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Prebiotic galactooligosaccharides production from lactose and lactulose by *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL450†

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Galactooligosaccharides (GOS) are useful dietary ingredients recognized worldwide as prebiotics. In the present study, we evaluated the β-galactosidase (β-gal) activity of a panel of lactic acid bacteria (LAB) in order to select strains for the synthesis of oligosaccharides from lactose (GOS) and lactulose (OsLu) with a potential prebiotic effect. Fifteen strains out of 20 were able to grow on lactose and showed β-gal activities between 0.03 and 2.06 U mg^{-1}, whereas eleven were able to synthesize GOS. *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL450, the strain with the highest β-gal activity, synthesized a maximum of 41.3% GOS and 21.0% OsLu from lactose and lactulose, respectively, with β-(1 → 6) and secondary β-(1 → 3) linkages. When these compounds were tested without purifying, as carbon sources for the development of recognized probiotics and the producer strain, high growth was observed compared to non-prebiotic sugars like glucose and lactose. When the purified oligosaccharides were tested, the bacterial growth decreased, but the microorganisms displayed metabolic activity evidenced by the consumption of carbohydrates and the production of lactic acid. Additionally, the purified oligosaccharides demonstrated a bifidogenic effect. The obtained results support the potential of *L. delbrueckii* subsp. *bulgaricus* CRL450 for the production of the prebiotics GOS and OsLu and encourage the optimization of their synthesis for the design of new functional food ingredients.

1. Introduction

Lactic acid bacteria (LAB) are a diverse group of microorganisms that have been extensively studied because of their prominent role in food fermentation and human health.[1] In these microorganisms, the presence of the β-galactosidase (β-gal) enzyme (EC 3.2.1.23) becomes particularly relevant for their application to the manufacture of dairy products and for favor-

ing the metabolism of lactose in the gut with the consequent alleviation of intolerance symptoms.[2,3] These technological and probiotic properties are based on the hydrolysis of milk sugar by β-gal, which releases its monosaccharides glucose and galactose. However, this enzyme also catalyzes transgalactosylation reactions that allow the synthesis of galactooligosaccharides (GOS).[4,5] GOS are defined as nondigestible carbohydrates, formed by two to five galactose monomers and often a unit of terminal glucose linked by glycosidic bonds that exert prebiotic effects in consumers.[6,7] Prebiotics are substrates that are selectively utilized by host microorganisms conferring a health benefit.[8] Among them, GOS have proven to be useful for the modulation of the colonic microbiota toward a healthy balance, which usually involves the increase of bifidobacteria and lactobacilli and the decrease of less desirable microorganisms such as clostridia, bacteroides and enterobacteria pathobionts. Other health benefits ascribed to GOS include the inhibition of the attachment of pathogenic bacteria to the colonic epithelium, reduction of serum cholesterol and blood pressure, prevention of colon cancer and enhancement of immunity.[8,9] In addition, GOS have other useful properties as food ingredients/sweeteners: low-glycemic index, no-cariogenic, low-calorie and resistant to acidic pH values and high

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†Electronic supplementary information (ESI) available: Table S1: Growth parameters of *L. bulgaricus* CRL 450 after 24 h of incubation with purified Vivinal-GOS, *A. oryzae*-OsLu, CRL450-GOS and CRL450-OsLU. Fig. S1: Monitoring of ONPG hydrolysis (pH 6.5, 40 °C) by β-galactosidase enzyme contained in the CFE of representative lactobacilli from collection of CERELA-CONICET (CRL), (codes in Table 1). Fig. S2: Synthesis of oligosaccharides by lactobacilli enzymes evidenced qualitatively by TLC. See DOI: 10.1039/d0fo00942c

temperatures (pasteurization and baking).[10,11] Besides, GOS can protect probiotic strains during preservation processes like freeze-drying, obtaining a self-protected symbiotic formulation.[7] In view of all of these properties and the high demand for healthy foods by consumers, there is a growing commercial interest in the food industry for producing GOS as functional ingredients for a wide range of products such as infant foods, dairy products and fruit-based drinks.

In addition to GOS, lactulose and its derived oligosaccharides (OsLu) can be enzymatically synthesized by microbial β-gal[12–16] but, depending on the enzyme origin and the reaction conditions, the yield, composition and linkages between the monomers of the formed oligosaccharides may vary affecting their properties.

Previous studies have shown the synthesis, structural characterization, and prebiotic effects of GOS and OsLu produced by fungal β-gal from *Aspergillus* and *Kluyveromyces* species[12,13,15–18] and bacteria like *Bacillus circulans*,[19,20] *Propionibacterium acidipropionici*[21] and *Bifidobacterium* spp.[22–24] LAB have been studied intensively with respect to their enzymes because of their general recognition as safe (GRAS) and qualified presumption of safety (QPS) status that allow the use of the enzymes derived from these organisms without extensive purification in food-related applications. However, only a few studies have focused on the transgalactosidase activity of the β-gal from *Lactobacillus* strains for GOS production. This ability has been reported for *Lactobacillus reuteri*,[25] *L. acidophilus*,[26,27] *L. plantarum*[28,29] and *L. bulgaricus*.[30] In this respect, it has been suggested that the β-gal from probiotics might produce GOS structures that have special prebiotic effects, specifically targeting selected probiotic strains.[31] Therefore, the aim of this work was to assess the transgalactosidase activity of different *Lactobacillus* strains isolated from Argentinian products in order to determine which

strain leads to the highest GOS and OsLu yields. Then, the prebiotic potential of these oligosaccharides was tested on recognized probiotic strains by assessing their growth and metabolic activity evidenced by carbohydrate consumption and lactic acid production.

