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ORIGINAL ARTICLE

Polyol production during heterofermentative growth of the plant isolate *Lactobacillus florum* 2F

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Abstract

Aims: This study examined the fermentative growth and polyol production of *Lactobacillus florum* and other plant-associated lactic acid bacteria (LAB).
Methods and Results: Sugar consumption and end-product production were measured for *Lact. florum* 2F in the presence of fructose, glucose and both sugars combined. The genome of *Lact. florum* was examined for genes required for mannitol and erythritol biosynthesis. The capacity for other plant-associated LAB to synthesize polyols was also assessed.
Conclusions: *Lactobacillus florum* exhibited higher growth rates and cell yields in the presence of both fructose and glucose. *Lactobacillus florum* 2F produced lactate, acetate and ethanol as well as erythritol and mannitol. *Lactobacillus florum* 2F synthesized mannitol during growth on fructose and erythritol during growth on glucose. Gene and protein homology searches identified a mannitol dehydrogenase in the *Lact. florum* 2F genome but not the genes responsible for erythritol biosynthesis. Lastly, we found that numerous other heterofermentative LAB species synthesize erythritol and/or mannitol.
Significance and Impact of the Study: *Lactobacillus florum* is a recently identified, plant-associated, fructophilic LAB species. Our results show that *Lact. florum* growth rates and heterofermentation end-products differ depending on the sugar substrates present and growth yields can be improved when combinations of sugars are provided. *Lactobacillus florum* 2F produces erythritol and mannitol, two polyols that are relevant to foods and potentially also in plant environments. The capacity for polyol biosynthesis appears to be common among plant-associated, LAB species.

Introduction

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive bacteria characterized by their production of lactic acid as the primary end-product of fermentative growth. LAB are largely found in (fermented) meats, dairy and plant-based foods as well as the digestive tracts of animals and insects. LAB are also members of the indigenous population of microbes that inhabit the aerial surfaces of plants (Muller and Seyfarth 1997; Di Cagno *et al.* 2013; Williams *et al.* 2013). LAB that are most commonly isolated from plants include species from the *Lactobacillus, Leuconostoc, Weissella, Enterococcus* and *Pediococcus* genera (Di Cagno *et al.* 2013). Although typically detected in low numbers on living plant tissues, those LAB are responsible for the spontaneous fermentation of leaves, grains and fruits yielding food products such as sauerkraut and sourdough as well as a wide-variety of foods consumed by different ethnic groups world-wide (e.g. gundruk, khalpi, mesu, soidon and sinki) (Tamang and Sarkar 1996; Tamang *et al.* 2005; Jeyaram *et al.* 2010; Tamang and Tamang 2010; Chilton *et al.* 2015).

Plant-associated LAB possess a variety of traits specific to plant environments. For example, certain LAB

produce levansucrase (Tieking *et al.* 2005), tannase (Jimenez *et al.* 2014) and phytase (Fischer *et al.* 2014) involved in the breakdown of plant tissues. These bacteria likely express other novel traits relevant to plants as indicated by comparative genomics of plant- and dairy-associated strains of *Lactococcus lactis* (Siezen *et al.* 2008, 2011) and transcriptome and metabolome profiling of *Lact. lactis* grown on *Arabidopsis thaliana* leaf tissues (Golomb *et al.* 2015). However, more generally, a common feature of plant-associated and other LAB is their synthesis of high quantities of lactic and acetic acids by fermentation. Rapid acidification caused by the production of these organic acids is lethal to foodborne pathogens as well as commensal bacteria. Additionally, a variety of other fermentative metabolic end-products made by LAB might also have important roles in the ecological interactions of these bacteria in their natural habitats.

Many of the known plant-associated LAB species are heterofermentative and possess the phosphoketolase pathway for sugar metabolism and ATP generation (Zaunmuller *et al.* 2006). Pentoses are typically the preferred carbon sources for these bacteria. The use of glucose or other hexoses results in the synthesis of an additional two NAD(P)H that need to be reoxidized at the expense of ATP synthesis. End-products of heterofermentation are lactic acid, acetic acid, ethanol and CO_2. At least some heterofermentative LAB also possess other NAD(P)H oxidation pathways leading to the production of the polyols mannitol and erythritol (Ortiz *et al.* 2013). The reduction of fructose to mannitol is an efficient way for cells to regenerate NAD(P)+ and enables acetyl-P formed during heterofermentation to be channelled to acetate kinase with the gain of extra ATP. Mannitol is a known metabolic end-product for a variety of LAB, and numerous investigations have aimed to increase mannitol biosynthesis by these bacteria for industrial-scale production (Patra *et al.* 2009; Saha and Racine 2011). In contrast, knowledge on erythritol synthesis by LAB is largely limited to strains of *Oenococcus oeni* and *Leuconostoc mesenteroides* (Veiga da Cunha *et al.* 1992, 1993; Richter *et al.* 2001). NAD(P)H is reoxidized by the synthesis of erythritol instead of ethanol, when these species are grown on glucose and acetyl-CoA precursors are limited (Ortiz *et al.* 2013). Although the enzymatic pathway for erythitol biosynthesis has been described (Zaunmuller *et al.* 2006), and corresponding genes have yet to be identified and the extent to which other heterofermentative LAB produce erythritol is not well known.

