loaded into the wells of a 1% (wt/vol) pulsed-field gel electrophoresis (**PFGE**) agarose gel (Bio-Rad Laboratories Inc., Hercules, CA) prepared in 0.5× TRIS borate EDTA buffer (Sigma-Aldrich). The PFGE was run at 6 V/cm for 22 h at 14°C with the pulse ramped from 1 to 20 s on a CHEF-DR III unit (Bio-Rad). Gels were stained for 2 h with ethidium bromide (0.5 μg/mL) made in distilled water, followed by destaining in distilled water for 30 min, and visualized using the Alpha Imaging System (Alpha Innotech, BioSurplus, San Diego, CA). Images were imported to Bionumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium), and PFGE profiles were compared.

PCR to Detect mdh Gene

Primers were designed using the *mdh* gene sequence of *Leuconostoc pseudomesenteroides* KCTC 3652 (accession number AJ486977) as a reference with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), using set parameters, with predicted product size range between 150 and 200 bp. The primer sequences F-5′CTGCAAGCTTATGGCATTCA-3′ and R-5′ AATTGCGGCTTCTTGTGTCT-3 were synthesized by Sigma-Aldrich (Wicklow, Ireland). Genomic DNA samples were used as template DNA for *mdh* PCR. Gradient PCR was run with *mdh*-positive *Limosilactobacillus reuteri* DSM 20016, using temperature gradient of 55 to 65°C, by Prime Thermocycler (Techne Prime, Staffordshire, UK). The *mdh* PCR protocol consisted of the initial denaturation steps of 94°C for 5 min, followed by 35 cycles of 45 s at 94°C, 35 s annealing at 58°C, 30 s extension at 72°C, and a final extension at 72°C for 10 min.

Evaluation of Fructose Tolerance

Fructose tolerance of the *mdh*-positive LAB strains was determined as per the method of Gustaw et al. (2018), with some modifications. The LAB cultures were inoculated at 3% (vol/vol) into each of 10 mL FMRS broth comprising 0, 10, 20, 30, 40, and 50% (wt/vol) D-fructose, followed by incubation at 30°C for 20 h. Following incubation, the cultures were serially diluted in MRD and plated onto FMRS agar and incubated at 30°C for 48 h. Visible colonies on FMRS agar were counted after 48 h and denoted as initial viable count (N0) for growth in broth in the absence of fructose and final viable count (N1) for growth in broth containing fructose. Percentage of fructose tolerance was calculated using the following formula:

$$\text{Fructose tolerance (\%)} = \text{Log cfuN1/Log cfuN0} \times 100.$$

Screening of FLAB for Mannitol Production

Mdh-positive FLAB strains were screened for mannitol production via enzyme assay. Based on the preliminary trials, cultures were inoculated at 3% (vol/vol) into 10 mL of FMRS broth and incubated in a shaking incubator (Sartorius Stedim Certomat BS-1, Labequip Ltd., Ontario, Canada) at 30°C for 20 h with constant speed of 100 rpm. The fermented FMRS broth was centrifuged at 10,000 × *g* for 10 min at room temperature, and the supernatant was collected. Mannitol production was determined using a D-Mannitol/L-Arabitol Assay Kit (Megazyme, Wicklow, Ireland). A calibration curve, using mannitol standards, was prepared in a standard 96-well flat-bottomed microplate (Sarstedt, Wexford, Ireland). For sample analysis, solution 1 (buf-

fer) and solution 2 (NAD^+) from the Megazyme kit, along with distilled water, were added to the sample supernatants. The microplate was loaded onto a Synergy HT microplate reader (Biotek, Mason Technology Ltd., Dublin, Ireland), allowing shaking option for 10 s, and absorbance readings (A_1) were noted after 2 min at 340 nm. Then, suspension 3 from the Megazyme kit, comprising mannitol dehydrogenase (MDH), was added to the sample mixtures. The microplate was again loaded onto the reader with shaking for 10 s, and absorbance readings (A_2) were taken at 340 nm after 4 min. The absorbance difference ($A_2 - A_1$) was determined for a water blank (A_1) and sample (A_2). $\Delta A_{\text{D-mannitol}}$ was obtained by subtracting absorbance difference of the blank from the absorbance difference of the sample. Mannitol concentration (g/L) was determined from the calibration curve equation ($y = 0.042x + 0.150$), where $y = \Delta A_{\text{D-mannitol}}$ and x = concentration of mannitol in the samples.

Determination of Mannitol Content by HPLC

Batch fermentation was performed with selected mannitol-producing FLAB strains in each of 200 mL FMRS broth and 1% fructose milk (**FM**). Fermented milk was prepared by dissolving 12% skim milk powder (wt/vol) and 1% fructose (wt/vol) in distilled water at 45°C, followed by sterilization at 115°C for 5 min. The fermentation parameters were set as follows: culture inoculums of 3%, (vol/vol) were used, with temperature (30°C), agitation (100 rpm), and shaking time (20 h) set as described. Centrifugation followed, to obtain supernatant. In the case of FM, samples were first deproteinized by adding an equal volume of ice-cold 1 *M* perchloric acid, and the supernatant was obtained by centrifugation at 1,500 × *g* for 10 min. The supernatants from FMRS and FM were passed through a 0.2-μm nylon filter.

Fructose and mannitol content were analyzed by HPLC (Alliance 2695, Waters Corp., Milford, MA), using an Aminex HPX-87C column (Bio-Rad), following the protocol of Carvalheiro et al. (2011) with some modifications. The HPLC column was maintained at 60°C, and the mobile phase used was 0.009 *N* H_2SO_4 at flow rate of 0.5 mL/min. Single standard solutions of both fructose and mannitol were prepared at a concentration of 1 mg/mL to establish elution times. Quantification was based on the external standard method, and calibration curves for the 2 sugars using different concentrations (10, 20, 50, and 100 μg/mL) were generated in a linear response. All the compounds were analyzed and quantified with a refractive index detector (Waters Corp.).

Calculation of Parameters

The fructose consumption rate, expressed in g/L, was obtained by subtracting the fructose content after fermentation from the fructose content before fermentation in the sample. Fructose consumption rate (qs, g/L per h) was calculated by dividing the fructose consumed by fermentation time (h). The yield of mannitol (Y_{Man}) from fructose is expressed in grams per gram and calculated as follows: Y_{Man} = mannitol produced divided by fructose consumed in the sample. Volumetric mannitol production rate, Q_{Man} (g/L per h), was calculated as mannitol produced in the sample divided by the fermentation time (h). Specific mannitol production rate (q_{Man}) was calculated by dividing Q_{Man} by wet biomass (g/L).