2. Materials and methods

2.1. Reagents

The standards (glucose, galactose, fructose, lactose, lactulose, raffinose and stachyose), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), β-gal from *Aspergillus oryzae*, activated charcoal and thin layer chromatography (TLC) plates were purchased from Sigma-Aldrich (St Louis, MO, USA). Lactozym®Pure 6500L (Lactozym), a β-gal from *Kluyveromyces lactis* was provided by Novozymes (Dittingen, Switzerland). Vivinal®-GOS syrup (Vivinal, Friesland Campina Domo, The Netherlands) contains approximately 75% dry matter (DM), 59% of GOS, 21% of glucose, 19% of lactose and 1.4% of galactose respect to quantified carbohydrates. *Aspergillus oryzae*-OsLu (*Ao*-OsLu) synthesized from lactulose were composed by 81% DM, 58% *Ao*-OsLu (23% disaccharides different to lactulose, mainly 6-galactobiose; 28% trisaccharides, mainly 6′-galactosyl-lactulose and 8% tetrasaccharides), 24% lactulose, 14% galactose and 4% lactose respect to quantified carbohydrates.[10,13,17] The culture media MRS and acetonitrile HPLC grade were obtained from Merck (Darmstadt, Germany).

2.2. Bacterial strains and culture media

Twenty *Lactobacillus* strains belonging to the culture collection of CERELA-CONICET (CRL), Argentina, were tested in the present study (Table 1). The probiotic strains *Lactobacillus*

Table 1 Growth parameters, β-gal activity and qualitative GOS production by lactobacilli developed 24 h at 37 °C on MRS containing lactose as carbon source. Data are the means of two independent assays (±SD)

CODE	Strain[a]	Origin	OD600 max	pH	β-Gal activity (U mg⁻¹)	OD420 slope	GOS synthesis
CRL 43	*L. acidophilus*	Tafi cheese	—	—	—	—	—
CRL 450	*L. bulgaricus*	Yoghurt	0.54 ± 0.09[a,b]	4.20 ± 0.10[a,b]	2.06 ± 0.02[a]	0.238[a]	+
CRL 494	*L. bulgaricus*	Yoghurt	0.47 ± 0.02[a,b]	4.53 ± 0.04[a,b]	0.24 ± 0.00[a,b]	0.084[c,d]	+
CRL 468	*L. bulgaricus*	Yoghurt	0.28 ± 0.03[b]	4.17 ± 0.06[a,b]	0.40 ± 0.00[a,b]	0.079[c,d]	+
CRL 958	*L. bulgaricus*	Yoghurt	0.37 ± 0.00[a,b]	4.23 ± 0.06[a,b]	0.27 ± 0.00[a,b]	0.038[e,f]	+
CRL 574	*L. fermentum*	Infant feces	—	—	—	—	—
CRL 76	*L. paracasei*	Commercial cheese	0.58 ± 0.03[a,b]	4.07 ± 0.06[a,b]	0.25 ± 0.00[a,b]	0.059[d,e]	+
CRL 143	*L. paracasei*	Commercial cheese	0.58 ± 0.02[a,b]	5.00 ± 0.03[a,b]	0.03 ± 0.00[b]	0.003[g]	—
CRL 200	*L. paracasei*	Tafí cheese	0.99 ± 0.01[a]	4.09 ± 0.08[a,b]	0.86 ± 0.01[a,b]	0.092[c]	+
CRL 1501	*L. paracasei*	Tomato extract	—	—	—	—	—
CRL 41	*L. plantarum*	Tafí cheese	0.55 ± 0.02[a,b]	5.34 ± 0.08[a]	0.26 ± 0.02[a,b]	0.032[f]	—
CRL 58	*L. plantarum*	Tafí cheese	—	—	—	—	—
CRL 93	*L. plantarum*	Tafi cheese	0.56 ± 0.03[a,b]	4.42 ± 0.12[a,b]	0.03 ± 0.00[b]	0.004[g]	—
CRL 972	*L. plantarum*	Cabbage	0.66 ± 0.00[a,b]	4.90 ± 0.11[a,b]	0.11 ± 0.00[b]	0.020[f,g]	+
CRL 1076	*L. plantarum*	Pea	—	—	—	—	—
CRL 1234	*L. plantarum*	Cabbage	0.82 ± 0.10[a,b]	3.81 ± 0.10[b]	0.13 ± 0.02[b]	0.005[g]	+
CRL 1775	*L. plantarum*	Olive	0.48 ± 0.01[a,b]	4.45 ± 0.04[a,b]	0.20 ± 0.02[a,b]	0.093[c]	—
CRL 2211	*L. plantarum*	Pea	0.25 ± 0.04[b]	4.07 ± 0.06[a,b]	0.65 ± 0.02[a,b]	0.162[b]	+
CRL 1101	*L. reuteri*	Adult human gut	0.46 ± 0.01[a,b]	4.15 ± 0.04[a,b]	0.06 ± 0.01[b]	0.014[f,g]	+
CRL 1880	*L. sakei*	Sausage	0.54 ± 0.00[a,b]	4.72 ± 0.04[a,b]	0.05 ± 0.00[b]	0.002[g]	+