Lactobacillus florum is a recently described species and is thus far exclusively associated with the aerial surfaces of plants (Endo *et al.* 2010; Mtshali *et al.* 2012). This species is a heterofermentative, fructophilic LAB that is unable to consume pentoses (Endo *et al.* 2010). The purpose of this study was to examine the metabolic features of *Lact. florum* 2F, a strain for which the genome sequence is known (Kim *et al.* 2013). Fermentative growth of *Lact. florum* 2F on glucose and fructose was examined along with the resulting end-products which included erythritol and mannitol. The capacity for other LAB plant isolates to produce these polyols was also explored.

Materials and methods

Bacterial strains

Lactobacillus florum strain 2F was isolated from Valencia orange leaves along with other LAB from leaves and fruits collected at a farm in Fallbrook, California in October, 2011 (Table 1). For taxonomic identification, the 16S rRNA gene from each of the isolates was sequenced as previously described (Golomb *et al.* 2013) and compared

Table 1 LAB screened for erythritol and mannitol production

Species	UCD #/isolate	Source
Carnobacterium maltaromaticum	3B5	Brown Turkey Fig (R)
Fructobacillus sp.	3H5	Brown Turkey Fig (L)
Lactobacillus florum	2F	Valencia Orange (L)
Lactobacillus plantarum	1B1	Cactus (R)
Lactococcus lactis	1A9	Cactus (R)
Lact. lactis	2B6	Banana (L)
Leuconostoc citreum	3D6	Sapote (R)
Leuc. citreum	1A5	Cactus (R)
Leuconostoc pseudomesenteroides	1C7	Cactus (R)
Leuc. pseudomesenteroides	1D1	Brown Turkey Fig (R)
*Oenococcus oeni**	154, 1041	Wine, Sauvignon Blanc
*O. oeni**	164, AM22	Wine, Cabernet Sauvignon
*O. oeni**	167, 1081	Wine, Pinot Noir
*O. oeni**	176, IS-1	Wine, Spain
*O. oeni**	193, 3N:D1	Wine, Cabernet Sauvignon
*O. oeni**	199, 1N:F1	Wine, Merlot
*O. oeni**	224	Wine, Chile
*O. oeni**	261, IOEB 9306	Cider, France
*Oenococcus kitaharae**	311, NRIC 0645	Shochu residue compost
Weissella confusa	1B5	Cactus (R)
W. confusa	1B3	Cactus (R)
Weissella paramesenteroides	1A8	Cactus (R)

R, ripe fruit; L, leaves.
*UC Davis Viticulture and Enology Culture Collection.

against the National Center for Biotechnology Information (NCBI) database using the nucleotide Basic Local Alignment Search Tool (BLASTN) program (http://blast.ncbi.nlm.nih.gov). Isolates with the same species designations were confirmed to be genetically distinct by Random Amplified Polymorphic DNA (RAPD) analysis according to methods described in (Endo *et al.* 2010). Strains of *O. oeni* from diverse wine sources were selected from the University of California, Davis Viticulture and Enology culture collection (http://wineserver.ucdavis.edu/industry/enology/culture/index.html) (Table 1).

Culture conditions for sugar consumption and fermentation end-product analysis

Erythritol and mannitol biosynthesis was measured in four different laboratory culture media: (i) MRS (BD, Franklin Lakes, New Jersey); (ii) MLAB, MRS containing 10% filtered V8 tomato juice (Campbell's, Camden, NJ) and supplemented with 0·5% w/v (27·8 mmol l^{-1}) fructose and 0·1% (7·5 mmol l^{-1}) w/v malic acid (Bae *et al.* 2006; Endo and Okada 2006); (iii) FT-80 supplemented with 2% w/v (111 mmol l^{-1}) glucose (Cavin *et al.* 1989); (iv) and Zhang Block Mills media (ZMB-1) (Zhang *et al.* 2009). For MLAB, V8 juice was sterilized by filtration through a 0·22-μm Millipore Express Plus filter (EMD Millipore, Billerica, MA). Sugar consumption and end-product analysis were also measured for *Lact. florum* 2F in a modified MRS medium (mMRS) lacking beef extract and containing 2% w/v (111 mmol l^{-1}) fructose, 2% w/v (111 mmol l^{-1}) glucose or a mixture of 1% w/v (55·6 mmol l^{-1}) glucose and 1% w/v (55·6 mmol l^{-1}) fructose. The pH of the laboratory culture media was measured using a SevenEasy pH meter with InLab Routine Pro probe (Mettler Toldeo, Columbus, OH) and adjusted with either 10 N NaOH or 1 N HCl. Cultures were incubated at 30°C for 24–72 h prior to end-product analysis. Optical density (OD600) was measured on a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Growth rates of *Lact. florum* 2F were measured over 72 h in mMRS with each of the three supplemented sugar conditions (2% w/v fructose, 2% w/v glucose or a mixture of 1% w/v glucose and 1% w/v fructose) using a Biotek Synergy 2 plate reader (BioTek, Winooski, VT).

