Food Microbiology 77 (2019) 93–105

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Synthesis of prebiotic galactooligosaccharides from lactose and lactulose by dairy propionibacteria

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ARTICLE INFO

Keywords:
Propionibacteria
β-galactosidase
Transgalactosylation
Oligosaccharides
Prebiotics

ABSTRACT

The potential of probiotic bacteria to produce prebiotic oligosaccharides by transgalactosylation has been minimally studied. In this work, we screened the β-galactosidase (β-gal) activity of dairy propionibacteria (PAB) isolated from Argentinean foods to select strains for the synthesis of oligosaccharides from lactose (GOS) and lactulose (OsLu). PAB, when grown in a medium with lactose as a carbon source, were disrupted, and the cell-free extracts were assayed for β-gal activity. Nine strains grew on lactose and showed β-gal activities from 0.27 to 2.60 U mL^{-1}. *Propionibacterium acidipropionici* LET 120, the strain with the highest activity, was able to synthesize, using 30% lactose and lactulose at pH 6.5 and 45 °C, 26.8% of LET 120-GOS and 26.1% of LET 120-OsLu after 24 h. When they were tested as carbon sources for growth, *P. acidipropionici* LET 120 attained higher biomasses, μ_{max} and β-gal activities at the expense of *Aspergillus oryzae*-OsLu, Vivinal®-GOS and lactulose compared to lactose or glucose. In addition, LET 120-GOS and LET 120-OsLu synthesized by PAB were prebiotic for some probiotic strains. For the first time, our results show the production of GOS and OsLu by dairy PAB, and these results encourage further studies on the optimization of the synthesis and structure characterization of the obtained oligosaccharides.

1. Introduction

In the last several decades, functional foods, i.e., those that supply health benefits beyond basic nutrition, have received great attention from the food industry due to the high demand of healthy products by consumers worldwide. In this respect, probiotic microorganisms and prebiotic compounds are the functional ingredients most widely studied and the only two recognized by Argentinean legislation and the National Food Code of the country (Código Alimentario Argentino, 1969, www.anmat.gov.ar).

Probiotics are live microorganisms that when administered in adequate amounts improve host health through different mechanisms (Hill et al., 2014). One of the first beneficial effects ascribed to probiotics is their ability to contribute to lactose metabolism in the gut and the alleviation of intolerance symptoms. This effect is mediated by the β-galactosidase (β-gal) enzyme (EC 3.2.1.23) of the microorganisms that are included in the probiotic product and which hydrolyzes lactose in food before consumption and *in situ* in the intestine (Vonk et al., 2012). However, β-gal have more biotechnological applications, and depending on the reaction conditions (substrate concentration, a_w, pH, temperature, time), the enzyme may display hydrolase or transferase activities. In particular and due to transferase activity, there is a production of prebiotic oligosaccharides by the transgalactosylation of galactose moieties to a carbohydrate acceptor instead of water (Moreno et al., 2014; Vera et al., 2016; Zárate et al., 2013). Prebiotics are defined as "non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of desirable bacteria in the body" (Gibson et al., 2017). Among the prebiotics, fructans (inulin and fructooligosaccharides), galactooligosaccharides (GOS) and lactulose are recognized as useful for the modulation of colonic microbiota toward a healthy balance, which usually involves the increase of bifidobacteria and lactobacilli at the expense of less desirable organisms such as Clostridia, Bacteroides and Enterobacteria (Cardelle-Cobas et al., 2009a; Roberfroid et al., 2010).

As mentioned, some prebiotics, such as GOS, lactulose and its derived oligosaccharides (OsLu), can be obtained enzymatically by using microbial β-gal (Cardelle Cobas et al., 2008a, 2008b; Guerrero et al.,

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https://doi.org/10.1016/j.fm.2018.08.014
Received 9 March 2018; Received in revised form 28 July 2018; Accepted 27 August 2018
Available online 27 August 2018
0740-0020/

2011; Martínez Villaluenga et al., 2008a, 2008b). However, depending on the origin of β-gal and reaction conditions, the amount of GOS obtained, composition of monomers and linkages between D-galactose units may vary, affecting their properties and prebiotic potential. Previous studies have shown the synthesis, structural characterization and prebiotic effect of GOS and OsLu produced by fungal β-gal from *Aspergillus oryzae* (Cardelle-Cobas et al., 2016; Urrutia et al., 2013; Vera et al., 2012), *A. aculeatus* and *Kluyveromyces lactis* (Cardelle-Cobas et al., 2009b, 2008a; 2008b; Hernández-Hernández et al., 2011; Martínez-Villaluenga et al., 2008b). In the same manner, GOS synthesized by bacteria such as *Bacillus circulans* (Corzo Martínez et al., 2013; Yin et al., 2017), *Lactobacillus reuteri* (Splechtna et al., 2006), *L. plantarum* (Benavente et at., 2015; Iqbal et al., 2010) and *Bifidobacterium* species (Hsu et al., 2007; Osman et al., 2010) have been reported.

Dairy propionibacteria (PAB) are microorganisms that are traditionally used by industry for the manufacture of Swiss-type cheeses and the biologic production of propionic acid. However, research on their potential application to the production of relevant biomolecules, such as vitamins, conjugated linoleic acid (CLA), exopolysaccharides (EPS), trehalose, and bacteriocins, has significantly increased in recent years. In addition, several probiotic effects have been reported for dairy PAB, which could be due to their ability to modulate gut physiology, microbiota composition and host immunity in a beneficial manner (Rabah et al., 2017; Zárate and Pérez Chaia, 2015). As result, *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* have been included in the list of agents recommended for the Qualified Presumption of Safety (QPS) of the European Food Safety Authority (EFSA, 2013).

Regarding lactose metabolism, both *P. freudenreichii* and *P. acidipropionici* hydrolyze lactose by β-gal activity since no phospho-β-gal was detected (Hartley and Vedamuthu, 1975; Zárate et al., 2003). The main biochemical characteristics of the enzyme and its regulation in the presence of lactose and lactate were determined to decide on an appropriate vehicle to deliver PAB to the host (Zárate and Pérez Chaia, 2012; Zárate et al., 2003). Since β-gal of *P. acidipropionici* was able to resist both the manufacture of a Swiss-type cheese and gastrointestinal conditions, it could contribute to lactose digestion included in a probiotic product (Pérez Chaia and Zárate, 2005; Zárate et al., 2000). Mice fed with *P. acidipropionici* CRL 1198 that was included in a milk or cheese, showed increased levels of β-gal in the small bowel and high propionic acid concentrations in the caecum that could favor the recovery of water and electrolytes involved in osmotic diarrhea induced by unabsorbed lactose (Pérez Chaia and Zárate, 2005). Although β-gal from dairy PAB has shown properties for probiotic purposes, its transglycosidase activity for prebiotic synthesis was not investigated. In the present study, we assessed the ability of PAB isolated from Argentinian dairy products to produce GOS and OsLu and their potential prebiotic activity.

2. Materials and methods

2.1. Chemical and reagents

All chemicals and reagents used were of analytical grade (glucose, galactose, fructose, lactose, lactulose, raffinose, and stachyose used as standards and enzyme substrates) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile of high-performance liquid chromatography (HPLC) grade was purchased from Merck (Darmstadt, Germany). The commercial preparation Lactozym®Pure 6500L (Lactozym) was a gift from Novozymes (Dittingen, Switzerland) and was used as enzyme control. Vivinal®-GOS syrup (Friesland Campina Domo, The Netherlands) was kindly provided by Dr. A. Illanes (EIB, PUCV, Chile) and it contained 75% dry matter (DM), and a carbohydrate composition of 59% of GOS, 19% of lactose, 21% of glucose and 1.4% of galactose. OsLu were synthesized using a commercial lactulose preparation (670 g L^{-1}; Duphalac®, Abbott Biologicals B.V., Olst, The Netherlands) and β-gal from *Aspergillus oryzae* (16 U mL^{-1}; Sigma, St. Louis, MO) according to the protocol described in Algieri et al. (2014). The analysis of OsLu showed a DM of 81% and a carbohydrate composition of 14% of galactose, 4% lactose, 24% lactulose, and 58% OsLu (23% disaccharides, 28% trisaccharides and 8% tetrasaccharides (López-Sanz et al., 2015). MRS culture medium for microbial growth was purchased from Pronadisa (Madrid, Spain) and Britania (Buenos Aires, Argentina).