Evaluation of FLAB for Milk Gelation Ability

Milk gelation by FLAB strains was evaluated as per the method of Famelart et al. (2004), with some modifications. The elastic (**G′**) and viscous (**G″**) moduli were monitored at 30°C for FLAB and at 37°C for DSM 20016 as a function of time in an AR2000 rheometer (TA Instruments, Waters, St. Quentin en Yvelines, France), in oscillatory mode with coaxial cylinder geometry at a frequency of 1 Hz and a strain of 0.02. Twenty milliliters of FM inoculated with 3% (vol/vol) of the FLAB strain of interest was placed in the cup. Measurements were recorded dynamically at 30-s intervals. The gel time was defined as the point at which $G' > 0.1$ Pa.

Statistical Analysis

Enzymatic mannitol production experiments were performed in triplicate, and the results are expressed as mean ± standard error of the mean (SEM). The data were statistically analyzed using Prism, version 7 (GraphPad Software, San Diego, CA), by one-way ANOVA with Tukey's post hoc test. Differences were considered statistically significant at least at $P < 0.01$. Fructose tolerance values (mean ± SEM) were obtained from 3 independent trials. Milk gelation experiments were carried out in duplicate.

RESULTS

Isolation and Identification of LAB from Fructose-Rich Sources

A total of 141 individual colonies isolated from samples of flowers, fruits, vegetables, leaves, and honey

were selected from FYP and FMRS agar (Table 1). Out of these, 45 colonies grew well once purified in both FYP and FMRS broth. Pure isolates that showed turbidity in fructose-containing broth and appeared microscopically as LAB were considered as putative FLAB. Approximately 60% of the isolates were obtained from 4 flower types (foxglove, dandelion, white clover, and cotoneaster), whereas 40% were from 9 other samples (Table 1). Euphorbia, Cerinthe, and all fruit and vegetable samples tested did not yield any isolates.

For taxonomic identification of the isolates, 16S rRNA gene sequence analysis was performed. The obtained 16S rRNA sequences were aligned and compared with the 16S rRNA database in the GenBank library. Of the 45 isolates recovered, 41 were identified as LAB and 4 as non-LAB species (*Enterococcus*, *Staphylococcus*, *Serratia*, and *Ewingella*). Based on the sequence similarities, 32 of the 41 LAB were identified as strains of *L. mesenteroides* (including 2 strains of subspecies *L. mesenteroides* ssp. *jonggajibkimchii*), 5 as *F. fructosus*, 2 as *Lactococcus lactis*, and 1 each as *Fructobacillus durionis* and *Lactiplantibacillus plantarum* (Supplemental Table S1, https://doi.org/10.3168/jds.2020-19120). The cultures were deposited in the Teagasc DPC Culture Collection, strains DPC 7230 to DPC 7274 (Supplemental Table S1).

PFGE Characterization of Strains from Fructose-Rich Sources

Two restriction enzymes, *Sma*I and *Sfi*I, were used for the discrimination of 32 *L. mesenteroides* and 6 *Fructobacillus* strains, respectively. Cluster analysis revealed a similarity range between 30 and 100% for *Leuconostoc* and 70 to 100% for *Fructobacillus* strains (Figure 1). The *Leuconostoc* strains showed high variability, with similarity <90%, whereas the *Fructobacillus* strains were less variable. According to the profiles generated by *Sma*I, *L. mesenteroides* isolates were grouped into 25 pulsotypes: 5 with similarity ≥90% and 20 singletons (Figure 1A). According to the profiles generated by *Sfi*I, 2 pulsotypes of *Fructobacillus* strains were identified. *Fructobacillus fructosus* DPC 7267, DPC 7235, and DPC 7239, DPC 7266, and *F. fructosus* DPC 7238 showed >90% identity, whereas *F. fructosus* DPC 7237 showed 70% identity with the other genetic profiles (Figure 1B).

Table 1. Description of samples, numbers, and distribution of putative lactic acid bacteria (LAB) isolates

Name and type of sample	Botanical name	Designation[1]	Selected colonies[2]	Putative LAB isolates	
				No.[3]	% Distribution
Farm, Co. Tipperary, Ireland					
Herb robert, flower	*Geranium robertianum*	HRG	11	3	7
White clover, flower	*Trifolium repens*	WCG	15	5	11
Blackberry blossoms, flower	*Rubus fruticosus*	BBG	13	4	9
Limnanthes douglasii, flower	*Limnanthes douglasii*	LDG	7	2	5
Cotoneaster, flower	*Cotoneaster horizontalis*	CG	14	6	13
Abutilon, flower	*Abutilon pitcairnense*	AG1	4	1	2
Foxglove, flower	*Digitalis purpurea*	FGG	10	8	18
Dandelion, flower	*Taraxacum officinale*	DG	12	8	18
Laurel leaves	*Laurus nobilis*, *Lauraceae*	LLG	7	1	2
Dandelion leaves	*Taraxacum officinale*	DLG	8	2	5
Raw honey	—	RHG	5	1	2
Garden center, Kilworth, Co. Cork, Ireland					
Euphorbia, flower	*Euphorbia milii*	EP	5	—[4]	—
Erysimum, flower	*Erysimum cheiri*	EPP	4	2	4
Cerinthe, flower	*Cerinthe major*	CBP	5	—	—
Lupin, flower	*Lupinus graecus*	HBP	5	2	4
Local market, Fermoy, Co. Cork, Ireland					
Grapes, fruit	*Vitis vinifera*	GFM	3	—	—
Banana, fruit	*Musa acuminate*	BFM	5	—	—
Cauliflower, vegetable	*Brassica oleracea*	CVM	5	—	—
Spinach, vegetable	*Spinacia oleracea*	SVM	3	—	—
Total (n = 19)			141	45	100

[1]Designations relate to source of isolate and are detailed in Supplemental Table S1 (https://doi.org/10.3168/jds.2020-19120).

[2]Combined colonies from fructose yeast peptone and fructose de Man, Rogosa, and Sharpe agar.

[3]Pure isolates from mixed cultures.

[4]—: not recovered.

Detection of the mdh Gene

To identify strains with the genetic potential to produce mannitol, gradient PCR was standardized with an *mdh*-positive strain *Limosilactobacillus reuteri* DSM 20016, which generated an amplified product of 190 bp (Supplemental Figure S1, https://doi.org/10.3168/jds.2020-19120). Of the 45 strains tested, 26 *L. mesenteroides* and 5 *Fructobacillus* strains were found to possess the *mdh* gene (Figure 2). The *mdh* gene was not detected in 4 *L. mesenteroides* strains (DPC 7242, DPC 7256, DPC 7271, DPC 7273), 2 *L. mesenteroides* ssp. *jonggajibkimchii* strains (DPC 7262 and DPC 7272), and *F. fructosus* strain DPC 7239. Other LAB species such as *L. lactis* (DPC 7230 and DPC 7236), *Lactiplantibacillus plantarum* (DPC 7247), and 4 non-LAB species were also *mdh*-negative according to PCR analysis.