High performance liquid chromatography

For detection of residual sugars and fermentation end-products, cells were collected by centrifugation at 10 000 ***g*** for 10 min and the supernatant was filtered through a 0·2-μm Millipore syringe filter (Millipore) prior to storage at −20°C. Levels of glucose, fructose, erythritol, mannitol, lactate, acetate and ethanol were measured on an Agilent Hewlett Packard liquid chromatograph HP 1100 series high-performance liquid chromatography (HPLC) system coupled to Diode Array and 1047A Refractive Index detectors (Agilent Technologies, Santa Clara, CA) in series. For carbohydrate analysis, a Bio-Rad Aminex HPX-87C calcium form column, 300 × 7·8 mm (Bio-Rad, Hercules, CA) was used at a temperature of 80°C with a mobile phase of 30% acetonitrile and an isocratic flow rate of 0·6 ml min^{-1} and injection volume of 20 μl. For measurement of organic acids and ethanol, the column type used was an Agilent Hi-Plex H column, 300 × 7·7 mm at 65°C. The mobile phase was 5 mmol l^{-1} sulphuric acid with an isocratic flow rate of 0·6 ml min^{-1} and sample injection volume of 20 μl. Organic acids were detected by UV at 210 nm and both carbohydrates and ethanol were measured by refractive index.

Lactate dehydrogenase assay

Lactobacillus florum 2F was harvested after 24, 48 and 72 h of growth in mMRS supplemented with 2% w/v (111 mmol l^{-1}) fructose, glucose or equal quantities (1% w/v, 55·6 mmol l^{-1}) of fructose and glucose. Cells were prepared by centrifugation at 10 000 ***g*** for 10 min followed by two consecutive washes in phosphate-buffered saline (PBS) (8 g l^{-1} NaCl, 0·2 g l^{-1} KCl, 1·44 g l^{-1} NaH_2PO_4 and 0·24 g l^{-1} KH_2PO_4), pH 7·1, and the cell pellets were frozen at −20°C until analysis. For protein extraction, frozen cell pellets were thawed and suspended in enzyme assay buffer (25 mmol l^{-1} HEPES and 3 mm $MgCl_2$, pH 7·1) (Veiga da Cunha *et al.* 1993) in tubes containing 0·5 g of 0·1 mm zirconia/silica beads (BioSpec, Bartlesville, OK). Cells were mechanically lysed in an MP Bio FastPrep 24 bead beater (MP Biomedicals, Santa Ana, CA) at a speed setting of 6·5 m s^{-1} for 40 s for two cycles, and the cells were placed on ice for one min between cycles. Cell debris was collected by centrifugation at 10 000 ***g*** for 10 min at 4°C and then the protein concentration in the supernatant was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Quantities of 0·75–1·0 μg total protein were used for the Lactate Dehydrogenase Activity (LDH) Assay (BioVision Inc., Milpitas, CA) LDH measurements were carried out in 96-well optical bottom plates (Thermo Scientific, Waltham MA) read in a BioTek Synergy 2 plate reader (BioTek). Samples were incubated at 37°C for 90 min with absorbance readings taken every 5 min at 450 nm with 5 s of mixing immediately prior to each measurement. Statistical analysis of LDH activity levels was performed by ANOVA and the

unpaired Student *t*-test in GRAPHPAD PRISM 5 (GraphPad Software, Inc. La Jolla, CA)

In silico analysis for proteins coding for mannitol and erythritol biosynthesis

Protein sequences for erythrose reductase and mannitol dehydrogenase were compared against the predicted proteins in the genome of *Lact. florum* 2F (Kim *et al.* 2013) using the BLASTP algorithm provided by the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov). Mannitol 2-dehydrogenase from *Leuconostoc pseudomesenteroides* ATCC 12291 (accession number CAD31644) (Hahn *et al.* 2003) was used to confirm the presence of a similar protein-encoding gene in *Lact. florum*. Erythrose reductase(s) (ER) used for comparison included three isozymes of ERs (ER1, 2 and 3) from *Trichosporonoides megachiliensis* SNG-42 (in NCBI as *Moniliella megachiliensis*) (accession numbers BAD90687, BAD90688, BAD90689 respectively), two isozymes of ERs (MsER1 and MsER3) from *Moniliella sp* BH010 (accession number AGB07592 and AGB07593, respectively) and a single ER from *Candida magnoliae* JH110 (accession number ACT78590) (Ookura *et al.* 2005; Lee *et al.* 2010; Deng *et al.* 2012).