[a] *Lactobacillus* strains, codes and origin. [a,b,c,d,e,f,g] Statistically significant differences between strains.

casei CRL431® (CERELA-CONICET, Chr.Hansen) and *Bifidobacterium animalis* subsp. *lactis* BB-12® (Chr.Hansen) and the enterobacteria *Escherichia coli* C3 (Institute of Microbiology "Luis Verna" of the University of Tucumán) were used to assay the prebiotic activity of oligosaccharides. The strains, stored at −20 °C in 10% (w/v) reconstituted skim milk containing 5% yeast extract and 10% glycerol, were activated in MRS broth at 37 °C by three successive transfers every 24 h before their experimental use.

2.3. Determination of β-galactosidase activity of lactobacilli

The β-gal activity (U mg^{-1} protein) of LAB was assessed in cell-free extracts (CFE) obtained by mechanical disruption. The hydrolytic activity was determined with a colorimetric method (OD$_{420}$) that measures the hydrolysis rate of the synthetic substrate ONPG according to the experimental protocol described in our previous work.[21] The transglycosidase activity of β-gal was determined qualitatively by TLC. Each CFE was mixed with 60% (w/v) lactose (1:1) and incubated for 8 h. Then a sample (2 μL) containing 20 g L^{-1} sugar was spotted in TLC silica gel plates and eluted with a mixture of butanol, methanol and water in a 70:20:10 ratio as mobile phase. Detection was achieved visually by spraying with 5% sulfuric acid and 0.5% α-naphtol in ethanol and heating for 30 min at 100 °C. Vivinal, glucose, lactose, raffinose and stachyose were used as standards.

2.4. Synthesis of oligosaccharides from lactose (GOS) and lactulose (OsLu) by *Lactobacillus bulgaricus* CRL450

The ability of *Lactobacillus delbrueckii* subsp. *bulgaricus* (hereinafter named as *L. bulgaricus* CRL450) to produce GOS and OsLu was studied according to the experimental protocol used in a previous work.[21] The reaction mixtures, containing β-gal (1.3 U mL^{-1}) and the substrates (lactose or lactulose at 300 g L^{-1}), were incubated at 45 °C, pH 6.5, during 1, 3, 5, 7, 24 h, and inactivated by heating. For comparison, Lactozym was used as control at the same conditions. All the samples were stored at −20 °C until their corresponding analytical determinations. The reactions were conducted in duplicate, and each sample was analyzed twice.

2.5. Partial purification of GOS

The oligosaccharides synthesized by *L. bulgaricus* CRL450 (CRL450-GOS and CRL450-OsLu), Vivinal and *Ao*-OsLu were partially purified by selective adsorption onto activated charcoal to decrease their monosaccharide contents.[32] One mL (≅300 mg) of carbohydrates and 3 g of activated charcoal (100–400 mesh) were mixed in 100 mL of 10% ethanol and stirred for 30 min. The mixtures were filtered through Whatman No. 1 paper, and the charcoal was stirred with 100 mL of 50% ethanol for 30 min in order to remove the oligosaccharides. After a new filtration, the solution were evaporated under vacuum at 40 °C, dissolved in 1 mL of deionized water and stored at −20 °C. Prior to use, the purified GOS samples were analyzed by gas chromatography (GC-FID) to assess their carbohydrate contents.

2.6. Growth of *Lactobacillus bulgaricus* CRL450 and probiotics on different oligosaccharides

L. bulgaricus CRL450, *L. casei* CRL431, and *B. animalis* subsp. *lactis* BB-12 were cultured in MRS broth for 24 h at 37 °C and 5% CO$_2$, harvested by centrifugation (10 000g for 10 min at 4 °C), washed twice and resuspended in sterile saline solution (0.85%) to prepare carbon-free inoculum. These bacterial suspensions were inoculated at 2% (v/v) (initial OD$_{600}$ ≅ 0.1) in MRS which does not contain glucose and was supplemented with glucose, lactose, lactulose, not purified and purified oligosaccharides from Vivinal-GOS, *Ao*-OsLu, CRL450-GOS and CRL450-OsLu at a final concentration of 0.5% (w/v). The growth in a medium without any carbon source was taken as negative control. The cultures were incubated at 37 °C for 24 h and the growth was determined by the increase in the absorbance of bacterial biomass at 600 nm (OD$_{600}$) in an automated microplate reader (Varioskan Flash). The maximum growth rate (μ_{max}), maximum OD$_{600}$ (OD$_{max}$) and lag parameter (lag) of strains were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.3.5 (http://www.ifr.ac.uk/safety/DMfit/default.html). The bacterial counts (CFU mL^{-1}), pH and lactic acid concentrations were determined at 0 h, and 24 h for LAB and at 48 h for bifidobacteria. The colony forming units (CFU) were counted after plating ten-fold diluted samples on MRS agar that was incubated for 48 h at 37 °C under microaerophilic conditions (5% CO$_2$ atmosphere). The pH-values were determined with a pHmeter Altronix TPXI and lactic acid was quantified by liquid chromatography (HPLC-RID).