2.2. Microorganisms and culture conditions

Seventeen strains belonging to *P. freudenreichii, P. jensenii* and *P. acidipropionici* isolated in CERELA-CONICET from raw milk and Swiss-type cheeses made in Argentina were used in this study. *P. freudenreichii* subsp. *shermanii* ATCC 13673 and *P. acidipropionici* ATCC 25562 from American Type Culture Collection were used as a reference (Table 1). *P. acidipropionici* LET 120 was molecularly identified by sequencing the 16S rDNA as described by Lorenzo Pisarello et al. (2010). The obtained sequence was submitted to the European Nucleotide Archive database (Accession number PRJEB25502). *Bifidobacterium animalis* subsp. *lactis* BB-12®, *Lactobacillus casei* CRL 431® (from Chr.Hansen) and *Escherichia coli* C3 (from the Institute of Microbiology "Luis Verna" of the University of Tucumán) were used to test the prebiotic activity of oligosaccharide mixtures. The strains were stored at −20 °C in 10% (w/v) reconstituted skim milk (RSM) containing 5 g L^{-1} of yeast extract and 10% glycerol and were activated by three successive transfers every 24 h in MRS broth or LB broth for *E. coli*.

2.3. Assessment of the β-galactosidase activity of dairy propionibacteria (PAB)

The β-gal activity of PAB was determined in cell-free extracts (CFE)

Table 1
Growth parameters and β-gal production by dairy PAB developed after 24 h of incubation at 37 °C on MRS containing lactose as carbon source. Data are the means of two independent assays (± SD). The r^2 for fit of data to sigmoid curves ranged from 0.985 to 0.998 for all the strains.

Strain	Maximum CFU mL^{-1}	Growth rate (μ; h^{-1})	Final pH	Activity (U mL^{-1})	Specific activity (U mg^{-1})
*P**. *acidipropionici* LET 113	2.50 x $10^{9a,b}$ (0.11)	$0.09^{a,b}$ (0.01)	$4.80^{a,b}$ (0.04)	$0.680^{a,b}$ (0.131)	$0.283^{a,b}$ (0.054)
P. acidipropionici LET 116	2.26 x $10^{9a,b,c}$ (0.05)	$0.08^{a,b,c}$ (0.00)	$4.85^{a,b}$ (0.04)	0.270^{b} (0.055)	0.085^{b} (0.017)
P. acidipropionici LET 117	2.16 x $10^{9a,b,c,d}$ (0.01)	$0.09^{a,b}$ (0.00)	$4.81^{a,b}$ (0.06)	$0.380^{a,b}$ (0.067)	0.081^{b} (0.014)
P. acidipropionici LET 119	2.29 x $10^{9a,b,c}$ (0.04)	$0.09^{a,b,c}$ (0.01)	$4.80^{a,b}$ (0.08)	$0.720^{a,b}$ (0.195)	0.104^{b} (0.028)
P. acidipropionici LET 120	3.01 x 10^{9a} (0.08)	0.10^{a} (0.01)	4.75^{b} (0.04)	2.593^{c} (0.156)	2.553^{c} (0.153)
P. acidipropionici ATCC 25562**	2.06 × $10^{9b,c,d}$ (0.20)	$0.08^{a,b,c}$ (0.01)	$4.80^{a,b}$ (0.03)	1.950^{d} (0.077)	0.641^{e} (0.025)
P. freudenreichii LET 114	1.41 x $10^{9c,d,e}$ (0.04)	$0.07^{b,c}$ (0.01)	$4.95^{a,b}$ (0.10)	$0.670^{a,b}$ (0.220)	$0.214^{a,b}$ (0.070)
P. freudenreichii LET 125	1.31 × $10^{9d,e}$ (0.22)	$0.06^{c,d}$ (0.01)	$4.90^{a,b}$ (0.11)	$0.676^{a,b}$ (0.133)	$0.248^{a,b}$ (0.049)
P. freudenreichii LET126	1.08 x 10^{9e} (0.06)	$0.07^{b,c}$ (0.01)	$5.01^{a,b}$ (0.11)	$0.627^{a,b}$ (0.064)	$0.307^{a,b}$ (0.031)
P. freudenreichii ATCC 13673**	9.63 x 10^{8e} (1.11)	$0.06^{c,d}$ (0.01)	$4.95^{a,b}$ (0.01)	0.835^{a} (0.126)	$0.396^{a,e}$ (0.060)
P. jensenii LET128	8.85 x 10^{8e} (0.65)	0.04^{d} (0.00)	5.05^{a} (0.04)	$0.624^{a,b}$ (0.051)	0.915^{d} (0.075)

* Propionibacterium. [a, b, c, d, e] Statistically significant differences between groups. **References strains.

obtained by mechanical disruption. Active cultures of PAB (5 mL) were harvested by centrifugation (10,000 × g, 15 min, 4 °C), washed and suspended to the original volume in 0.9% NaCl and incubated at 37 °C for 2 h to deplete intracellular reserves. These suspensions were inoculated at a 2% (v/v) rate in a basic MRS fermentation broth without glucose and meat extract (Pronadisa), enriched with 0.2% Tween 80, 0.8% casein acid hydrolysate and 0.05% L-cysteine, and supplemented with 0.5% lactose as carbon and energy source to induce β-gal synthesis. After 24 h of incubation at 37 °C, the bacterial cells were harvested by centrifugation (10,000 × g, 15 min, 4 °C), washed and resuspended in 500 μL of 50 mM sodium phosphate buffer, pH 6.5, and mixed (1:1, w/v) with sterile glass beads (diameter, 150–212 mm; Sigma-Aldrich, St. Louis, MO, USA). The cells were disrupted in FastPrep equipment (Bio101; Savant Instruments, Holbrook, NY, USA) through physical beating, and then, the insoluble fraction and glass beads were removed by centrifugation (12,000 × g, 10 min, 4 °C). The supernatant fraction (CFE) containing β-gal for all of the assays was stored at −80 °C until ready for use. The protein contents of CFE were determined by using a Bradford Bio-Rad protein kit (Bio-Rad, Munich, Germany) and bovine serum albumin for preparation of a standard curve. The β-gal activity was determined by measuring the hydrolysis rate of the synthetic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma-Aldrich) with a colorimetric method (Hartley and Vedamuthu, 1975). The enzymatic reaction was conducted in 96-well microplates by mixing 10 μL of CFE with 200 μL of 1 mg mL^{-1} ONPG (3.2 mM final concentration) and dissolved in 50 mM sodium phosphate buffer at pH 6.5 containing 1 mM $MgCl_2$. The mixtures were incubated for 10 min at 40 °C, and the amount of released *o*-nitrophenol (ONP) was monitored continuously by measuring absorbance at 420 nm in an automated microplate spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). Enzymatic activities were expressed as U mL^{-1}, where one unit of β-gal was defined as the amount of enzyme that released 1 μmol of ONP per minute under defined assay conditions. The specific activity was calculated based on the mg of protein present in the CFE.

2.4. Enzymatic synthesis of oligosaccharides from lactose (GOS) and lactulose (OsLu)

The capacity of PAB β-gal to synthesize oligosaccharides was assessed by incubating CFE of *P. acidipropionici* LET 120 and Lactozym with solutions of lactose and lactulose. The reactions were performed in a final volume of 1.0 mL in microtubes by mixing the enzyme and substrates at the following conditions: pH 6.5, 45 °C, 300 g L^{-1} final concentration of lactose or lactulose, and 1.3 U mL^{-1} of enzyme. Aliquots (200 μL) were withdrawn at specific time intervals (1, 3, 5, 7 and 24 h) and immediately immersed in boiling water for 2 min to inactivate the enzyme. The samples were stored at −80 °C for subsequent analysis. All of the reactions were conducted in duplicate, and the corresponding analytical measurements were completed twice for each enzymatic synthesis condition.

2.5. Growth of P. acidipropionici LET 120 on lactose, lactulose and their derived oligosaccharides

P. acidipropionici LET 120 cultured 24 h at 37 °C in MRS was harvested by centrifugation (10,000 × g for 10 min at 4 °C), washed twice and resuspended in sterile saline solution (0.85%). These suspensions were inoculated (2%) in basic MRS broth supplemented with the tested carbohydrates (lactose, lactulose, Vivinal-GOS and *A. oryzae*-OsLu) at a final concentration of 1% and then pipetted in triplicate into 300-μL wells of sterile 96-well microplates with a lid (Sarstedt Inc., Newton, USA). The glucose and the medium without any carbon source were used as growth controls. The plates were incubated at 37 °C for 48 h in an automated microplate reader (Varioskan Flash), and the optical densities at 600 nm (OD_{600}) were recorded at 60 min intervals. The maximum growth rates (μ_{max}) of *P. acidipropionici* LET 120 were calculated by fitting the curves to a sigmoid model using Microcal Origin 6.0 (OriginLab Corporation, Northampton, MA, USA). The samples were taken at 0, 24 and 48 h for pH, bacterial enumeration and short chain fatty acids (SCFA) determinations. Colony-forming units (CFU) were counted after plating tenfold diluted samples on MRS agar incubated for 120 h at 37 °C in anaerobiosis (Gas-Pack, Anaerogen; Oxoid Ltd., Hampshire, England).