Fructose Tolerance by LAB Isolates

In the presence of 10% and 20% fructose, all 31 *mdh*-positive strains grew well, exhibiting from 80.96 ± 1.79% to 100.91 ± 0.12% fructose tolerance. The highest values were observed in *L. mesenteroides* DPC 7234 (100.91%) at 10% fructose and in *F. fructosus* DPC 7235 (100.61%) at 20% fructose (data not shown). Figure 3 shows the results of fructose tolerance tests at higher concentrations of fructose (30, 40, and 50%) in FMRS. At each fructose concentration, different strains exhibited the highest fructose tolerance (e.g., DPC 7240 at 30%, and DPC 7238 at 40 and 50%), but strains such as *L. mesenteroides* DPC 7232 and DPC 7261, *F. fructosus* DPC 7237, and *F. fructosus* DPC 7238 consistently showed better tolerance. *Lactococcus lactis* DPC 6665, *Limosilactobacillus reuteri*

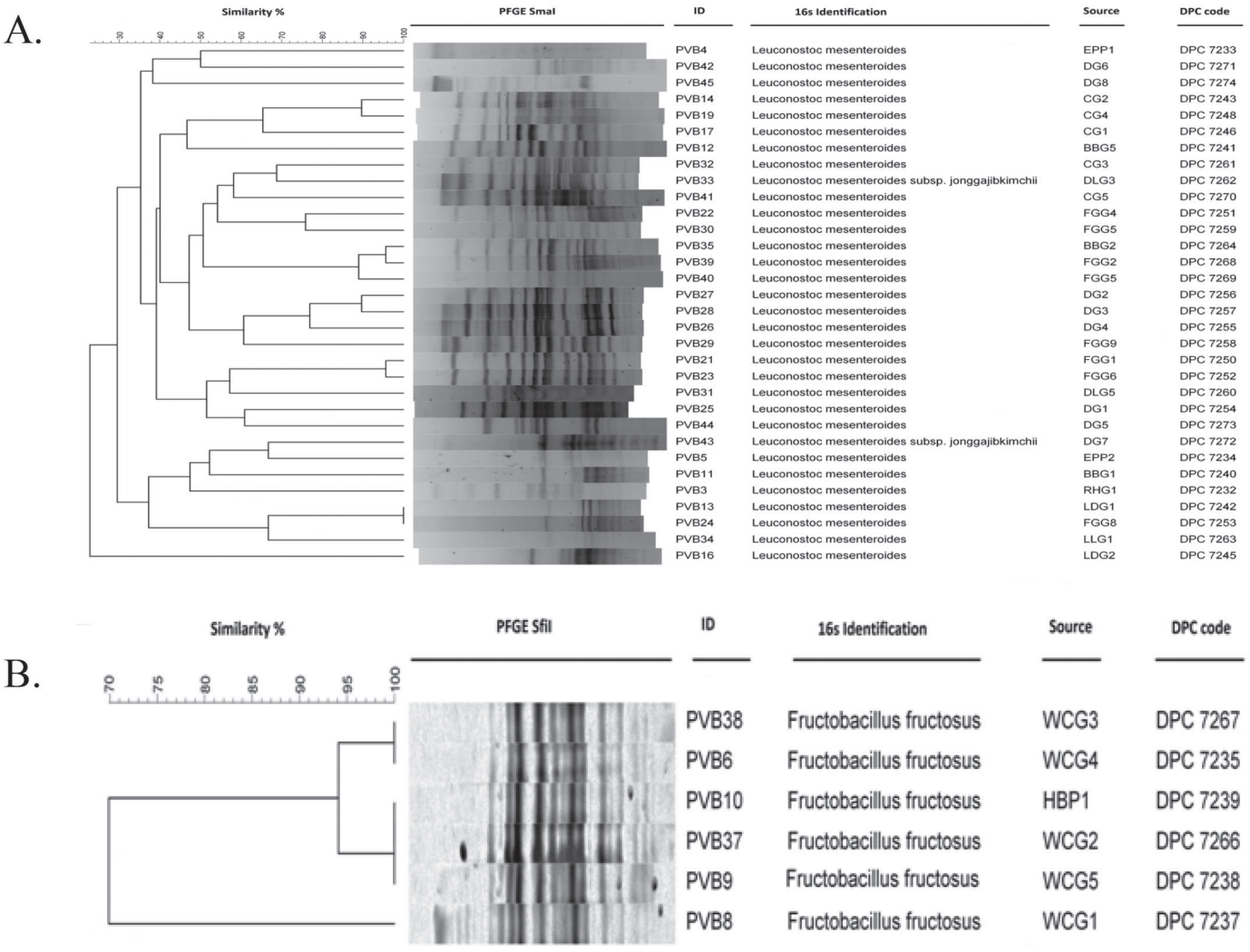

Figure 1. Dendrograms based on unweighted pair group method with arithmetic mean (UPGAMA) clustering (Dice coefficient) of pulsed-field gel electrophoresis (PFGE) profiles. (A) *Leuconostoc mesenteroides* strains; (B) *Fructobacillus fructosus*. DPC = Dairy Production Centre, from the Teagasc Moorepark Culture Collection; SmaI and SfiI = restriction enzymes.