2.7. Determination of the prebiotic activity score (PAS) of CRL450-GOS and CRL450-OsLu

The prebiotic potential was determined by the quantitative PAS based on the change in cell biomass after 24 h of growth of a probiotic strain on the prebiotic under study or glucose, relative to the biomass change of an enteric strain grown under the same conditions.[33] With this aim, the growth of *Escherichia coli* C3 was assessed in the conditions described in section 2.6 for LAB and bifidobacteria. The PAS of Vivinal-GOS, *Ao*-OsLu, CRL450-GOS and CRL450-OsLu was calculated as:

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

where PP$_{24}$ and PP$_0$ is the probiotic biomass (OD$_{600}$) on selected prebiotics after 24 h and 0 h of fermentation, respectively. PG$_{24}$ and PG$_0$ is the probiotic OD$_{600}$ on glucose after 24 h and 0 h fermentation, respectively. EP$_{24}$, EP$_0$ and EG$_{24}$, EG$_0$ are the *E. coli* biomass on prebiotics and glucose after 24 h and 0 h, respectively. Sugars concentration before and after fermentation was monitored by GC-FID.

2.8. Chromatographic analysis of carbohydrates and organic acids

2.8.1 HPLC with refractive index detection (RID).
Carbohydrates and lactic acid were analyzed in a HPLC-RID system (Knauer, Germany) equipped with a Kromasil® column

100-NH$_2$ (250 mm × 4.6 mm, 5 μm particle size, Akzo Nobel, Brewster, NY, USA). The elution was isocratic with a flow rate of 1 mL min^{-1} for 30 min using acetonitrile/water (70 : 30 v/v) as mobile phase. For lactic acid analysis a Rezex ROA organic acids column (300 mm × 7.8 mm and 8 μm particle size, Phenomenex Torrance, USA) was used and the separation was performed at 55 °C in an isocratic mode with 0.01 M H$_2$SO$_4$ as the mobile phase at a flow rate of 0.6 mL min^{-1}. The data acquisition and processing were performed using EuroChrom for Windows Basic Edition v.3.05 software. The carbohydrates and lactic acid were identified by comparing their retention times (t_R) with those of standards and quantified by the external standard method, using calibration curves of each standard (0.05–5 mg mL^{-1}).[21] Carbohydrate standards included fructose and galactose for monosaccharides, lactose and lactulose for disaccharides, raffinose for trisaccharides and stachyose for tetrasaccharides quantification. The amounts of the different carbohydrates present in the mixtures were expressed as percentage of the total carbohydrate content. Lactic acid concentrations were reported as mg mL^{-1} of grown culture medium. All HPLC analyses were performed in duplicate, obtaining relative standard deviation values below 10% in all cases.

2.8.2. Gas chromatography with flame ionization detector (GC-FID). The carbohydrates composition of (i) the reaction mixtures of *L. bulgaricus* CRL450 with lactose and lactulose at selected time, (ii) the unpurified and purified GOS and OsLu and (iii) the culture media supplemented with the purified GOS or OsLu (0 and 24 h) after the growth of *L. bulgaricus* CRL450 and probiotics, were determined by GC-FID as described previously.[21] The carbohydrates were analyzed as trimethyl silylated oximes (TMSO) in an Agilent Technologies 7890A Gas Chromatograph (Wilmington, DE, USA) equipped with a commercial fused silica capillary column DB-5HT, bonded, cross-linked 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 : 20) using nitrogen at 1 mL min^{-1} as the carrier gas. The data acquisition and integration were done using Agilent ChemStation Rev. B.03.01 software.

2.9. Statistical analysis

All analyses were carried out at least in duplicate and the data were expressed as mean ± SD. The one-way analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) were applied to all of the assays. In addition, Pearson correlation coefficient was calculated for all the variables. All statistical analyses were performed on R v3.5.0.

3. Results and discussion

3.1. Determination of β-galactosidase activity of lactobacilli

In recent years, functional foods containing probiotics and prebiotics have gained a lot of attention from the food indus-

try. LAB are the most frequently used probiotics, whereas GOS are well-recognized prebiotics. Therefore, it was in our interest to study the ability of potential probiotics to produce prebiotics. With this aim, we screened the hydrolytic and transglycosidase β-gal activity of Argentinian lactobacilli strains isolated from different origins.