2.6. Determination of the prebiotic capacity of GOS and OsLu synthesized by PAB on probiotic cultures

Recognized strains, such as *B. animalis* subsp. *lactis* BB-12 and *L. casei* CRL 431, were selected for the evaluation of the prebiotic capacity of GOS and OsLu synthesized by *P. acidipropionici* LET 120 from lactose and lactulose, since these microorganisms are representative of the intestinal microbiota and proven probiotics (Aragon et al., 2014; Jungersen et al., 2014). PAB-oligosaccharides were partially purified by selective adsorption onto activated charcoal followed by yeast fermentation to decrease the monosaccharide and lactose contents (Hernandez et al., 2009). In brief, 1 mL of PAB-oligosaccharides formed after 24 h reactions (carried out as described in Section 2.4), and 3 g of activated charcoal (100–400 mesh, Sigma–Aldrich), was mixed in 100 mL of 10% ethanol and stirred for 30 min. The mixtures were filtered through Whatman No. 1 paper under a vacuum and washed with 25 mL of the same ethanolic solution. Desorption of the oligosaccharides from the activated charcoal was conducted by stirring with 100 mL of 50% ethanol for 30 min and further filtration (as before). The filtrates were evaporated under a vacuum at 40 °C, dissolved in 1 mL of deionized water and mixed with 1 mL of *Saccharomyces cerevisiae* (Levex®, Lesaffre, Argentina) in PBS (≅ 10^9 CFU mL^{-1}). Fermentation was conducted with agitation at 37 °C for 24 h, and the mixtures were finally filtered through 0.22 μm filters (Millipore Corp.) to remove the yeasts before using them as carbon sources in the assays. Chromatography analysis (which was performed as described in Section 2.7.1) showed that the procedure removed 100% of the monosaccharides and ≥95% of the disaccharides. Probiotic strains that were grown for 24 h in MRS with 0.05% L-cysteine hydrochloride (MRS-cys) were washed with 0.9% NaCl and inoculated (initial OD_{600} ≅ 0.05) in basic MRS-cys broth supplemented with Vivinal-GOS, *A. oryzae*-OsLu, LET 120-GOS or LET 120-OsLu at 1% and as the sole carbohydrate source. *P. acidipropionici* LET 120 and *E. coli* C3 were tested for comparison. Probiotic cultures and PAB were incubated at 37 °C for 24 h under anaerobiosis, while *E. coli* was incubated in aerobiosis. The cell growth was determined based on the increase in bacterial biomass measured by turbidimetry at 600 nm and the change in pH of the culture media. The prebiotic activity score (PAS) was calculated according to the following equation of Huebner et al. (2007).

$$PAS = \frac{PP_{24} - PP_0}{PG_{24} - PG_0} - \frac{EP_{24} - EP_0}{EG_{24} - EG_0}$$

where PP_{24} and PP_0 are the probiotic biomass (OD_{600}) after 24 h and 0 h of fermentation of selected prebiotics, respectively. PG_{24} and PG_0 are the probiotic OD_{600} on glucose after 24 h and 0 h fermentation, respectively. EP_{24}/EP_0 and EG24/EG0 are the *E. coli* biomass on prebiotics and glucose after 24 h and 0 h fermentation, respectively. Each assay was replicated twice.

2.7. Analysis of GOS and OsLu

2.7.1. HPLC with refractive index detection (HPLC-RID)

The samples from the enzymatic synthesis were diluted with acetonitrile/water (50:50 v/v) at a total carbohydrate concentration of ~10 mg mL^{-1}, filtered onto 0.2 mm pore size PVDF membranes (Millipore, Massachusetts, USA) and analyzed in an HPLC system (equipped with Smartlinepump 100, refractive index detector K-2301 and smart line autosampler AS 3800, Knauer, Germany). The separation

of carbohydrates was conducted on a Kromasil® column (100-NH_2; 250 mm × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY, USA) using acetonitrile/water (70:30 v/v) as the mobile phase and eluted in the isocratic mode at a flow rate of 1 mL min^{-1} for 30 min. The injection volume was 20 μL (~800 μg of total carbohydrates). Data acquisition and processing were performed using EuroChrom for Windows Basic Edition v.3.05 software. The carbohydrates in the reaction mixtures were initially identified by comparing their retention times (t_R) with those of standard sugars. Quantitative analysis was performed by the external standard method using calibration curves of each pure standard (including galactose and fructose for monosaccharides, lactose and lactulose for disaccharides, raffinose for trisaccharides and stachyose for tetrasaccharides quantification) in the range 0.05–5 mg mL^{-1}. All of the analyses were performed in duplicate, and relative standard deviation (RSD) values below 10% were obtained in all cases. The amounts of different carbohydrates present in the reaction mixtures were expressed as the percentage of the total carbohydrate content (w/w).

2.7.2. Gas chromatography with flame ionization detector GC-FID

The composition of the GOS and OsLu mixtures synthesized by *P. acidipropionici* LET 120 and Lactozym from lactose and lactulose was determined by GC-FID. The carbohydrates were analyzed as trimethyl silylated oximes (TMSO). First, 15 μL of the reaction mixture (4.5 mg of sugars) was added to 0.4 mL of the internal standard (I.S.) solution (0.5 mg mL^{-1} phenyl-β-glucoside). Afterwards, the mixture was dried at 38–40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The sugar oximes were formed by adding 250 μL of hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min and then silylating with hexamethyldisilazane (250 μL) and TFA (25 μL) and kept at 50 °C for 30 min. The reaction mixtures were centrifuged at 10,000 rpm for 2 min at room temperature. The supernatants were injected or stored at 4 °C prior to analysis.

Gas chromatography (GC) analysis was performed on an Agilent Technologies 7890A gas chromatograph (Wilmington, DE, USA) equipped with a commercial fused silica capillary column DB-5HT, bonded, crosslinked phase (30 m × 0.25 mm i.d. and 0.25 μm film thickness) (J&W Scientific, Folson, California, USA). The oven temperature was initially 180 °C increased at a rate of 3 °C min^{-1} to 350 °C and then held for 25 min. The injector and detector temperatures were set at 280 and 355 °C, respectively. The injections were conducted in split mode (1:30) using nitrogen at 1 mL min^{-1} as the carrier gas. Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software. The response factors were calculated relative to the I.S. using solutions containing galactose, glucose, lactose, raffinose and stachyose and were prepared over the expected concentration range in the samples. The identities of the carbohydrates were confirmed by comparison with relative retention times of standard samples. Bimuno® (Clasado Ltd., Reading United Kingdom), with 65% GOS formed mainly by β-(1 → 3), as well as β-(1 → 4) and β-(1 → 6) (Gibson et al., 2011); and 4′-galactosyl-lactose previously synthesized and identified in our laboratory (Cardelle-Cobas, 2009), were used as standards to tentatively identify GOS synthetized by PAB β-gal. All of the analyses were conducted in duplicate, and the data were expressed as (% total carbohydrates w/w) mean ± standard deviation (SD).

2.8. Analysis of short chain fatty acids (SCFA)

Determination of SCFA originated during the growth of *P. acidipropionici* LET 120 on carbohydrates (lactose, lactulose, Vivinal-GOS and *A. oryzae*-OsLu) was conducted as previously described (Zárate et al., 2017). Before analysis, the culture supernatants were deproteinized with 0.01 mol L^{-1} H_2SO_4 (15 min, 4 °C), centrifuged for 10 min (10,000 × g and 4 °C) and filtered using a 0.45 μm syringe filter. The separation of SCFA were conducted in an HPLC system equipped with Smartlinepump 100, refractive index detector K-2301 and smart line autosampler AS 3800 (Knauer, Germany). The elution of SCFA was performed using a Rezex ROA organic acids column (300 × 7.8 mm and 8 μm particle size) (Phenomenex Torrance, USA) thermostatized at 55 °C. The separation was in the isocratic mode, and the mobile phase was 0.01 M H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Quantification of the organic acids was conducted by the external standard method (Snyder et al., 1997) using standard solutions of acetic acid and propionic acid prepared in concentrations ranging from 0.05 to 5.0 mg mL^{-1}. The linear regression curves for each standard were higher than 0.99. The product concentrations were reported as mg mL^{-1} of grown culture medium.

2.9. Statistical analysis

ANOVA tests and Tukey's test for $p < 0.05$ were applied to all of the data generated including ONPG hydrolysis by β-gal enzymes contained in the CFE of dairy PAB, carbohydrates released with β-gal of *P. acidipropionici* LET 120 and Lactozym, the growth parameters of *P. acidipropionici* LET 120, and probiotic strains at the expense of different carbohydrates such as the carbon source. In addition, a Pearson correlation coefficient was calculated for all of the variables. The differences between the cultures and the substrates employed were studied. All of the statistical analyses were performed on R (R Core Team, 2017).