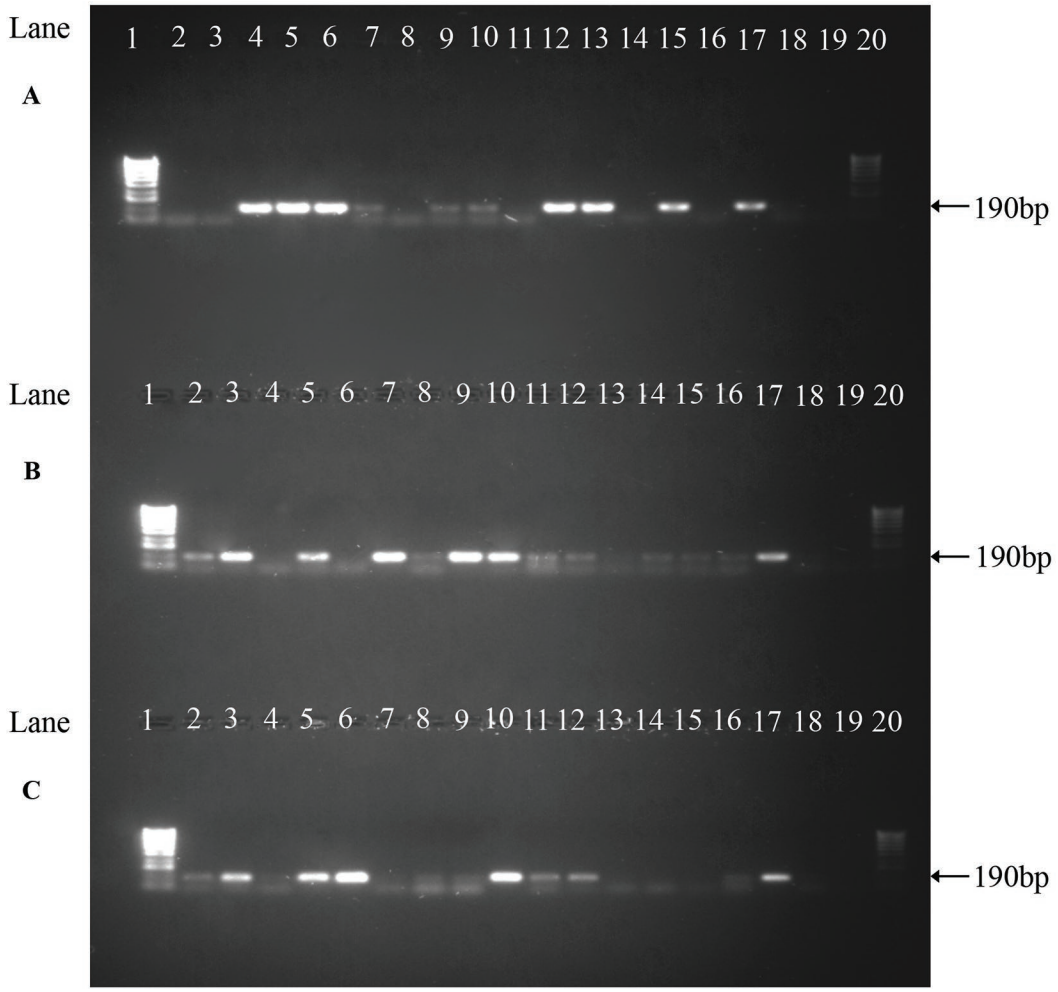

Figure 2. Detection of *mdh* gene by PCR in fructophilic lactic acid bacteria (FLAB) isolates. (A) Lanes: 1 = 100-bp ladder, 2 = DPC 7230, 3 = DPC 7231, 4 = DPC 7232, 5 = DPC 7233, 6 = DPC 7234, 7 = DPC 7235, 8 = DPC 7236, 9 = DPC 7237, 10 = DPC 7238, 11 = DPC 7239, 12 = DPC 7240, 13 = DPC 7241, 14 = DPC 7242, 15 = DPC 7243, 16 = DPC 7244, 17 = DSM 20016, 18 = water control, 19 = buffer control, 20 = 100-bp ladder. (B) Lanes: 1 = 100-bp ladder, 2 = DPC 7245, 3 = DPC 7246, 4 = DPC 7247, 5 = DPC 7248, 6 = DPC 7249, 7 = DPC 7250, 8 = DPC 7251, 9 = DPC 7252, 10 = DPC 7253, 11 = DPC 7254, 12 = DPC 7255, 13 = DPC 7256, 14 = DPC 7257, 15 = DPC 7258, 16 = DPC 7259, 17 = DSM20016, 18 = water control, 19 = buffer control, 20 = 100-bp ladder. (C) Lanes: 1 = 100-bp ladder, 2 = DPC 7260, 3 = DPC 7261, 4 = DPC 7262, 5 = DPC 7263, 6 = DPC 7264, 7 = DPC 7265, 8 = DPC 7266, 9 = DPC 7267, 10 = DPC 7268, 11 = DPC 7269, 12 = DPC 7270, 13 = DPC 7271, 14 = DPC 7272, 15 = DPC 7273, 16 = DPC 7274, 17 = DSM20016, 18 = water control, 19 = buffer control, 20 = 1-kb ladder. DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DPC = Dairy Production Centre, from the Teagasc Moorepark Culture Collection.

DSM 20016, and *L. mesenteroides* DPC 7274 did not grow at concentrations higher than 10%, 30%, and 40% fructose, respectively.

Screening of mdh-Positive FLAB for Mannitol Production

Mannitol production ranged from 0.18 ± 0.003 to 16.24 ± 0.08 g/L and 2.19 ± 0.04 to 14.98 ± 0.25 g/L for *Leuconostoc* and *Fructobacillus* cultures, respectively (Figure 4). We observed a difference ($P < 0.001$) between the positive control, *Limosilactobacillus reuteri* DSM 20016, and the 22 strains under investigation. Six strains (DPC 7232, DPC 7237, DPC 7238, DPC 7255, DPC 7260, and DPC 7261) produced higher amounts of mannitol (>12.57 ± 0.14 g/L) than DSM 20016 (10.79 ± 0.20 g/L), with the remainder showing similar or lower levels of mannitol. In general, *L. mesenteroides* DPC 7261 and *F. fructosus* DPC 7238 produced significantly higher mannitol concentrations ($P < 0.0001$) in *Leuconostocs* and *Fructobacillus* groups respectively. *Leuconostoc mesenteroides* DPC 7232 and *F. fructosus*

DPC 7237 were the second highest mannitol producers. Two *L. mesenteroides* strains (DPC 7234 and DPC 7241) were unable to produce mannitol.

Based on the results of the fructose tolerance and mannitol production assays, 4 fructophilic strains, 2 *L. mesenteroides* (DPC 7232 and DPC 7261) and 2 *Fructobacillus* species (*F. fructosus* DPC 7237 and *F. fructosus* DPC 7238) were selected for further study.

Mannitol Production, Yield, and Productivity in FMRS and FM

Table 2 depicts the FLAB directed fermentation parameters, such as fructose consumption, mannitol production, productivity, and yield in FMRS and FM. All 4 strains displayed high fructose utilization (>9 g/L), consumption rate (>0.45 g/g per h) and produced mannitol at high yields (>0.89 g of mannitol/g of fructose) with relatively greater volumetric productivities (>0.021) in FMRS. These values were much higher than those observed for *Limosilactobacillus reuteri* DSM 20016. The best yields were obtained for *F. fructosus* DPC 7238, followed by *L. mesenteroides* DPC 7261. *Fructobacillus fructosus* DPC 7238 utilized most of the fructose (9.27 g) present in the FMRS, with consumption rate of 0.46 g/g per h and produced as much as 9.19 g/L of mannitol with yield of 0.99 g/g of fructose.

Although the yields of mannitol and other parameters were lower in FM than in FMRS, they were higher for all fructophilic strains isolated in this study compared with the positive control *L. reuteri* DSM 20016 (Table 2). *Leuconostoc mesenteroides* DPC 7261 demonstrated the highest values for all calculated fermentation parameters in FM, exhibiting higher fructose consumption, mannitol production, and yield than the other strains (Table 2). *Fructobacillus fructosus* DPC 7238 was slightly less efficient, utilizing 8.57 g/L fructose and producing 4.25 g/L of mannitol with yield of 0.50 g/g of fructose in FM.