To evaluate hydrolytic activity, 20 *Lactobacillus* strains belonging to the culture collection of CERELA-CONICET were inoculated into a basic broth containing lactose as the sole carbon source (*L. plantarum*, $n = 8$; *L. paracasei*, $n = 4$; *L. bulgaricus*, $n = 4$; *L. reuteri*, $n = 1$; *L. fermentum*, $n = 1$; *L. sakei*, $n = 1$; *L. acidophilus*, $n = 1$) (Table 1). Most of the strains (15 out of 20) expressed β-gal activity and were able to develop at the expense of lactose. Although the growth varied among the strains, the highest biomasses were achieved by *L. paracasei* CRL 200 (OD$_{600}$max: 0.99), significantly higher than those reported for the rest of the strains. However, *L. bulgaricus* strains showed the highest specific enzyme activity determined in CFE by the hydrolysis of ONPG, especially ($p<0.05$) *L. bulgaricus* CRL450 (2.06 U mg^{-1}). A wide range of kinetics of hydrolysis was observed among lactobacilli, and *L. bulgaricus* CRL450 showed the significantly highest slope (Table 1 and Fig. S1 of the ESI†). These results suggest that β-gal activity does not significantly depend on the species but on the strain. On the other hand, β-gal activity was independent of OD$_{600}$ values. Thus, in general, the maximum growth rates of *L. paracasei* were significantly higher than those of *L. bulgaricus*. In addition, no significant differences in the pH or enzymatic activity were observed according to the species and origin of the microorganism, while those strains isolated from cabbage and cheese showed significantly higher OD$_{600}$ values than those isolated from yoghurt and pea. In summary, no relevant correlations could be found between OD$_{600}$, pH and β-galactosidase activity. Regarding trangalactosylation capability, the reaction mixtures of 11 strains showed sugars that appeared in the TLC chromatogram at the position corresponding to oligosaccharides when compared to commercial Vivinal used as standard (Fig. S2†).

At present, most of the GOS-containing products commercially available are manufactured with the fungal enzymes of *A. oryzae* or *K. lactis* or the bacteria *B. circulans*.[6] However, depending on the source of β-gal and the reaction conditions, the amounts of GOS obtained, the monomer composition and the linkages between D-galactose units may vary, affecting their prebiotic potential.[6,34,35] In this regard, it has been proposed that β-gal from probiotic microorganisms would be more appropriate for the production of selective prebiotic oligosaccharides.[24,31] Since *L. bulgaricus* CRL450 displayed the highest hydrolytic activity and also transglycosidase capacity, it was selected to further study its ability to synthesize GOS and OsLu using lactose and lactulose as substrates, respectively.

3.2. Synthesis of oligosaccharides from lactose and lactulose

Fig. 1 shows the enzymatic synthesis of galactosyl derivatives from lactose and lactulose, respectively, using the β-gal of *L. bulgaricus* CRL450 (CRL450-β-gal) and Lactozym as a refer-
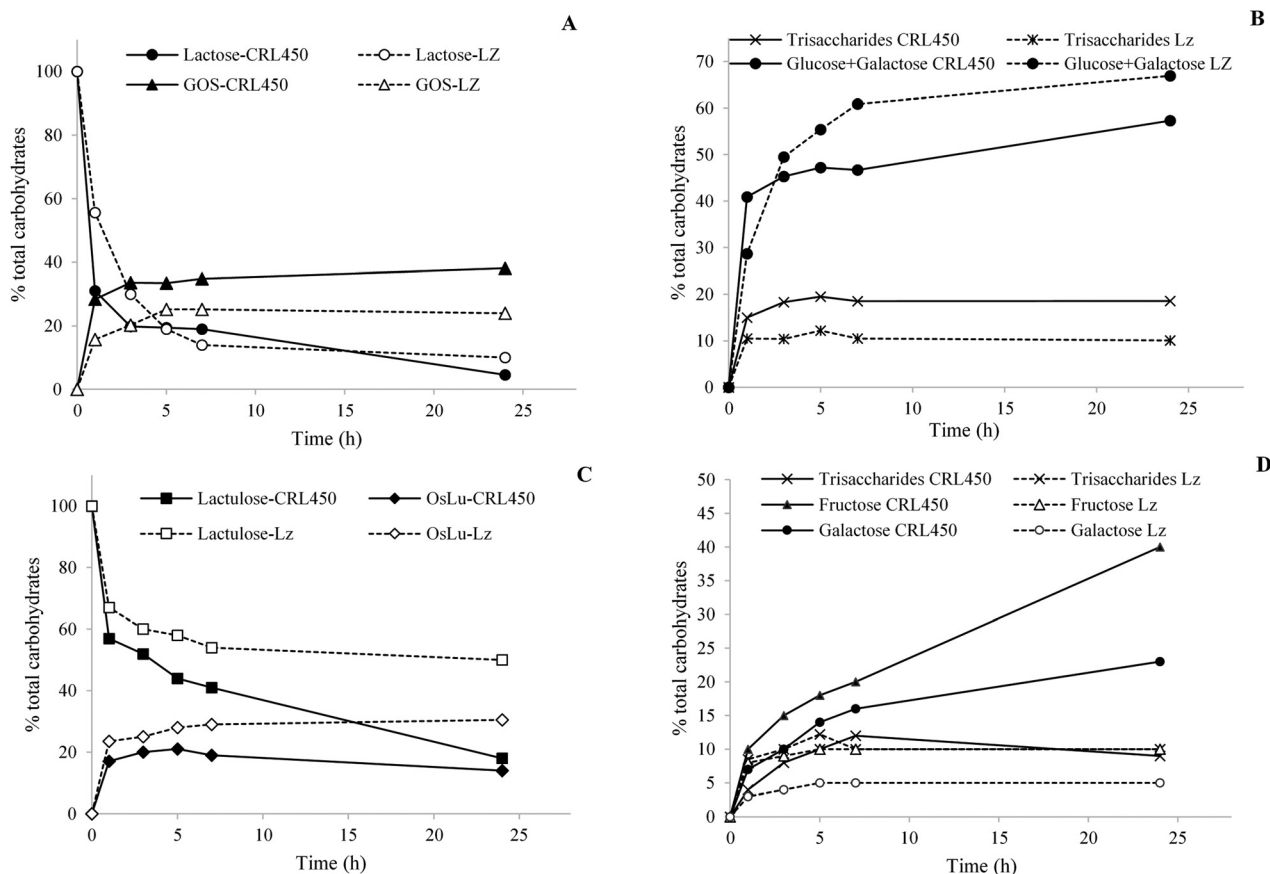
Fig. 1 Time course of reactions catalyzed with β-galactosidase of *Lactobacillus bulgaricus* CRL 450 and Lactozym Pure 6500L (Lz) at 45 °C, pH 6.5 and 300 g L^{-1} of lactose as substrate: (A) lactose and galacto-oligosaccharides (GOS) (B) mono- and trisaccharides, or 300 g L^{-1} of lactulose as substrate: (C) lactulose and oligosaccharides derived from lactulose (OsLu) (D) mono- and trisaccharides. Carbohydrates in reaction mixtures were quantified by HPLC-RID.