3. Results and discussion

3.1. Assessment of the β-gal activity of dairy PAB

At present, non-digestible oligosaccharides (NDO), such as GOS, have gained great interest for health and industrial applications, since they are well-recognized prebiotics and food additives (Vera et al., 2016; Zárate et al., 2013). In this regard, it is proposed that β-gal from probiotic strains would be more appropriate for the synthesis of selective prebiotic oligosaccharides (Osman et al., 2010; Rabiu et al., 2001). Then, as a first step in the evaluation and selection of PAB with suitable β-gal for GOS synthesis, we screened the growth and enzyme activity of 17 strains of dairy PAB at the expense of lactose as the sole carbon source. Eleven strains, including the references *P. freudenreichii* ATCC 13673 and *P. acidipropionici* ATCC 22562, expressed β-gal and were able to develop on lactose (*P. acidipropionici,* n = 5; *P. freundereichii,* n = 3; and *P. jenseni,* n = 1 strains). As a general result, *P. acidipropionici* strains grew faster, showed statistically significant differences, and attained higher cell counts and lower pH than the *P. freudenreichii* and *P. jensenii* strains (Table 1).

The β-gal activity of microorganisms quantified on CFE by determining their specific activities varied widely between 0.081 and 2.553 U mg^{-1} for *P. acidipropionici* LET 116 and LET 120, respectively. However, there has been less variation observed in specific activities of *P. freudenreichii strains* (0.214–0.396 for LET 114 and ATCC 13673, respectively) (Table 1). The statistical analysis of the parameters reported in Table 1 shows that maximum cell count, growth rate and the final pH were strongly correlated.

Fig. 1 shows three kinetics of ONPG hydrolysis; most of the strains were distributed among a low and intermediate rate of hydrolysis, whereas only *P. acidipropionici* ATCC 25562 and LET 120 displayed high hydrolysis rates. Statistically significant differences were found between *P. acidipropionici* LET 120 activity and the rest of the strains. Similarly, *P. acidipropionici* ATCC 25562 activity was also significantly different from the rest, whereas no statistically significant differences were observed between strains showing an intermediate rate of hydrolysis (*P. freudenreichii* LET114, LET125 and LET126 and *P. jenseni* LET128) and between strains with a low rate of hydrolysis (*P. acidipropionici* LET113, LET116, LET117 and LET119). These data could indicate that the hydrolysis rate of ONPG does not depend on the

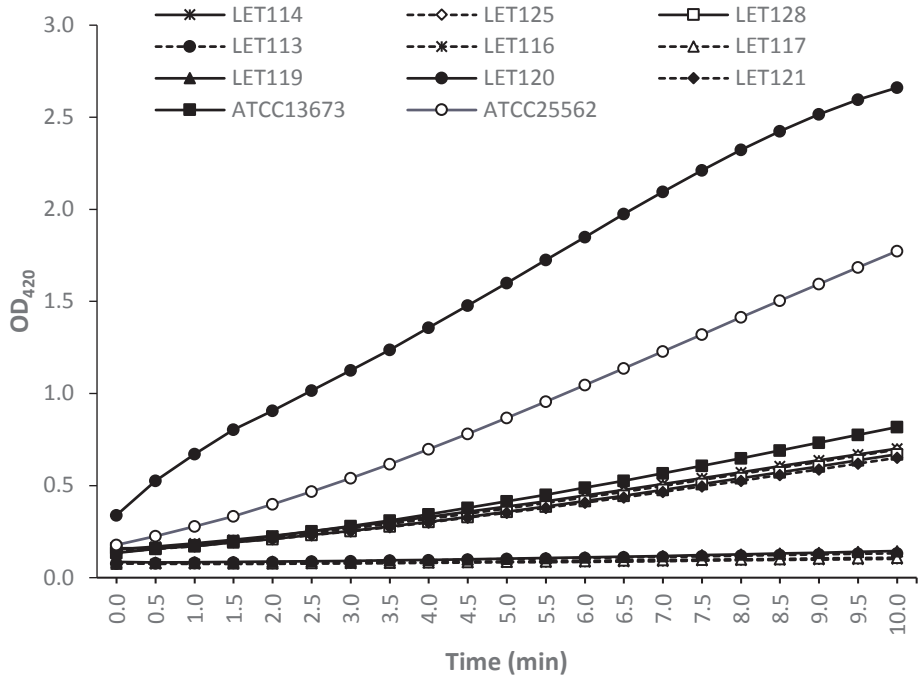

Fig. 1. Monitoring of ONPG hydrolysis (pH 6.5, 40 °C) by the β-galactosidase enzyme contained in the CFE of dairy PAB (codes in Table 1). The data are the means of two determinations of two independent assays (n = 4).

species but on the strain. Since *P. acidipropionici* LET 120 produced a high amount of β-gal with the highest specific activity, it was selected to assess its ability to synthesize GOS and OsLu.

3.2. Synthesis of GOS and OsLu by P. acidipropionici LET 120

Lactose has been mainly used as substrate for the production of GOS (Cardelle-Cobas et al., 2008b, 2009b); however, many studies have proven that other carbohydrates, such as lactulose, are also good substrates for the enzymatic synthesis of prebiotic oligosaccharides using microbial β-gal (Cardelle-Cobas et al., 2008a; Guerrero et al., 2011, 2015; Hernández-Hernández et al., 2011; Martínez Villaluenga et al., 2008b). The CFE of *P. acidipropionici* LET 120 containing the β-gal activity enzyme (2.5 U mL^{-1}) was mixed with lactose or lactulose at a final concentration of 300 g L^{-1} and incubated at 45 °C at a pH 6.5 for 24 h, and samples were taken at intervals for carbohydrate analysis. For a comparison, reactions were also performed with the commercial preparation Lactozym that contains β-gal from *Kluyveromyces lactis*. Figs. 2 and 3 show the time course of β-gal-catalyzed reactions with lactose and lactulose as the substrate, respectively. The oligosaccharides in the reaction mixtures were quantified by HPLC-RID. The reactions with both disaccharides produced, besides hydrolysis into their glucose and galactose monosaccharides (Fig. 2b), the synthesis of GOS by transgalactosylation. As can be observed in Fig. 2a, a similar behavior towards lactose was observed with both enzymes, although the purified enzyme of *K. lactis* was more efficient than the unpurified extract containing β-gal of *P. acidipropionici* LET 120, since it degraded more lactose and released a higher amount of products. The lactose concentration decreased rapidly during the first hours of the reaction, which resulted in GOS (di-, tri- and tetrasaccharides) in concentrations that increased with the progress of the reaction up to 24 h (Fig. 2a). However, the β-gal of *P. acidipropionici* LET 120 produced in the first hour of the reaction almost the maximum levels of GOS, which increased slightly in the following hours. In the optimal conditions using Lactozym, lactose hydrolysis, the release of monosaccharides and GOS formation were significantly higher. Maximum GOS formation by β-gal of *P. acidipropionici* LET 120 was achieved after 24 h of reaction and accounted for 23.6% (w/w) of total sugars, whereas only 5 h of reaction was needed to reach a maximum GOS formation of 25.0% (w/w) of the total sugars with Lactozym. Other studies on the transgalactosylation of the lactose solutions by β-gal from other bacteria, such as *L. reuteri* and *Bifidobacterium* species using different reaction conditions, reported yields of GOS ranging from 26.8 to 47.6% (Rabiu et al., 2001; Splechtna et al., 2006; Hsu et al., 2007; Iqbal et al., 2010).

Lactulose was also hydrolyzed by both β-gal into galactose and fructose (Fig. 3b) and transgalactosylated into OsLu (di- and tri-saccharides). Similar curves of lactulose degradation and production of OsLu were observed for *P. acidipropionici* LET 120 β-gal and Lactozym (Fig. 3a). The maximum production of LET-120 OsLu was achieved after 5 h of reaction and corresponded to 27.0% of the total carbohydrates, whereas a maximum OsLu of 30.5% was obtained with Lactozym at 24 h, and no significant differences were found. However, in OsLu synthesis reactions, lactulose hydrolysis was significantly lower with *P. acidipropionici* LET 120. Although lactose hydrolysis was significantly higher than that of lactulose, a significantly higher amount of oligosaccharides was obtained when lactulose was used as the substrate.