Results

Growth of *Lactobacillus florum* 2F on glucose and fructose

To examine the sugar substrate preferences of *Lact. florum* 2F, this strain was incubated in the presence of fructose, glucose or an equal quantity of both sugars. The average growth rate of *Lact. florum* 2F was significantly higher in mMRS containing both fructose and glucose ($0{\cdot}28 \pm 0{\cdot}01\ h^{-1}$) than fructose alone ($0{\cdot}26 \pm 0{\cdot}01\ h^{-1}$) ($P < 0{\cdot}05$, Student's *t*-test) (data not shown). Growth rates were reduced ($0{\cdot}15 \pm 0{\cdot}01\ h^{-1}$) and a slower reduction in culture pH was observed when glucose was provided as the sole carbon source in the culture medium (Fig. 1).

Fructose was the preferred sugar substrate for *Lact. florum* 2F in mMRS (Fig. 2). When incubated in the presence of fructose, *Lact. florum* 2F consumed the majority of that sugar within 48 h (Fig. 2a). By comparison, only 50% w/v of the glucose was consumed after 72 h incubation (Fig. 2b). Moreover, two-fold more fructose than glucose was consumed during the first 24 h incubation of *Lact. florum* 2F in mMRS with equal starting quantities of those sugars (Fig. 2c).

Organic acid and polyol production of *Lactobacillus florum* 2F during growth on glucose and fructose

End-products of *Lact. florum* 2F metabolism differed depending on the sugar substrates available for growth. The highest quantities of acetate were produced by exponential phase *Lact. florum* 2F in mMRS-containing fructose (22·5 mmol l^{-1} ($1{\cdot}28 \pm 0{\cdot}02$ g l^{-1})) (Fig. 3a). The most lactate was produced in mMRS containing both fructose and glucose (36·1 mmol l^{-1} ($3{\cdot}20 \pm 0{\cdot}01$ g l^{-1})) (Fig. 3c). However, lactate concentrations increased as cells reached stationary phase, independent of whether glucose or fructose was provided as a carbon source. The increase in lactate was consistent with the increases in lactate dehydrogenase (LDH) activity over time (Table 2). Notably, ethanol was also detected as an end-product of *Lact. florum* metabolism (17 mmol l^{-1} ($0{\cdot}76 \pm 0{\cdot}03$ g l^{-1})) when glucose was provided as the sole carbon source.

Mannitol was produced by *Lact. florum* in higher quantities than either acetate or lactate (Fig. 3). Mannitol was detected during growth in mMRS-containing fructose or fructose and glucose, but not in the presence of glucose alone (Fig. 3). Approximately 50% of the fructose was converted to mannitol by *Lact. florum* 2F when fructose was the sole carbon source (62 mmol l^{-1} (11·32 g l^{-1})) (Fig. 3a). In the presence of both fructose and glucose, mannitol concentrations reached 45·9 mmol l^{-1} (8·4 g l^{-1}), and assuming that only

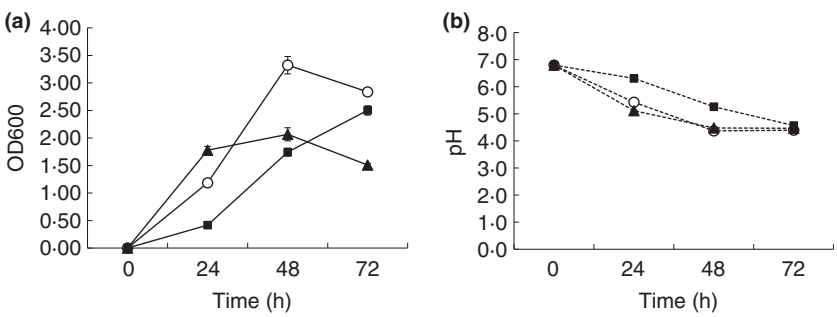

Figure 1 Growth of *Lact. florum* 2F on fructose, glucose and a 1 : 1 mixture of fructose and glucose. The (a) OD600 (—) and (b) pH (---) were measured during growth in mMRS containing 111 mmol l^{-1} (20 g l^{-1}) fructose (▲), glucose (■) or equal 10 g l^{-1} (55·6 mmol l^{-1}) quantities of fructose and glucose (○). All measurements are the avg ± SD of triplicate cultures.