ence. The time course of lactose hydrolysis and GOS synthesis showed a rapid decrease in the lactose concentration in the first 3 h of reaction with both enzymes, being significantly faster with CRL450-β-gal. After 24 h of reaction, the degradation of 95.4% and 90.0% of lactose (w/w, with respect to total sugars) was achieved with CRL450-β-gal and Lactozym, respectively showing no significant differences among them (Fig. 1A). Although a lower amount of lactose remains with the unpurified extract containing β-gal of lactobacilli, Lactozym was more hydrolytic, releasing significantly higher amounts of monosaccharides (66.9% *vs.* 57.3% at 24 h) (Fig. 1B). Regarding transgalactosylation, in correlation to hydrolytic activities, the CRL450-β-gal was more active than Lactozym after 3 h of reaction, reaching 33.6% of oligosaccharides *vs.* 20.3% synthesized by Lactozym. Maximum GOS formation by CRL450-β-gal was achieved after 24 h (38.1%, with 18.6% corresponding to trisaccharides), whereas only 24% of GOS were obtained with Lactozym (Fig. 1A).

When lactulose was used as the substrate, an opposite pattern was observed: CRL450-β-gal was more hydrolytic than Lactozym and released significantly higher amounts of galactose and fructose, whereas Lactozym displayed significantly

higher transgalactosidase activity and synthesized more OsLu (Fig. 1C and D). Maximum production of OsLu by Lactozym was attained after 24 h of reaction and corresponded to 30.5% of total carbohydrates, whereas a maximum of 21.0% OsLu was obtained with CRL450-β-gal at 5 h (Fig. 1C). Finally, although a similar hydrolysis of both substrates was observed with CRL450-β-gal, a significantly larger amount of oligosaccharides was obtained from lactose.