To better understand the oligosaccharides formed in enzymatic hydrolysates of lactose and lactulose using β-gal from *P. acidipropionici* LET 120 and Lactozym, GC-FID analyses of the reaction mixtures at optimal conditions for GOS and OsLu formation were performed. Additionally, a comparison with the GC-FID profile of oligosaccharides found in hydrolysates using β-gal of *K. lactis* was conducted. Thus, different profiles were obtained with both β-gal and a good separation of mono-, di-, tri- and tetrasaccharides (Fig. 4a). The compounds formed during lactose reactions with β-gal from *P. acidipropionici* LET 120 were compared with GOS synthesized by our research group in previous studies (Martínez Villaluenga et al., 2008a; Cardelle-Cobas, 2009) and Bimuno, which is a commercial GOS product. Thus, the disaccharides present in the reaction mixtures were identified as allolactose (β-D-Gal*p*-(1 → 6)-D-Glu; peak 7) and 6-galactobiose (β-D-Gal*p*-(1 → 6)-D-Gal; peak 9). The main trisaccharide (peak 11) was coincident with that previously reported by Martínez-Villaluenga et al. (2008b) as 6′-galactosyl-lactose (*β*-D-Gal*p*-(1 → 6)-lactose). Other trisaccharides were also detected, but only two of them were identified as 3′-galactosyl-lactose (*β*-D-Gal*p*-(1 → 3)-lactose) (peak 12), which is the

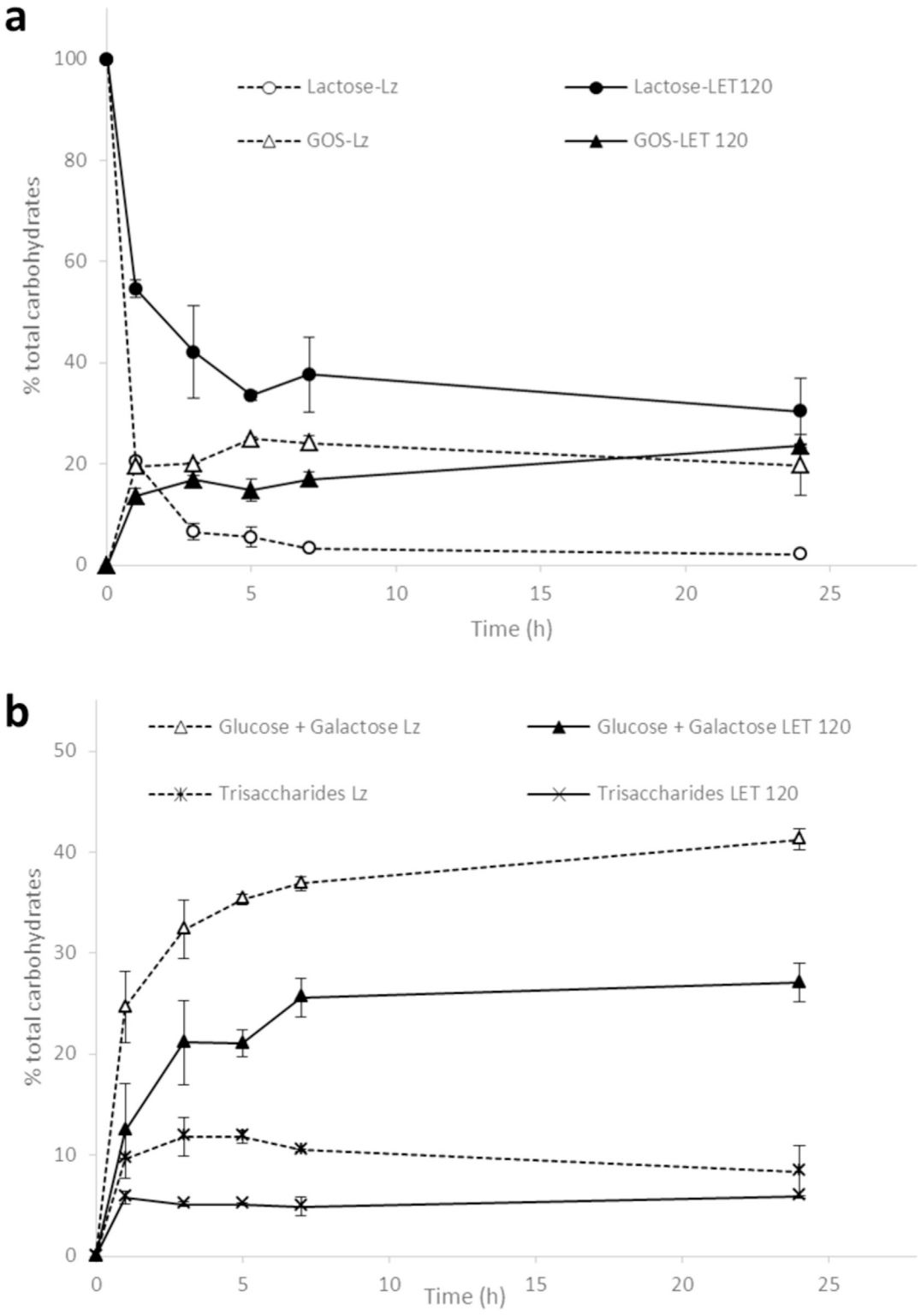

Fig. 2. Time course of the reactions catalyzed with the β-gal of *P. acidipropionici* LET 120 (LET 120) and Lactozym Pure 6500L (Lz) at 45 °C, pH 6.5 and 300 g L^{-1} of lactose as substrate: a) lactose and galactooligosaccharides (GOS) b) mono- and trisaccharides. Carbohydrates in the reaction mixtures were quantified by HPLC-RID.

most abundant trisaccharide found in commercial GOS-Bimuno, and 4′-galactosyl-lactose (peak 10) (Cardelle-Cobas, 2009). Additionally, tetrasaccharides were detected, but none of them could be identified.

Oligosaccharides formed during the lactulose reaction with β-gal of *P. acidipropionici* LET 120 were also studied. As is shown in Fig. 4b, di-, tri- and tetrasaccharides were detected in the GC profile. By comparison with the OsLu synthesized by our research group in previous studies (Martínez Villaluenga et al., 2008b), disaccharides, such as allolactulose (*β*-D-Gal*p*-(1 → 6)-D-Fru) (peaks 8) and 6-galactobiose (peaks 13), could be identified as well as the trisaccharide 6′-galactosyl-lactulose (*β*-D-Gal*p*-(1 → 6)-lactulose). Other trisaccharides as well as tetrasaccharides were also detected, but they could not be identified.

Oligosaccharide quantification in enzymatic mixtures using β-gal from *P. acidipropionici* LET 120 and Lactozym was also conducted with

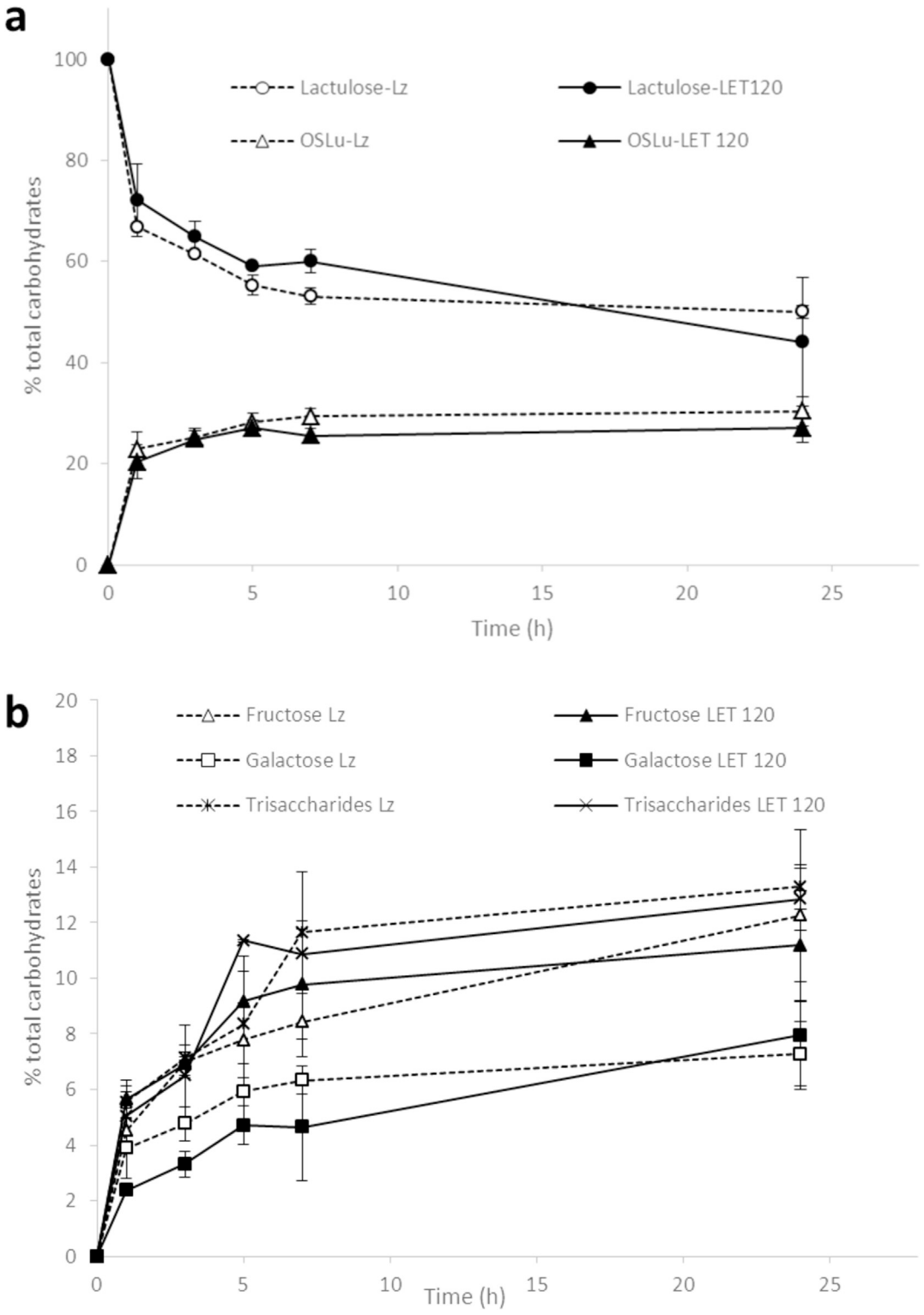

Fig. 3. Time course of the reactions catalyzed with the β-gal of *P. acidipropionici* LET 120 (LET 120) and Lactozym Pure 6500L (Lz) at 45 °C, pH 6.5 and 300 g L^{-1} of lactulose as substrate: a) lactulose and oligosaccharides derived from lactulose (OsLu) b) mono- and trisaccharides. Carbohydrates in the reaction mixtures were quantified by HPLC-RID.