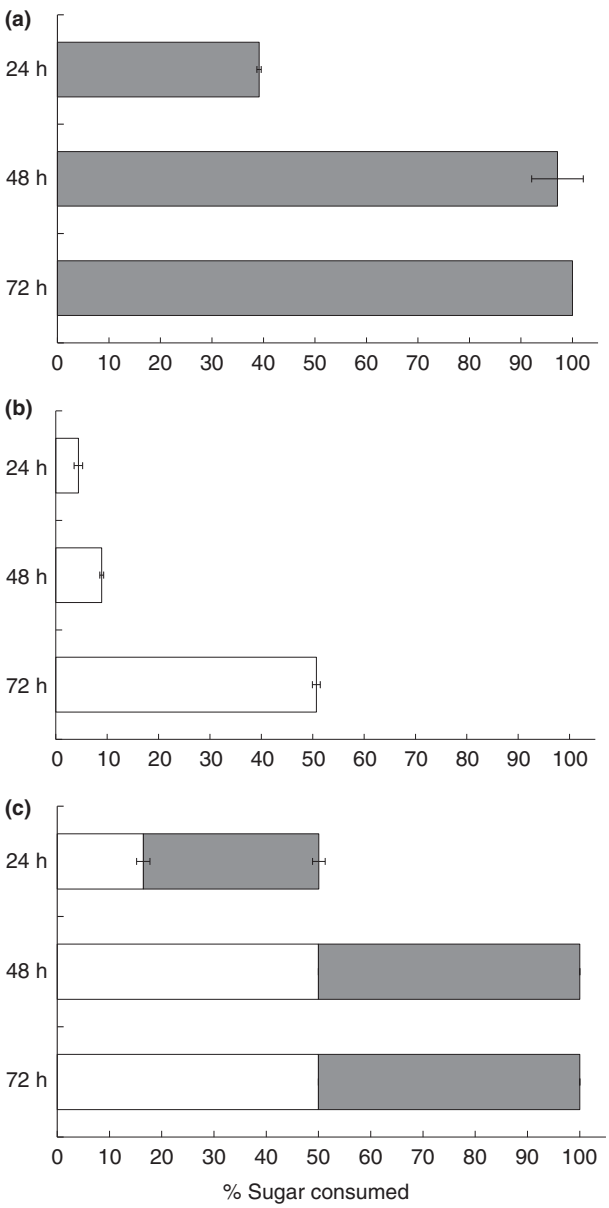

Figure 2 Sugar consumption by *Lact. florum* 2F. *Lact. florum* 2F was grown in mMRS supplemented with 20 g l^{-1} (111 mmol l^{-1}) (a) fructose (closed bars), (b) glucose (open bars) or (c) equal 10 g l^{-1} (55·6 mmol l^{-1}) quantities of fructose and glucose. Cultures were measured at 24, 48 and 72 h. All measurements are the avg ± SD of triplicate cultures.

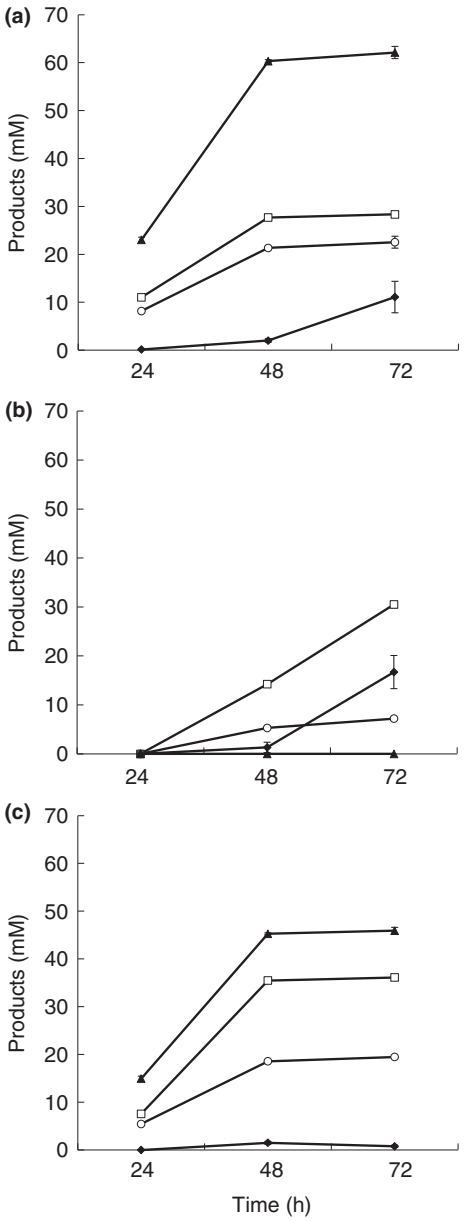

Figure 3 The effects of different carbohydrate fermentation on the amount of lactate (□), acetate (○), erythritol (♦) and mannitol (▲) produced by *Lact. florum* 2F. The strain was grown in mMRS supplemented with 20 g l^{-1} (111 mmol l^{-1}) fructose (a), glucose (b) or equal 10 g l^{-1} (55·6 mmol l^{-1}) quantities of fructose and glucose (c). Erythritol and mannitol were not detected in the MRS culture medium used to grow *Lact. florum*. Lactate and acetate were detected at quantities of 0·4 and 1·5 g l^{-1} respectively. These values were subtracted from the quantities of lactate and acetate measured during *Lact. florum* growth. At the time of inoculation (0 h), the concentrations of all metabolites shown here were not above background levels. All measurements are the avg ± SD of triplicate cultures.

fructose was used for mannitol biosynthesis, nearly 80% of the fructose was converted to mannitol within the first 48 h of growth (Figs 2c and 3c).