Milk Gelation by FLAB

The milk gelation ability demonstrated by FLAB strains is presented in Figure 5. The gelation point expressed in time (s) was considered when the stor-

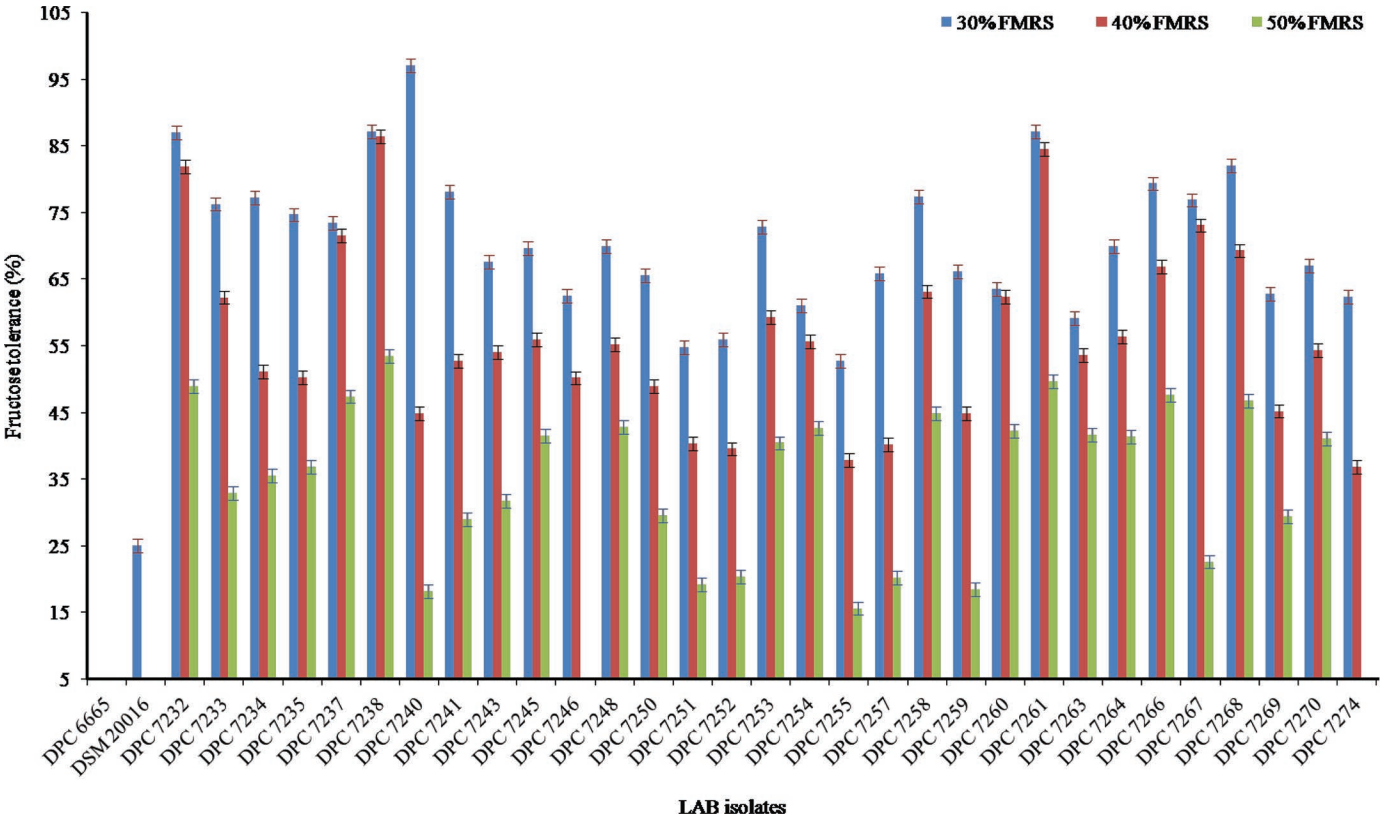

Figure 3. Fructose tolerance (%) by lactic acid bacteria (LAB) isolates (mean ± SEM). FMRS = fructose de Man, Rogosa, and Sharpe broth. Fructose tolerance (%) values for LAB isolates were between 80.96 and 100.36%, whereas DSM 20016 tolerance values were between 80.74 and 96.70% at 20 and 10%, respectively; DSM 20016 growth was not observed at 40% FMRS. DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DPC = Dairy Production Centre, from the Teagasc Moorepark Culture Collection.

Table 2. Fructose consumption and mannitol production, productivity, and yield in FMRS and FM[1]

FLAB isolates[2]	Fructose consumption (g/L)	qs (g/g per h)	Biomass (g/L)	Mannitol (g/L)	Y_{Man} (g/g)	Q_{Man} (g/L per h)	q_{Man} (g/g per h)
FMRS							
DSM 20016	6.05	0.30	14.0	4.13	0.68	0.21	0.015
DPC 7232	9.06	0.45	14.6	8.21	0.91	0.41	0.028
DPC 7261	9.25	0.46	15.5	9.02	0.96	0.45	0.029
DPC 7237	9.19	0.46	19.7	8.15	0.89	0.41	0.021
DPC 7238	9.27	0.46	9.5	9.19	0.99	0.46	0.048
FM							
DSM 20016	6.30	0.32	17.5	1.79	0.28	0.01	0.001
DPC 7232	8.79	0.44	20	3.20	0.36	0.02	0.001
DPC 7261	8.99	0.45	21	6.50	0.72	0.04	0.002
DPC 7237	8.44	0.42	20.5	2.60	0.31	0.02	0.001
DPC 7238	8.57	0.43	19.5	4.25	0.50	0.03	0.002

[1]FMRS = 1% fructose de Man, Rogosa, and Sharpe broth; FM = 1% fructose milk; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DPC = Dairy Production Centre, from the Teagasc Moorepark Culture Collection. Fructose consumption (g/L) = fructose content in samples before fermentation − fructose content in samples after fermentation. Fructose consumption rate (qs, g/L per h) = fructose consumption/time. Yield of mannitol (Y_{Man}) from fructose (g/g) − mannitol produced in sample/fructose consumed in sample. Volumetric mannitol production rate (Q_{Man}, g/L per h) = mannitol produced in samples/time. Specific mannitol production rate (q_{Man}, g/g per h) = Q_{Man}/wet biomass (g/L).