CG-FID. Table 2 shows the maximum value attained for oligosaccharides (di-, tri- and tetrasaccharides) in the two studied hydrolysates containing lactose and lactulose. In hydrolysates with lactose, the main disaccharide formed was allolactose followed by 6-galactobiose. In the case of hydrolysate from β-gal of *P. acidipropionici* LET 120, three trisaccharides were quantified: 6′-galactosyl-lactose, 3′-galactosyl-lactose and 4′-galactosyl-lactose. However, 3′-galactosyl-lactose was not detected in hydrolysates from Lactozym. Unknown di-, tri- and tetrasaccharides were also quantified, and all of them were included in the GOS values (Table 2).

In hydrolysates from lactulose, quantification was also performed by GC-FID (Table 2). The main disaccharide formed was allolactulose

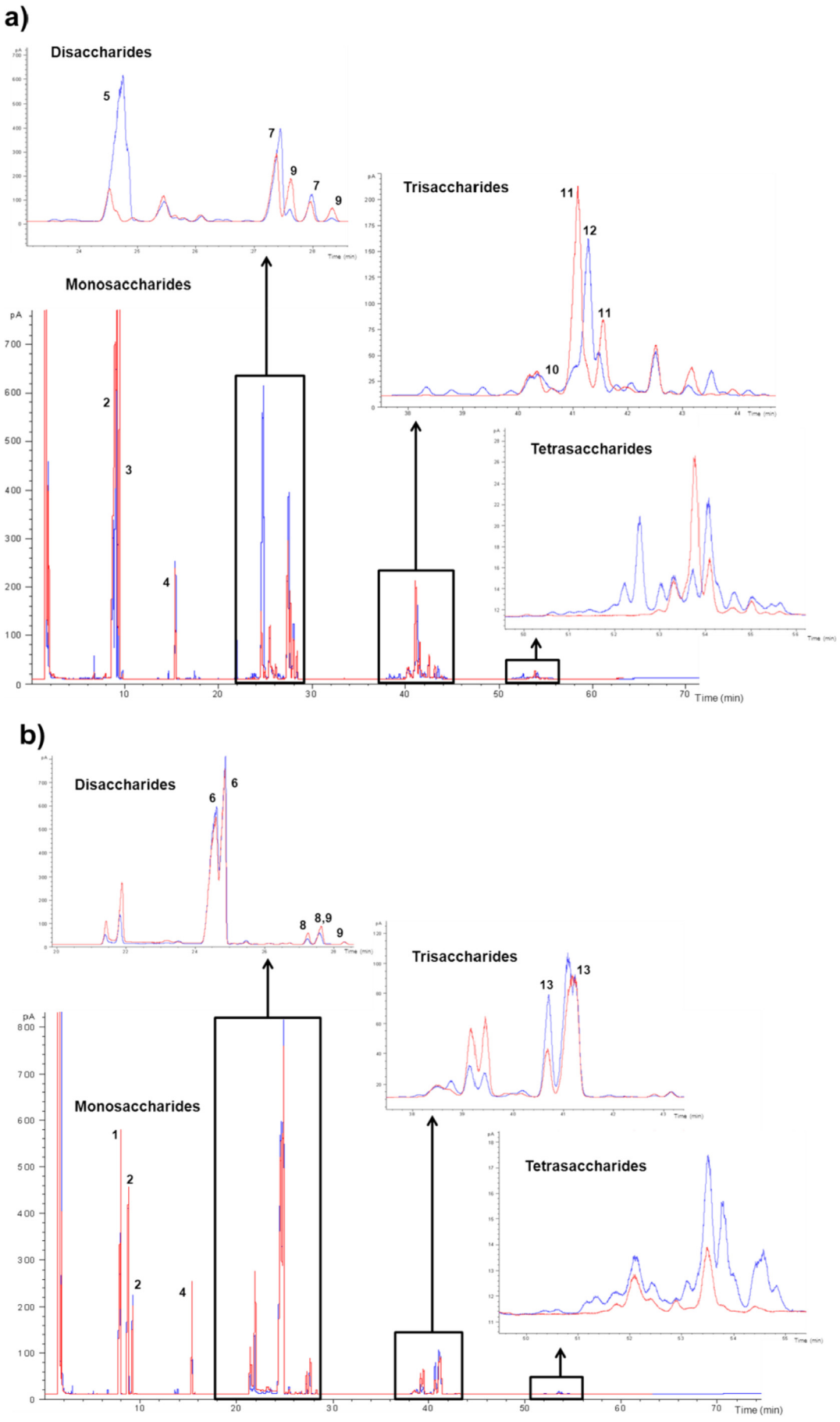

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Fig. 4. Gas chromatographic (GC-FID) profile of the carbohydrates present in the reaction mixtures of β-galactosidase (1.3 U mL^{-1}) from *P. acidipropionici* LET 120 (blue line) and Lactozym (red line) with 300 g L^{-1} of (a) lactose or (b) lactulose performed at 45 °C, pH 6.5 during 7 h. 1: fructose, 2: galactose, 3: glucose, 4: internal standard, 5: lactose, 6: lactulose, 7: allolactose, 8: allolactulose, 9: 6-galactobiose, 10: 4′-galactosyl lactose, 11: 6′-galactosyl lactose, 12: 3′-galactosyl lactose, 13: 6′-galactosyl lactulose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

followed by 6-galactobiose. Only a trisaccharide, 6′-galactosyl-lactulose, was quantified in the two hydrolysates. Unknown di-, tri- and tetrasaccharides were also quantified, and all of them were included in the OsLu values (Table 2). It has been previously reported that β-gal from potential probiotic genera, such as *Lactobacillus*, mainly produce β-(1 → 6) linkages in their transgalactosylation mode, whereas β-(1 → 3) linked products are formed to a lesser extent (Splechtna et al., 2006; Iqbal et al., 2010). Depeint et al. (2008) found that GOS mainly formed with β-(1 → 3) linkages using β-gal of *B. bifidum* NCIMB 41171. Accordingly, it has been demonstrated that linkages β-(1 → 6) are preferred and cleaved faster than other linkages by β-gal from bifidobacteria (Cardelle Cobas et al., 2011); thus, GOS produced by *P. acidipropionici* LET 120 could exhibit a good prebiotic potential.

3.3. Growth of P. acidipropionici LET 120 on lactose, lactulose and their derived oligosaccharides

Prebiotic GOS are mainly targeted to stimulate the development of Bifidobacteria in the colon. However, they could also beneficially influence the growth of potential probiotic strains of other genera such as *Lactobacillus* and *Streptococcus* (Cardelle-Cobas et al., 2011). We first assessed the ability of *P. acidipropionici* LET 120 to grow at the expense of recognized prebiotics such as Vivinal-GOS, lactulose and *A. oryzae*-OsLu. Fig. 5 shows growth curves of the *P. acidipropionici* LET 120 strain inoculated on the different assayed carbon sources. Statistically significant differences were found between the maximum absorbance reached in all of the studied growth curves with the exception of lactulose and *A. oryzae*-OsLu, where their maximum absorbance values were not significantly different.

Some relevant parameters related to growth are presented in Table 3. No growth was observed in the control culture without a carbohydrate source. In contrast, *P. acidipropionici* LET 120 was able to develop in the five substrates tested and reached a maximum OD_{600} of 1.1 at 13 h of growth and the highest μ_{max} (0.14 h^{-1}) with *A. oryzae*-OsLu as the carbon source. Similar lag times, growth rates and maximum OD_{600} were observed on *A. oryzae*-OsLu and lactulose. When *P. acidipropionici* LET 120 grew at the expense of Vivinal-GOS, a slightly lower μ_{max} (0.10 h^{-1}) and cell density were achieved (OD_{600} 0.94). Finally, lactose and glucose resulted in lower growth rates and biomasses and reached a maximum OD_{600} of 0.78 and 0.62 for each substrate, respectively, with statistically significant differences with respect to *A. oryzae*-OsLu and lactulose. The lowest μ_{max} but longer growth was obtained at the expense of glucose, although the lag time was similar to the observed with the other carbohydrates.