The highest quantities of erythritol were produced by *Lact. florum* 2F during growth on glucose (17 mmol l^{-1} (2·04 g l^{-1})) (Fig. 3b). Erythritol was first detected 48 h after the initiation of growth and increased during the subsequent 24 h (Fig. 3b). Erythritol was also produced by *Lact. florum* 2F in mMRS containing fructose but only after the majority of that sugar was consumed (Figs 2a and 3a). By comparison, only very low quantities of

Table 2 *Lactobacillus florum* 2F lactate dehydrogenase (LDH) activity

Carbohydrate	Time (h)	Specific activity (U mg^{-1} protein) LDH
Fructose	24	0·014 ± 0·002*,[a]
	48	0·103 ± 0·005[a]
	72	0·024 ± 0·002*,[a]
Glucose	24	ND
	48	0·110 ± 0·008[a]
	72	0·135 ± 0·054[b]
Fructose and Glucose	24	0·355 ± 0·007*,[b]
	48	0·499 ± 0·025*,[b]
	72	0·428 ± 0·023*,[c]

LDH, lactate dehydrogenase.
Cells were inoculated in mMRS (pH 6·8) with different carbon sources at 30°C.
Values are the avg ± SD of three replicates. Not determined (ND). LDH values were compared with ANOVA and *post hoc* (unpaired) *t*-tests.
Significant differences detected at each time point between cultures are indicated with letters (a, b, c) ($P < 0·05$).
*Significantly different LDH values for different time points in the same culture media ($P < 0·05$).

erythritol were detected during incubation of *Lact. florum* 2F in the presence of both sugars (Fig. 3c).

Protein-encoding genes for mannitol and erythritol production in *Lactobacillus florum* 2F

Mannitol dehydrogenase is responsible for the reduction of fructose to mannitol in heterofermentative LAB (Wisselink *et al.* 2002). One predicted protein in *Lact. florum* 2F (accession number EKK21208) is highly related to a protein annotated as mannitol-2-dehydrogenase in *Leuc. pseudomesenteroides* ATCC 12291 (98% amino acid sequence coverage) (Hahn *et al.* 2003). The *Lact. florum* 2F gene is annotated as a sorbitol dehydrogenase, however, because sorbitol is an isomer of mannitol, this protein is most likely responsible for both sorbitol and mannitol biosynthesis.

The genes required for erythritol biosynthesis in fungi but not bacteria are known (Moon *et al.* 2010). BLASTP searches of fungal erythritol reductases (ERs) against proteins encoded in the *Lact. florum* genome located a predicted aldo-keto reductase protein annotated as a 2,5-diketo-D-gluconic acid reductase (accession number EKK21190). *Candida magnoliae* JH110 ER shares 33% amino acid identity (94% sequence coverage) with this protein and similar levels of amino acid sequence identity were found for ERs produced by *Moniliella sp.* BH010 and *T. megachiliensis* SNG-42. Fungal ER and 2,5-diketo-D-gluconic acid reductases encoded by strains of *O. oeni* and *Lact. lactis* were also similar at the amino acid level (data not shown). These findings indicate that there is sequence conservation among aldo-keto reductase superfamily proteins in bacteria and fungi.

Erythritol and mannitol production by plant-associated LAB

A collection of 13 isolates from different LAB species was examined for the capacity to produce erythritol or mannitol in a variety of culture media. These isolates were recovered from fresh fruits and leaves of different plants at the same geographic location as *Lact. florum* 2F (Table 1). To serve as a reference, *Lact. florum* 2F, eight strains of *O. oeni* and one strain of *Oenococcus kitaharae* were also tested. The 22 LAB were screened for polyol production in MRS, MLAB, FT-80 and ZMB1 culture medium intended to reflect different nutritive environments. All isolates tested grew most rapidly and to the highest cell densities in MLAB and growth was poor in the defined culture medium ZMB1 (data not shown).

Seventeen out of the 22 LAB screened produced erythritol (Fig. 4). Erythritol was most frequently detected during growth in MLAB and was found at least once in all other culture media tested indicating that there is no single media type that induced or suppressed erythritol synthesis (Fig. 4). Isolates that most consistently synthesized erythritol on different media types were *Lact. florum* 2F, *Fructobacillus* sp. 3H5, *Leuconostoc citreum* 1A5, *O. kitaharae* 311, *O. oeni* 261 and *Weissella confusa* 1B3. In contrast, homofermentative LAB including isolates of *Carnobacterium maltaromaticum*, *L. plantarum* and *Lact. lactis* as well as *O. oeni* 164 did not produce erythritol under the conditions examined here.