In order to elucidate the composition of the oligosaccharide mixtures synthesized from lactose and lactulose by the CRL450-β-gal, the samples obtained at the time of maximum production were analyzed by GC-FID and compared with the profile of oligosaccharides found in the reaction mixtures performed with Lactozym. Fig. 2a shows the presence of mono-, di-, and trisaccharides that were compared with oligosaccharides previously synthesized by our research group[15,16,36] and the commercial GOS product Bimuno®. Among the oligosaccharides obtained from lactose, the following could be mentioned: the disaccharides 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 trisaccharides 6′-galactosyl-lactose (β-D-Gal*p*-(1 → 6)-lactose) (peak 10), and 3′-galactosyl-lactose (β-D-Gal*p*-(1 → 3)-
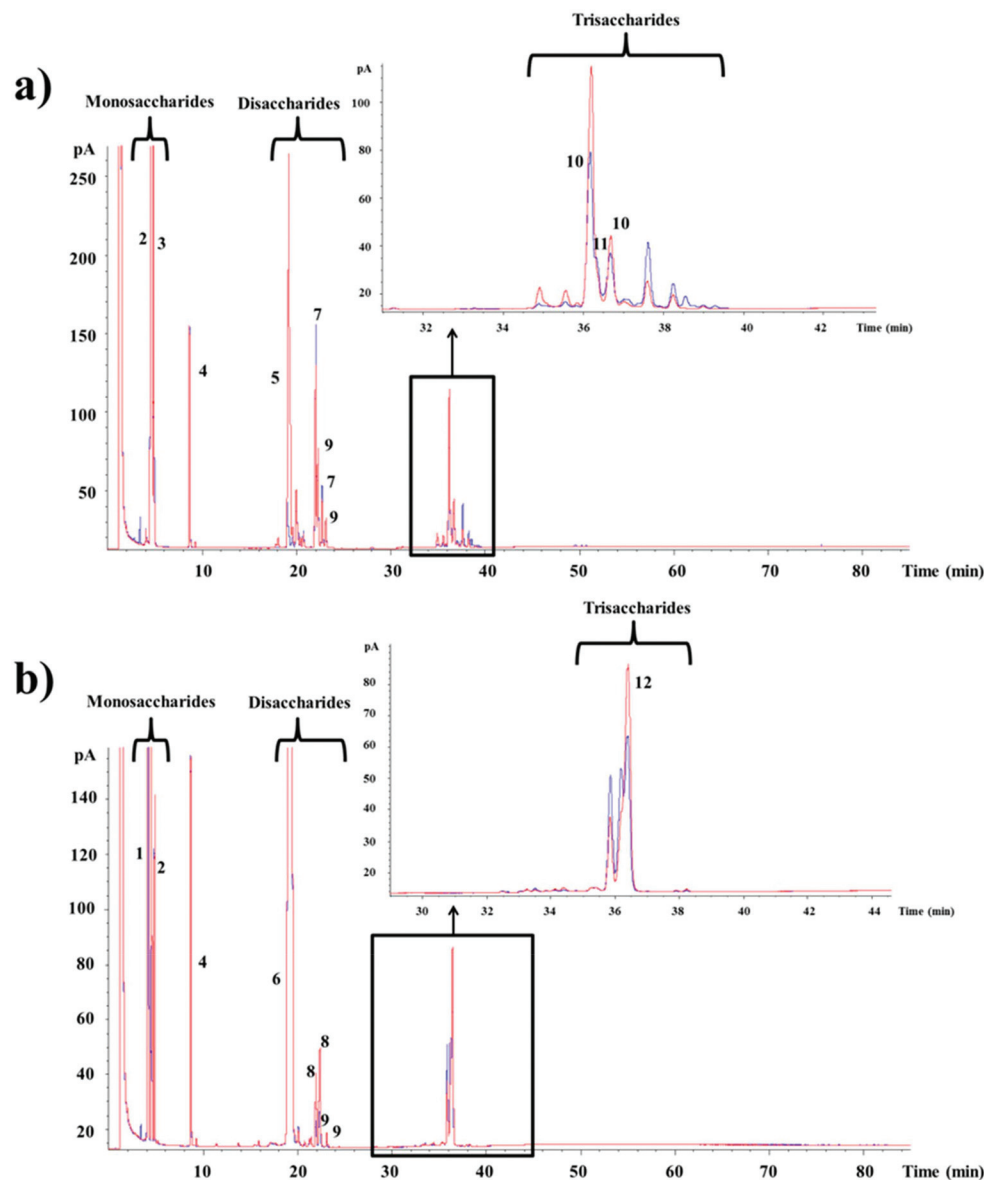
Fig. 2 Gas chromatographic (GC-FID) profile of the carbohydrates present in the reaction mixtures of β-galactosidase (1.3 U mL^{-1}) from *Lactobacillus bulgaricus* CRL 450 (blue line) with 300 g L^{-1} of (a) lactose or (b) lactulose performed at 45 °C, pH 6.5 during 7 h, compared with the obtained with Lactozym (red line). 1: fructose, 2: galactose, 3: glucose, 4: internal standard, 5: lactose, 6: lactulose, 7: allolactose, 8: allolactulose, 9: 6-galactobiose, 10: 6′-galactosyl lactose, 11: 3′-galactosyl lactose, 12: 6′-galactosyl lactulose.

lactose) (peak 11). Commercial GOS products are mixtures of monosaccharides, disaccharides and trisaccharides with different linkages: Vivinal and Oligomate® contain predominantly β-(l → 4) trisaccharides.[31,37] In the case of potential probiotic bacteria, it has been reported that bifidobacteria such as *B. bifidum* NCIMB 41171 synthesize GOS formed mainly with β-(1 → 3) linkages,[38] *P. acidipropionici* LET 120 produces GOS β(1 → 6), β(1 → 3) and β(1 → 4) linked trisaccharides,[21] whereas other *Lactobacillus* species have been shown to produce β-(1 → 6) and β-(1 → 3) linkages in their transgalactosylation mode.[25,29,39] This property is relevant since we have recently studied the digestibility of these compounds using brush border membrane vesicles from pig small intestine and

found that β(1 → 6) linkage had higher resistance than β(1 → 4) and β(1 → 3) linkages.[40] Therefore, the new synthetized GOS could be expected to reach the gut in large quantities.