SCFA production was related to the substrate preference and growth on each carbon source as it was much higher in cultures grown on *A. oryzae*-OsLu, Vivinal-GOS and lactulose than in glucose or lactose. Only fermentation of oligosaccharides maintained the molar ratio of 2:1 between propionic and acetic acids that is typical of this bacterial genus. Regarding β-gal, *P. acidipropionici* LET 120 displayed activity in the absence and presence of the different carbon sources tested, and this suggests the constitutive nature of the enzyme in this species as previously reported (Zárate et al., 2003). However, the production of this enzyme was induced by the presence of different carbohydrates, including glucose, with the highest significant activity found on *P. acidipropionici* LET 120 grown at the expense of *A. oryzae*-OsLu, followed by lactulose and Vivinal-GOS (Table 3) in accordance with the results of growth and organic acids.

Correlation analysis (determined by the Pearson coefficient) between the set of parameters (Table 3) shows that the maximum OD_{600} was strongly correlated with final pH, growth rate, β-gal activity and acetic acid production. Similarly, the maximum cell count, growth rate, enzymatic activity and SCFA production were strongly correlated.

Many GRAS microorganisms that are intended as probiotics are combined in foods with prebiotics. The probiotic potential of dairy PAB has been widely reported (Zárate and Pérez Chaia, 2015). However, to exert health benefits, they must persist in the gastrointestinal tract either by their ability to adhere to the intestinal mucosa or proliferate in the lumen. Thus, in addition to specific surface adhesins, the presence of appropriate enzymes for metabolizing NDO may confer them a competitive advantage over other members of the gut microbiota, and the possibility of increasing their number. The results showed that *A. oryzae*-OsLu and lactulose exerted on *P. acidipropionici* LET 120 had a higher stimulating growth effect than more easily usable sugars such as glucose or lactose and enhanced the production of SCFA (propionic and acetic acids). Previous studies have reported the relationship between the oligosaccharide chemical structure and its potential bioactivities (Cardelle-Cobas et al., 2011; García Cayuela et al., 2014; Rabiu et al., 2001).). It has been reported that some *Bifidobacterium* strains preferably ferment tri- and tetrasaccharide GOS over disaccharides, lactose and more simple carbohydrates, such as glucose or galactose (Amaretti et al., 2007 Gopal et al., 2001), and show a degree of polymerization-specific strain preferences (Barboza et al., 2009). In addition, potential probiotic strains of *Streptococcus, Lactobacillus* and *Bifidobacterium* were able to utilize lactulose and pure trisaccharides derived from lactulose and lactose with a general preference towards β-galactosyl residues β(1 → 6) and β(1 → 1) linked over β(1 → 4) linked. In addition, some strains achieved higher cell densities and rates of growth on 6′-galactosyl-lactulose than on 6′-galactosyl-lactose (Cardelle-Cobas et al., 2011).

Most commercially available GOS are mixtures containing monosaccharides, disaccharides and trisaccharides with different linkages. In the case of Vivinal-GOS, it contains predominantly β(1→4) trisaccharides (Chockchaisawasdee et al., 2005), whereas OsLu synthesized with β-gal of *A. oryzae* contains high percentages of 6′-galactosyl-lactulose (Hernandez-Hernandez et al., 2011). The results suggest that *P. acidipropionici* LET 120 also prefer β(1 → 6) linked residues present in *A. oryzae*-OsLu over the β(1 → 4) GOS present in Vivinal-GOS syrup, but further studies are needed to confirm this hypothesis.

3.4. Determination of the prebiotic capacity of GOS and OsLu synthesized by PAB in probiotic cultures

For consideration as prebiotic candidates, oligosaccharides synthesized by PAB should be able to stimulate the growth of beneficial microorganisms present in the gut such as bifidobacteria and lactobacilli. Then, the utilization of LET 120-GOS and LET 120-OsLu by recognized probiotic strains, such as *B. animalis* subsp. *lactis* BB-12 and *L. casei* CRL 431, was assessed to determine their prebiotic and symbiotic potential. Fermentations were conducted in batch cultures with glucose, Vivinal-GOS, *A. oryzae*-OsLu, LET 120-GOS or LET 120-OsLu as the sole carbohydrate source, and the growth of the strains was evaluated by recording the culture optical density and pH at 24 h. For a given sugar to have prebiotic activity, it should be better metabolized than glucose by a test strain. Both probiotics were able to grow on GOS and Oslu produced by LET 120 as evidenced by the increase in OD_{600} and the decrease in pH. As shown in Table 4, the increase in cell density of both probiotics grown on all of the tested GOS was higher than glucose.

Table 2
Carbohydrates content (% of total carbohydrates in the sample) determined by GC-FID, found in the reaction mixtures with 300 g L^{-1} of lactose or lactulose incubated at 45 °C and pH 6.5 with the β-gal of *P. acidipropionici* LET120 and Lactozym (β-gal from *K. lactis*) at maximum oligosaccharides formation times.

Source of β-galactosidase	Substrate	Time (h)	Carbohydrate concentration (% total carbohydrates w/w)												
			Fr	Ga	Gl	La	Lu	AloLa	AloLu	GaBio	3′GaLa	4′GaLa	6′GaLa	6′GaLu	GOS*	OsLu**
***P. acidipropionici* LET 120**	lactose	24	–	12.2^{a} (0.3)	28.4^{a} (2.2)	28.7^{a} (1.4)	–	7.1^{a} (1.0)	–	4.2^{a} (0.8)	2.1^{a} (0.0)	1.1^{a} (0.1)	5.4^{a} (0.2)	–	26.8^{a} (1.6)	–
	lactulose	24	14.8^{c} (0.8)	10.6^{c} (0.8)	–	–	46.8^{c} (5.0)	–	1.2^{c} (0.5)	0.6^{c} (0.2)	–	–	–	7.2^{c} (0.1)	–	26.1^{c} (1.3)
Lactozym	lactose	5	–	22.6^{b} (5.5)	28.8^{a} (2.7)	11.0^{b} (1.4)	–	8.0^{a} (0.5)	–	4.5^{a} (0.5)	–	0.9^{a} (0.2)	12.9^{b} (1.4)	–	36.9^{b} (3.4)	–
	lactulose	24	14.2^{c} (1.3)	8.8^{c} (0.6)	–	–	44.8^{c} (0.9)	–	2.7^{d} (0.5)	1.4^{a,d} (0.1)	–	–	–	5.5^{c} (1.7)	–	32.2^{d} (1.0)

Fr, fructose; Ga, galactose; Gl, glucose; La, lactose; Lu, lactulose; AloLa, allolactose; AloLu, allolactulose; GaBio, 6-galactobiose; 3′GaLa, 3′galactosyl-lactose; 4′GaLa, 4′galactosyl-lactose; 6′GaLa, 6′galactosyl-lactose; 6′GaLu, 6′galactosyl-lactulose.
a, b, c, d Statistically significant differences between rows for lactose (a,b) and lactulose reactions (c, d).
*GOS: Σ AloLa; GaBio; 3′GaLa; 4′GaLa; 6′ GaLa; ukwnown di-, tri- and tetrasaccharides.
**OsLu: Σ AloLu; GaBio; 6′GaLu; ukwnown di-, tri- and tetrasaccharides.

Fig. 5. Growth curves of *P. acidipropionici* LET120 at expense of different carbohydrates lactose, lactulose, Vivinal¯GOS (GOS) and *A. oryzae*-OsLu (OsLu) at 0.5% final concentration. WO/CH: without carbohydrates. Each curve is the average of three replicates from two independent assays.

However, the prebiotic effect of PAB-oligosaccharides was lower than that of Vivinal-GOS and *A. oryzae*-OsLu. No statistically significant differences were observed between the two probiotic strains in most cases. In spite of the higher growth, a lower decrease in pH was observed at the expense of all prebiotics. Otherwise, as expected and even with similar growth, the final pH values obtained with *B. animalis* subsp. *lactis* BB-12 were significantly higher than those obtained with *L. casei* CRL 431. Differences according to the substrate were observed, and in general, the final pH values were higher for these probiotics grown in LET 120-GOS and LET 120-OsLu.

Huebner et al. (2007) established a prebiotic quantitative score (PAS) to describe the extent to which prebiotics support the selective growth of lactobacilli and bifidobacteria. This prebiotic activity assay is based on the change in cell biomass after 24 h of growth of the probiotic strain on 1% prebiotic or 1% glucose relative to the biomass change of a commensal enteric strain grown under the same conditions. Fig. 6 shows the PAS of the tested oligosaccharides on *B. animalis* subsp. *lactis* BB-12, *L. casei* CRL 431 and *P. acidipropionici* LET 120. The growth of enteric *E. coli* C3 on the prebiotics was lower than growth on glucose; thus, all of the GOS demonstrated positive PAS for the probiotics and PAB. Oligosaccharides synthesized by PAB showed lower PAS than commercial Vivinal-GOS and *A. oryzae*-OsLu for the three strains tested; however, no significant differences were observed between the PAS values obtained with the four substrates. The observed trend could be due to their lower content of monosaccharides and lactose after purification.