Mannitol synthesis was also detected for 17 LAB isolates (Fig. 4). Most of the isolates produced mannitol strictly in fructose-containing MLAB and not in other laboratory culture media. With the exception of *O. oeni* 193, cultures that produced erythritol also produced mannitol.

Discussion

This study showed that the growth and fermentative metabolism of the plant-derived strain *Lact. florum* 2F is significantly altered depending on the availability of fructose and/or glucose. *Lactobacillus florum* 2F produced erythritol and mannitol and the synthesis of these polyols is a common feature among other plant-associated, heterofermentative LAB species.

The growth rate of *Lact. florum* 2F was highest in mMRS containing both fructose and glucose and resulted in the complete removal of those sugars from the culture medium. With access to fructose and

	Erythritol				Mannitol			
	MRS	MLAB	FT-80	ZMB1	MRS	MLAB	FT-80	ZMB1
Carnobacterium maltaromaticum 3B5								
Fructobacillus sp. 3H5	0·06	0·04	0·05	0·05	0·41	5·32		
Lactobacillus florum 2F	0·20	0·14	0·17		0·75	5·63		
Lactobacillus plantarum 1B1								
Lactococcus lactis 1A9								
Lactococcus lactis 2B6								
Leuconostoc citreum 3D6	0·02			0·13	0·43	5·65		
Leuconostoc citreum 1A5	0·02	0·02	0·03	0·24		6·03		
Leuconostoc pseudomesenteroides 1C7		0·02		0·07		5·62		
Leuconostoc pseudomesenteroides 1D1		0·03		0·04		5·10		
Oenococcus kitaharae 311	0·09	0·45	0·11			0·13		
Oenococcus oeni 154		0·04				1·00		
Oenococcus oeni 164						0·47		
Oenococcus oeni 167		0·03				4·77		
Oenococcus oeni 176		0·04				5·14		
Oenococcus oeni 193		0·10						
Oenococcus oeni 199		0·04				3·27		
Oenococcus oeni 224		0·04				5·11		
Oenococcus oeni 261		0·23	0·10		0·21	5·97		
Weissella confusa 1B5		0·04		0·05		0·08		
Weissella confusa 1B3	0·02	0·06		0·09		0·16		
Weissella paramesenteroides 1A8	0·02			0·03	0·51	5·90		

Figure 4 Erythritol and mannitol production by plant-associated LAB. Presence (grey squares) or absence (blank) of erythritol and/or mannitol production by 22 LAB. Erythritol was detected in a range from 0·02 to 0·45 g l^{-1} and mannitol was detected levels of 0·08–6·03 g l^{-1}. The isolates were incubated at 30°C until turbid (2–14 days) prior to measurement.

glucose, *Lact. florum* primarily used fructose as an external electron acceptor for mannitol biosynthesis and glucose for energy generation. The reduction of fructose to mannitol is energetically favourable because it can lead to the use of acetyl-P generated by the phosphoketolase pathway to the production of ATP by acetate kinase instead of NAD(P)H oxidation (Richter *et al.* 2003). This possibility is also supported by the conversion of approx. 80% of the fructose to mannitol by *Lact. florum* and a lack of production of either ethanol or erythritol from glucose. To this regard, high levels of mannitol synthesis in the presence of fructose and glucose were previously shown for other heterofermentative *Lactobacillus* species (Saha and Nakamura 2003; Saha 2006; Arskold *et al.* 2008). However, less mannitol was produced by *Lact. florum* compared to other LAB which were reported to produce up to 0·98 mol mannitol per mol fructose consumed (Yun and Kim 1998; von Weymarn *et al.* 2002; Saha and Racine 2011; Sung *et al.* 2012). Because the conditions used here were not intended to maximize mannitol biosynthesis by *Lact. florum* 2F, further increases in mannitol yields might be accomplished by altering the concentrations of fructose relative to other sugars and adjusting parameters such as pH and temperature to which the cells are exposed (Saha and Racine 2011; Sung *et al.* 2012).

Lactobacillus florum 2F grew more rapidly and reached higher cell densities when fructose was the sole carbon source. The fructophilic metabolism of strain 2F is in agreement with other *Lact. florum* strains (Endo *et al.* 2009). Fructose is the preferred substrate because this sugar can serve as both a growth substrate for energy production and an external electron acceptor. This multifaceted use of fructose was demonstrated by the finding that approx. 50% of the fructose available in mMRS was converted to mannitol. The majority of the remaining sugar was fermented for ATP synthesis resulting in lactic and acetic acids in a molar ratio of 1–0·6. Interestingly, erythritol was also produced by late stationary-phase cells in fructose. This finding is similar to the observed increase in erythritol biosynthesis by *O. oeni* during nutrient starvation (Richter *et al.* 2001).