[2]FLAB = fructophilic lactic acid bacteria.

age modulus (G′) was above 0.1 Pa. The onset of milk gelation (G′ > 0.1 Pa) for *Fructobacillus* strains occurred between 41,667 and 42,238 s, whereas for *L. mesenteroides* strains, it was observed between 52,256 and 53,370 s. In the case of milk inoculated with *L. reuteri* DSM 20016, gelation commenced at 31,428 s.

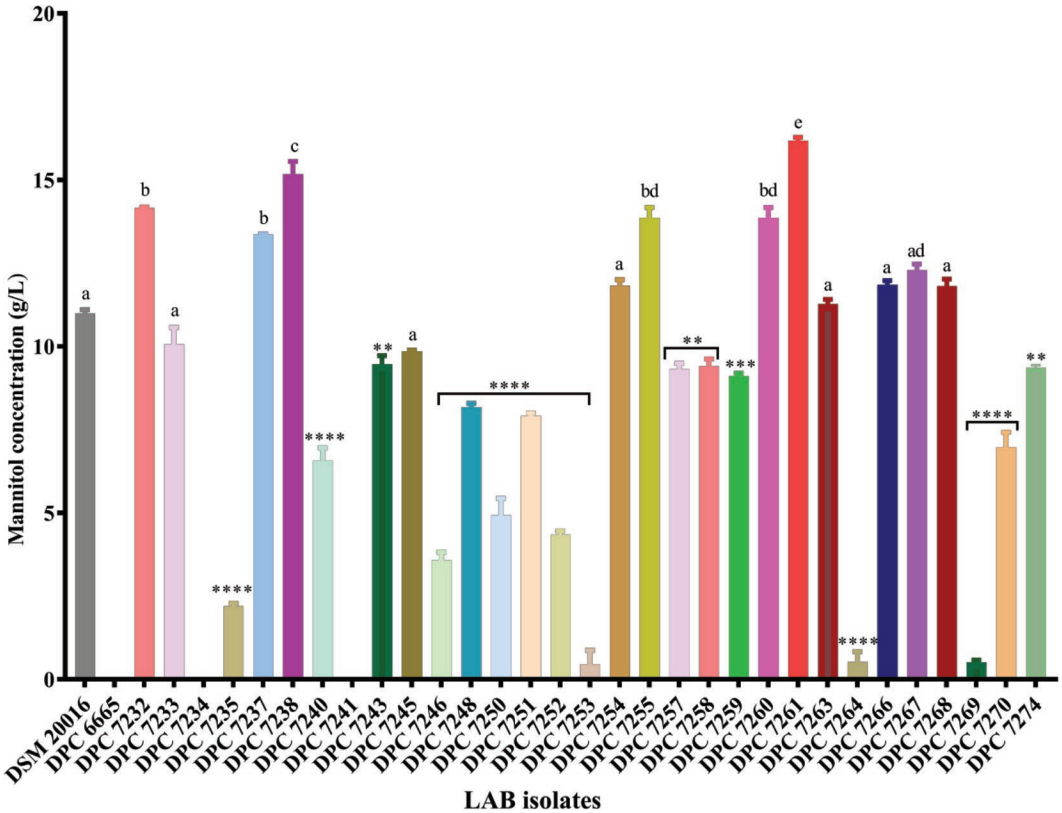

Figure 4. Mannitol production (mean ± SEM) by fructophilic lactic acid bacteria (LAB) using enzyme kit. Differences were compared with DSM 20016. DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DPC = Dairy Production Centre, from the Teagasc Moorepark Culture Collection. a–e: Different lowercase letters differ ($P < 0.01$ or $P < 0.0001$). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

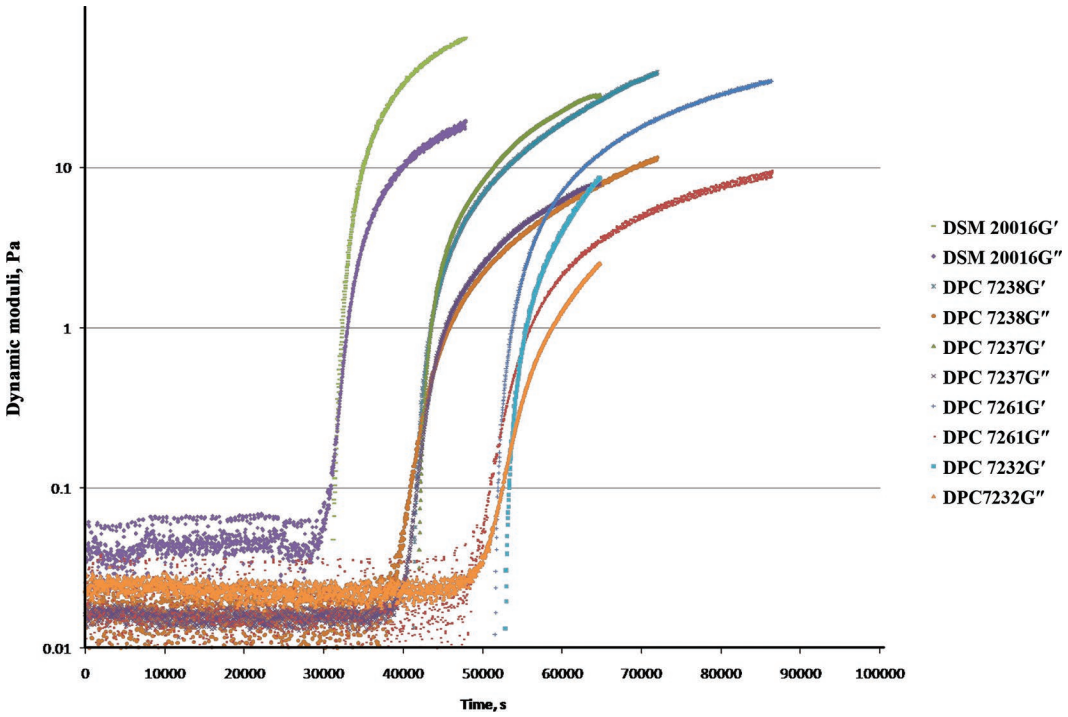

Figure 5. Changes of elastic modulus (G′) and viscous modulus (G″) of 1% fructose milk with *Limosilactobacillus reuteri* DSM 20016, *Fructobacillus fructosus* DPC 7238, *Fructobacillus fructosus* DPC 7237, *Leuconostoc mesenteroides* DPC 7261, and *L. mesenteroides* DPC 7232. Milk gelation point: G′ > 0.1 Pa.

Milk gelation times for various strains were in the order of DSM 20016 > DPC7238 > DPC7237 > DPC7261 > DPC7232. The viscous modulus (G″) values for the FLAB strains isolated in this study followed a trend quite similar to that of G′. However, the G″ values were observed to be smaller than the G′ values for all the strains. A G′ value that is higher than the G″ indicates the elastic character of a gel.