With respect to the different strains growing on Vivinal-GOS, *A. oryzae*-OsLu, LET 120-GOS and LET 120-OsLu, in general, PAS was significantly lower for *L casei* CRL 431, whereas no statistically significant differences were observed between *B. animalis* subsp. *lactis* BB-12 and *P. acidipropionici* LET 120. Additionally, the highest PAS of oligosaccharides from PAB was observed with the producer LET 120 (1.05 and 1.20 for LET 120-GOS and LET 120-OsLu, respectively). However, no significant differences were observed between PAS values obtained with the four substrates. Other studies previously determined the prebiotic effect of lactose and lactulose-derived oligosaccharides on pure cultures of potential probiotic strains by assessing growth, substrate consumption and/or metabolites production (Cardelle-Cobas et al., 2011; Garcia Cayuela et al., 2014; Hernández et al., 2012). However, PAS can be considered as a simple method for evaluating prebiotics utilization by beneficial bacteria and has been used by other

Table 3
Some relevant parameters related to the growth of *P. acidipropionici* LET 120 and SCFA formation at expense of lactose, lactulose, Vivinal-GOS and *A. oryzae*-OsLu. Data are the means of two independent assays (± SD). The r^2 for fit of data to sigmoid curves ranged from 0.974 to 0.995 for all the carbon sources tested in the study.

	Time for maximum growth	Maximum OD_{600}	CFU mL^{-1}	Final pH	μ (h^{-1})	β-gal (U mg^{-1})	Acetic acid (mg mL^{-1})	Propionic acid (mg mL^{-1})
No sugar	–	0.17^{a} (0.02)	1.5×10^{6a} (0.1)	5.85^{a} (0.04)	–	0.43^{a} (0.09)	–	–
Glucose	28 h	0.62^{b} (0.03)	1.1×10^{9a} (0.2)	4.93^{b} (0.04)	0.03^{a} (0.01)	$1.57^{a,b}$ (0.29)	0.19^{a} (0.05)	0.77^{a} (0.06)
Lactose	13 h	0.78^{c} (0.03)	1.0×10^{9a} (0.1)	4.95^{b} (0.07)	$0.07^{a,b}$ (0.00)	$2.26^{b,c}$ (0.39)	0.12^{a} (0.02)	0.39^{b} (0.04)
Lactulose	17 h	1.05^{d} (0.03)	2.1×10^{9b} (0.5)	4.90^{b} (0.03)	0.12^{b} (0.02)	2.78^{c} (0.15)	0.45^{b} (0.09)	0.72^{a} (0.05)
Vivinal-GOS	15 h	0.94^{e} (0.02)	3.35×10^{9b} (0.2)	5.02^{b} (0.01)	$0.10^{a,b}$ (0.01)	$2.67^{b,c}$ (0.46)	$0.54^{b,c}$ (0.04)	1.10^{c} (0.08)
***A. oryzae*-OsLu**	13 h	1.06^{d} (0.01)	3.5×10^{9b} (0.6)	5.00^{b} (0.06)	0.14^{b} (0.03)	2.95^{c} (0.15)	0.67^{c} (0.04)	1.59^{d} (0.07)

[a, b, c, d, e] Statistically significant differences between groups.

Table 4
Increase in the cell density between time 0 and time 24 h and final pH for pure cultures of selected probiotic strains grown on various oligosaccharide substrates.

	Lactobacillus casei CRL 431		*Bifidobacterium animalis* subsp *lactis* BB-12	
	Increase of biomass*	Final pH**	Increase of biomass	Final pH
Glucose	$0.55 \pm 0.12^{B,b,c}$	$4.04 \pm 0.06^{B,b}$	$0.39 \pm 0.18^{C,c}$	$5.02 \pm 0.01^{A,c}$
Vivinal-GOS	$0.76 \pm 0.10^{A,a}$	$3.74 \pm 0.08^{B,c}$	$0.66 \pm 0.07^{A,a,b}$	$5.74 \pm 0.09^{A,a,b}$
***A. oryzae*-OsLu**	$0.65 \pm 0.06^{A,a,b}$	$4.05 \pm 0.10^{B,b}$	$0.64 \pm 0.11^{A,a,b}$	$5.42 \pm 0.08^{A,b,c}$
LET 120-GOS	$0.60 \pm 0.14^{A,a,b,c}$	$5.14 \pm 0.01^{B,a}$	$0.57 \pm 0.16^{A,a,b,c}$	$6.29 \pm 0.54^{A,a}$
LET 120-OsLu	$0.62 \pm 0.12^{A,a,b}$	$5.05 \pm 0.11^{B,a}$	$0.54 \pm 0.15^{B,b,c}$	$6.13 \pm 0.28^{A,a,b}$

*Determination of biomass in the pure cultures was done by turbidimetry and reported as OD_{600}.
**Culture medium pH at the end of fermentation of probiotics with different carbohydrates as the only carbon source. Initial pH was 6.50 ± 0.11.
[A,B] Statistically significant differences between the two probiotic strains.
[a,b,c] Statistically significant differences between substrates.

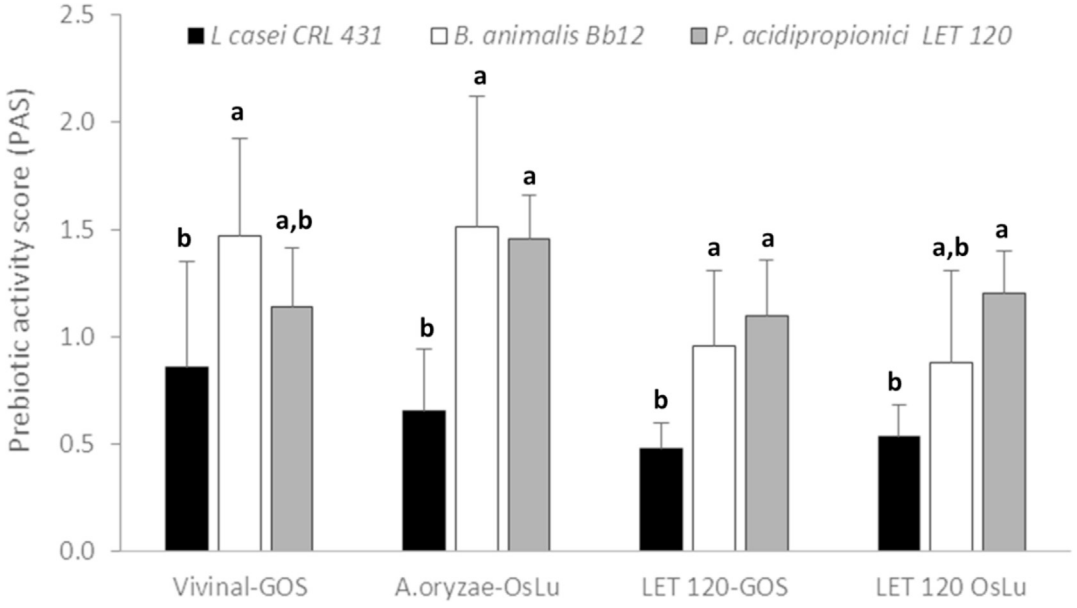

Fig. 6. Prebiotic activity scores (PAS) of oligosaccharides on selected probiotics and *P. acidipropionici* LET 120. ([a,b,c] Statistically significant differences between probiotic strains).

authors as a first approximation to establish the prebiotic condition of oligosaccharides on pure cultures (Guerrero et al., 2015; Huebner et al., 2007; Shi et al., 2018). Additional studies are needed to determine the physiological role of PAB oligosaccharides in the complex gut environment and their potential applications as food ingredients to improve intestinal health.

4. Conclusion

The production of new bioactive oligosaccharides has recently gained much attention for their potential use as functional ingredients. To our knowledge, no studies have addressed the transgalactosidase activity and oligosaccharides synthesis by dairy PAB or their ability to

grow at the expense of NDO. For the first time, the results show that *P. acidipropionici* LET 120 was able to produce GOS β(1 → 6), β(1 → 3) and β(1 → 4) linked trisaccharides and OsLu containing mainly β(1 → 6) linked trisaccharides, which showed prebiotic activity on beneficial *Bifidobacterium* and *Lactobacillus* strains. As additional information, *P. acidipropionici* LET 120 was able to utilize lactulose, GOS and OsLu as carbon sources, which contributes to the knowledge regarding the ability of this potential probiotic bacteria to metabolize these substrates. Further studies to increase insight into the structure of trisaccharides synthesized by *P. acidipropionici* LET 120 and the optimal conditions for their synthesis are in process.

Declaration of interest

None.

Acknowledgments

This work was supported by PICT 2014-3583 from ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica, Argentina), Project I-COOP_2014_COOPB20099 and EMHE-CSIC 2017 MHE-200047 from CSIC (Consejo Superior de Investigaciones Científicas, España); and AGL2017-84614-C2-1-R from MICINN. Carlos Sabater thanks his FPU Predoc contract from Spanish MECD (FPU14/03619).

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