Lactobacillus florum 2F grew poorly on glucose. In medium containing glucose and no exogenous electron acceptors such as fructose, O_2 or pyruvate, heterofermentative LAB are generally impaired at regenerating NAD(P)+ through the phosphoketolase pathway (Maicas *et al.* 2002). The metabolism of glucose to ethanol and erythritol could be used in lieu of generating additional ATP. Our detection of ethanol and erythritol in glucose-grown *Lact. florum* cultures indicates that those pathways served to regenerate NAD(P)+. This possibility is also supported

by the low levels of acetate produced by *Lact. florum* in the glucose-containing mMRS. Instead, after 72 h incubation in mMRS, erythritol and ethanol were detected in equal molar quantities (17 mmol l^{-1}). The production of erythritol is notable because similar quantities were also found for *O. oeni* GM (Veiga da Cunha *et al.* 1992, 1993).

Further increases in erythritol yields might be achieved by limiting levels of exogenous pantothenate, an essential precursor for acetyl-CoA biosynthesis and NAD(P)+ regeneration by acetaldehyde/ethanol dehydrogenase (Richter *et al.* 2001). Moreover, identification of the genes required for erythritol biosynthesis would enable a better understanding of the transcriptional control over that metabolic pathway. Erythritol production in bacteria is predicted to involve erythritol 4-phosphate dehydrogenase (EPDH) (Veiga da Cunha *et al.* 1993). By comparison, fungi use the erythrose reductase (ER) pathway (Moon *et al.* 2010). The fungal ER pathway has been genetically characterized for *Candida magnolia* JH110, *T. megachiliensis* SNG-42 and *M. megachiliensis* BH010 (Ookura *et al.* 2005; Lee *et al.* 2010; Deng *et al.* 2012). To this regard, we located a gene in the *Lact. florum* genome annotated as a 2,5-diketo-D-gluconic acid reductase that shares amino acid similarity to fungal ERs (Deng *et al.* 2012). Similar to fungal ERs, 2,5-diketo-D-gluconic acid reductases are aldo-keto reductases that result in the oxidation of NAD(P)H (Chotani *et al.* 2000; Kaswurm *et al.* 2013). However, because similar proteins were also predicted to be encoded by strains of *Lact. lactis*, a species not yet shown to produce erythritol, further effort is needed to elucidate the specific EPDH or ER responsible for erythritol biosynthesis in bacteria.

A total of 16 of the 22 LAB tested produced both mannitol and erythritol. Isolates of *Leuc.* (*pseudo*)*mesenteroides* and *O. oeni* made these polyols, and this result is in agreement with previous reports on other strains of these species (Veiga da Cunha *et al.* 1992; Richter *et al.* 2001; von Weymarn *et al.* 2002; Sung *et al.* 2012). However, it has remained unclear whether other heterofermentative LAB share this capacity. To that regard, we found that in addition to *Lact. florum,* isolates of *Fructobacillus, O. kitaharae, W. confusa* and *Weissella paramesenteroides* were also able to synthesize mannitol and erythritol. Interestingly, *O. oeni* 193 produced erythritol but not mannitol and *O. oeni* 164 produced mannitol, but not erythritol. These findings suggest that there is considerable intraspecies variation in heterofermentative pathways in *O. oeni*, possibly as a result of the hypermutability of this genus (Marcobal *et al.* 2008).

In conclusion, plants are colonized by diverse LAB species and these bacteria likely possess novel traits that can be harnessed by the food industry as a way to directly and naturally enhance the health benefits of (fermented) foods. Both mannitol and erythritol have applications in foods because they are both noncariogenic (Kawanabe *et al.* 1992; Soderling and Hietala-Lenkkeri 2010) and nonglycaemic (Bornet *et al.* 1996; Ishikawa *et al.* 1996). Erythritol is unique in that it has nearly zero calories (Livesey, 2003; Roper and Goossens 1993) and can be consumed in large quantities without a laxative effect (Storey *et al.* 2007). Moreover, the production of polyols on plant surfaces might have important roles in the microbe-microbe and host-microbe interactions of LAB on plants. For example, mannitol can serve as a compatible solute in bacteria (Kets *et al.* 1996; Sand *et al.* 2013), and certain bacterial species are able to consume mannitol (Wu *et al.* 2011) and erythritol (Geddes *et al.* 2013) for growth. Our findings for *Lact. florum* 2F can be used for improving and diversifying LAB applications in foods and beverages and also to support investigations into the ecology of LAB on plants.

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Conflict of Interest

Dr Hugenholtz was an employee of Coca-Cola, Inc when this research was conducted.

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