DISCUSSION

Although FLAB species have recently received renewed interest due to their possible use as mannitol producers, such strains have not yet been well characterized from a technological standpoint in dairy applications. The ability of LAB and FLAB species to produce high mannitol concentrations may provide an opportunity for the development of in situ mannitol-enriched fermented food products (Ruiz Rodríguez et al., 2017). In addition, mannitol production by these cultures as food or pharmaceutical compounds without sorbitol may be a promising alternative to chemical processes (Ruiz Rodríguez et al., 2017). Here, we generated a bank of mannitol-producing isolates from fructose-rich sources and investigated their potential for application in dairy. Our sources consisted of flowers, honey, fruits, and vegetables. Previously, FLAB have been isolated from fruits and vegetables (Edwards et al., 1998; Trias et al., 2008; Emerenini et al., 2013; Wu et al., 2014); however, we were unable to retrieve these organisms from the fruit and vegetable samples tested in this study. Environmental conditions and geographical locations that shape microbial communities, handling, storage conditions, and other factors may affect the occurrence of LAB in fruits and vegetables (Alvarez-Pérez et al., 2012; Samuni-Blank et al., 2014). By contrast, the flower and honey samples proved to be a rich source of LAB and FLAB. *Leuconostoc mesenteroides* was widely distributed among all the flower types. Interestingly, 2 strains of the subspecies *L. mesenteroides* ssp. *jonggajibkimchii* were found, one associated with dandelion flower and one associated with its leaves. Our PFGE analysis showed these as quite distinct strains genetically. The remaining *L. mesenteroides* strains from the various flower sources were also quite distinct by PFGE, with several different pulsotypes identified. *Fructobacillus* species such as *F. fructosus* were present mainly in white clover, with the exception of *F. fructosus* DPC 7239, which was present in lupin. Previously, few *Fructobacillus* species have been reported in flowers (Endo et al., 2009; Endo, 2012). The occurrence and dominance of specific species in plants and flowers is generally

dependent on the season, the flower, and also the pollinators visiting the flower. We found that samples of leaves, flowers housing honeybees, and raw honey gathered from the Tipperary farm provided large numbers of LAB isolates with fructophilic behavior compared with the wild flower samples gathered from the garden in Kilworth. This may well be explained by the fact that the Tipperary farm rears Native Irish Black Queen bees (*Apis mellifera mellifera*) for honey production, which potentially transfer fructophilic microflora onto the flowers. This is supported by other studies, which report that honeybees and insects are vital sources of FLAB (Holzapfel and Wood, 2014; Endo et al., 2018). Species of FLAB, especially *Apilactobacillus kunkeei* and *F. fructosus*, found as microbial components in the digestive tract of honeybees (Endo and Salminen, 2013; Filannino et al., 2016), were also present in flower inhabitants (Endo et al., 2009), suggesting that bees share their gut microorganisms with their diet sources. In contrast to the results obtained for *L. mesenteroides* and its subspecies, PFGE analysis of the *Fructobacillus* isolates revealed similarities between all the strains, irrespective of the source. *Fructobacillus fructosus* DPC 7239, obtained from *Lupinus graecus* flowers in Kilworth, and *F. fructosus* DPC 7266, obtained from *Trifolium repens* flowers collected from the Tipperary farm, showed 100% similarity when analyzed by Bionumerics. In the case of *F. fructosus*, DPC 7237 was 70% similar to other isolates from the same flower (white clover). Also, *F. fructosus* DPC 7238, obtained from *Trifolium repens* flowers collected in Kilworth, despite being a separate species, showed 100% similarity with other *F. fructosus* isolates. *Fructobacillus fructosus* and *F durionis* are very closely related species, and it may be the case that the discriminatory power of PFGE is insufficient to separate them.

Bacterial species harboring the *mdh* gene are often capable of producing mannitol from fructose, and this characteristic has been reported in *L. mesenteroides*, *L. pseudomesenteroides*, *Lactobacillus*, and *Fructobacillus* species (Aarnikunnas et al., 2002; Korakli and Vogel, 2003; Ruiz Rodríguez et al., 2017). Of the 41 strains of *Leuconostoc* and *Fructobacillus* strains tested, 31 were shown to harbor this gene, which may be attributed to their origin, a high fructose-rich environment. Previously, *mdh*-associated mannitol production has been reported in most FLAB isolated from fructose-rich flowers, fruits, and insect intestines (Endo et al., 2009; Filannino et al., 2016). As evidenced from the fructose tolerance assay, all *mdh*-positive LAB strains demonstrated high fructose-tolerating capabilities, with some growing well at 10 and 20% fructose in MRS broth. Fructose tolerance declined as the fructose concentration increased above 20%, but the decrease was strain-dependent. *Fructobacillus fructosus* DPC 7238, *F. fructosus* DPC 7237, *L. mesenteroides* DPC 7261, and *L. mesenteroides* DPC 7232 displayed relatively better tolerance in all fructose concentrations tested but particularly at the higher concentrations (50%). These strains were isolated from leaves, honey, and fructose-rich flowers that house honeybees, and therefore it was not unexpected that they tolerated fructose concentrations as high as 50% in the growth media. In agreement with our results, fructophilic *Lactiplantibacillus plantarum* FPL strain isolated from honeydew could tolerate 20 to 50% glucose or fructose in the MRS and FYP broth, as evidenced by visible growth as biomass and turbidity at the bottom of the tube after 24 to 48 h of incubation (Gustaw et al., 2018). As mentioned earlier, *Fructobacillus* and *L. mesenteroides* strains isolated in this study required fructose for their growth and showed delayed or no growth in the absence of fructose. This property is known for FLAB, especially *Fructobacillus* spp. and some *Lactobacillus* spp. (such as *Apilactobacillus kunkeei*, *Apilactobacillus apinorum*, and *Fructilactobacillus florum*), but is rarely seen in *L. mesenteroides* (Endo et al., 2018; Filannino et al., 2018, 2019; Maeno et al., 2019). It has been found that FLAB species prefer fructose over glucose, utilizing fewer carbohydrates, and have specific genome reductions uncommon to LAB (Endo et al., 2018). Recently, fructophilic-like growth characteristics (called pseudofructophilic activity) has been reported in *Leuconostoc* species, specifically *Leuconostoc citreum* F-192-5, isolated from the peel of the satsuma mandarin (Maeno et al., 2019). Unlike FLAB, strain F-192-5 possesses phenotypically and genetically rich carbohydrate metabolic systems, with a genome size comparable to those of nonfructophilic *L. citreum* strains. Although pseudofructophilic activity was strain-specific, it is not surprising that such a property can be observed in many other LAB species present in fructose-rich niches, as was the case in our isolated strains, henceforth called FLAB. However, a detailed investigation of specific carbohydrate utilization patterns and genomic arrangements in this strain of *L. citreum* and the *Leuconostoc* strains isolated in this study would be required to further understand their fructophilic behavior and niche adaptability.

Mannitol production varied among the strains tested, with 16 *Leuconostoc* and 5 *Fructobacillus* strains producing more than 5 g/L of mannitol from 10g/L of fructose. Similarly, Filannino et al. (2018) reported that 6 out of 24 mannitol-producing strains of the genera *Leuconostoc* and *Fructobacillus* synthesized mannitol in concentrations higher than 5 g/L from 10 g/L of fructose. Eighteen strains produced mannitol at concentrations higher than or similar to that of the control strain *Limosilactobacillus reuteri* DSM 20016,

originally isolated from human feces (Sriramulu et al., 2008; Carvalheiro et al., 2011). In previous studies, 8 of 13 FLAB strains, mainly fructobacilli isolated from fructose-rich fruits, produced 7.76 to 9.46 g/L of mannitol from 10 g/L of fructose (Ruiz Rodríguez et al., 2017). Fructophilic LAB strains isolated from bee intestines, including 5 strains of *F. fructosus*, produced significant quantities of mannitol in the fructose-rich medium (Filannino et al., 2016). We observed 2 *mdh*-positive strains, *L. mesenteroides* DPC 7234 and DPC 7241, that were unable to produce mannitol, which may be a consequence of gene mutation and merits further investigation. Notably, *L. mesenteroides* DPC 7261, *F. fructosus* DPC 7238, *F. fructosus* DPC 7237, and *L. mesenteroides* DPC 7232, which demonstrated higher fructose tolerance, were also the superior mannitol-producing strains, and were thus investigated further for mannitol production in batch method and milk fermentation.

Fermentation parameters, such as fructose consumption, mannitol production, yield, and volumetric productivities, calculated by chromatography methods, are reported in several LAB strains (Korakli et al., 2000; Wisselink et al., 2002; Ortiz et al., 2012). These parameters have been found to be rationale for the selection of superior mannitol-producing cultures for industrial applications (von Weymarn et al., 2002; Saha, 2006a). All the selected FLAB strains showed either similar or higher mannitol yields (0.89 to 0.99 g/g of fructose) compared with most of the LAB strains (0.70 to 0.96 g/g of fructose) studied by Carvalheiro et al. (2011), except *Lactobacillus fructosum*. Earlier, Saha (2006b) showed 200 g/L of mannitol production (0.67 g/g of fructose, yield) from *L. intermedius* NRRL B3693, using a simplified medium comprising 300 g/L fructose in the batch method. Upon comparison of the mannitol concentrations obtained by HPLC and the enzymatic method, we found some discrepancies, with the latter overestimating the mannitol level (>10 g/L from 10 g/L of fructose). However, the enzyme-based method is suitable as an initial screening tool. Previously, FLAB strains have been reported to utilize more fructose and produce higher mannitol concentrations because of their peculiar fructophilic metabolism (Ruiz Rodríguez et al., 2017; Filannino et al., 2018). Here, *F. fructosus* DPC 7238 and *L. mesenteroides* DPC 7261 consumed fructose at the highest rates and were also the best mannitol producers in FMRS. These strains also showed the highest volumetric productivities (0.46 and 0.45 g/L per h, respectively, by DPC 7238 and DPC 7261). A similar trend was observed for fructose consumption and mannitol production parameters in FM, but *L. mesenteroides* DPC 7261 performed better in FM, indicating that each strain differs in its mannitol-producing ability with respect to growth medium. *Fructobacillus fructosus* DPC 7238, producing 4.25g/L of mannitol in FM with a yield of 0.50 g/g of fructose, seems satisfactory for a nondairy-origin FLAB strain.

The onset of 1% FM gelation time varied with the FLAB strains, and was found to be shorter for DSM 20016 and *Fructobacillus* than for the *Leuconostoc* strains. These strains were also found to lower the pH of FM in a similar fashion, with the largest drop in pH observed for DSM 20016 (up to pH 4.72) followed by DPC 7238 (up to pH 4.9) after 20 h of incubation (Supplemental Figure S2, https://doi.org/10.3168/jds.2020-19120). However, the time required for complete gelation by these strains was quite long, and the gel formed, as observed visually, was not as firm as that produced by typical dairy strains (Gentès et al., 2011). Fructophilic LAB species are heterofermentative, producing lactate, acetate, and CO_2 (Chuah et al., 2016; Endo et al., 2018; Maeno et al., 2019), thus lowering the pH of the milk. It is also important to note that these cultures were subcultured repeatedly in fructose-containing milk, for their increased adaptability to the dairy environment. Weaker gel strength is associated with protein-protein interactions, and longer fermentation time with inherent capacities and proteolytic activity of the strains, which as of now, are poorly understood in these strains. Comparing mannitol production and milk gelation, *L. mesenteroides* DPC 7261 produced the highest mannitol concentration in the milk but required longer time than *F. fructosus* DPC 7238 to reach the gelling point in FM. Nevertheless, it is fascinating that the isolates obtained from fructose-rich niches may also be adapted to the milk system.

Although the milk gelation point varies from strain to strain (from 4 to 12 h, depending on the strain), the strains isolated in our study took longer than recognized dairy strains to reach the same gelation point. As functional starters in the production of fermented dairy products, these strains could have commercial potential for naturally produced mannitol in products such as stirred yogurt, cultured buttermilk, and dairy or nondairy beverages. For more set types of fermented dairy products, the weaker gel strength exhibited by these strains may require coculture with strains with enhanced capability in this regard. Further studies are required to focus on the technological and rheological properties of these strains in conjunction with other application-specific starters. Compatibility studies with dairy starters will provide an opportunity for novel dairy starter formulation, for development of innovative dairy products naturally sweetened with this low-calorie sugar.

CONCLUSIONS

Flowers housing honeybees were found to be a potential reservoir for fructophilic LAB species. *Leuconostoc mesenteroides* strains were widely distributed among the flowers, whereas fructobacilli were mainly isolated from white clover. The majority of the *L. mesenteroides* and *Fructobacillus* strains had the genetic and phenotypic capability to produce mannitol. With respect to fructose consumption, mannitol yield and volumetric mannitol productivities, *F. fructosus* DPC 7238 in FMRS and *L. mesenteroides* DPC 7261 in fermented milk showed the best results. These strains also took a relatively short time for gelation of milk. This finding suggests that *L. mesenteroides* DPC 7261 and *F. fructosus* DPC 7238, producing significant amounts of mannitol in milk and exhibiting milk gelation behavior, could be considered in starter or adjunct culture formulations for the development of in situ mannitol-enriched fermented dairy products.

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