Regarding the oligosaccharides formed from lactulose with the CRL450-β-gal (Fig. 2b), the di- and trisaccharides identified by comparison with OsLu synthesized by our research group[16] were allolactulose (β-D-Gal*p*-(1 → 6)-D-Fru) (peaks 8), 6-galactobiose (peaks 9) and 6′-galactosyl-lactulose (β-D-Gal*p*-(1 → 6)-lactulose) (peak 12). Other trisaccharides and tetrasaccharides that were also detected could not be identified. This type of oligosaccharides has been studied using the β-gal from fungi such as *K. lactis*, *A. oryzae*, and *A. aculeatus*.[12,16,17,35] Regarding bacteria, we previously reported the synthesis of 26.1% OsLu

containing mainly β(1 → 6) linked trisaccharides using β-gal of *P. acidipropionici* LET 120,[21] while β-gal from *B. circulans* was not able to hydrolyze the lactulose. As far as we know, this is the first report about the synthesis of lactulose-derived oligosaccharides with *Lactobacillus* β-gal. It should be noted that intestinal bacteria and probiotics belonging to *Bifidobacterium* and *Lactobacillus* spp. have shown the ability to use the trisaccharides derived from lactulose and lactose, but with a general preference towards β(1 → 6) and β(1 → 3) over β(1 → 4) linked products.[35,39,41] Then, it could be expected that CRL450-GOS/OsLu could exhibit good prebiotic potential.

Table 2 shows the concentration of oligosaccharides (di-, tri- and tetrasaccharides) obtained with CRL450-β-gal from both substrates. In the reaction mixtures containing lactose, the major disaccharide was allolactose (7.7%) and the main trisaccharides were 6′-galactosyl-lactose (11.2%) and 3′-galactosyl-lactose (7.1%). Unknown di-, tri- and tetrasaccharides were also quantified and all of them were included in GOS values. In the hydrolysates from lactulose, similarly the main di- and trisaccharides were allolactulose (2.4%) and 6′-galactosyl-lactulose (9.8%). Unknown di- and trisaccharides were included in OsLu values. The total amounts (as percentage of total sugars) of GOS and OsLu obtained with the unpurified CRL450-β-gal were 41.3% and 21.0%, respectively. GOS yield is in the same range of values (26.8 to 41%) reached with other LAB such as *L. reuteri*,[25] *L. acidophilus*[26] and *L. plantarum*[29] and within the more typical optimized GOS yields of around 30 to 40%.[42] Regarding *L. bulgaricus*, it has been reported that GOS yield by

L. bulgaricus L3 was increased by cloning the β-gal gene (bgaL3) and fusing with cellulose-binding domain using pET-35b(+) vector in *E. coli*. The resulting fusion protein was immobilized onto microcrystalline cellulose to produce up to 49% GOS.[30] Although these results were promising, the use of genetically engineered enzymes for food remains a concern in many countries.

3.3. Growth of *L. bulgaricus* CRL450 on prebiotic oligosaccharides

Prebiotics are mainly targeted to enhance the growth of beneficial bacteria such as bifidobacteria in the gut or alternatively in a symbiotic product.[41] Therefore, tailor-made GOS produced by lactobacilli-β-gal could be advantageous also for their own proliferation. Then, we tested the ability of *L. bulgaricus* CRL450 to develop at the expense of different carbohydrates that include no prebiotics (glucose and lactose), prebiotics (lactulose, Vivinal and *Ao*-OsLu) and potential prebiotics (CRL450-GOS and CRL450-OsLu). As negative control the growth in the absence of carbon sources was included for comparison. Fig. 3 shows the kinetics of growth on the different carbon sources whereas Table 3 presents some relevant parameters in the conditions assayed.

As expected, no growth was observed in the absence of a carbohydrate source but *L bulgaricus* CRL450 was able to grow to different extents in all the substrates tested. OD_{600} values for CRL450-OsLu and CRL450-GOS were significantly higher than those obtained for lactulose and *Ao*-OsLu (Fig. 3). Maximum growth was obtained in the oligosaccharides synthesized by their own β-gal, although these substrates also produced the longer lag phase (Fig. 3 and Table 3). Other studies have also reported that *Bifidobacterium* and *Propionibacterium* strains preferably ferment GOS over lactose and more simple carbohydrates like glucose or galactose.[21,43–45] After 12 h of incubation, *L. bulgaricus* CRL450 reached the stationary phase in all the substrates tested except in CRL450-OsLu, showing at

Table 2 Carbohydrate 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 β-galactosidase of *L. bulgaricus* CRL450 at maximum oligosaccharide formation times (5 h). Values are mean (±SD)

	GOS-CRL450	OsLu-CRL450
Fructose	—	16.92 (0.39)
Galactose	7.12 (0.24)	9.32 (0.20)
Glucose	15.62 (0.86)	—
Lactose	35.96 (2.28)	—
Lactulose	—	52.23 (2.14)
Allolactose	7.67 (0.57)	—
Allolactulose	—	2.39 (0.14)
6-Galactobiose	0.93 (0.06)	0.93 (0.22)
Others Di-GOS	4.22 (0.06)	2.07 (0.08)
6′GaLa	11.20 (0.00)	—
3′GaLa	7.14 (0.16)	—
6′GaLu	—	9.75 (1.08)
Others Tri-GOS	7.25 (0.32)	4.82 (1.13)
Monosaccharides	22.74 (1.10)	26.76 (0.61)
Disaccharides	48.78 (1.58)	57.62 (2.15)
Trisaccharides	25.58 (0.17)	14.57 (0.06)
Tetrasaccharides	2.90 (0.31)	1.05 (1.48)
GOS[a]	**41.30 (0.18)**	—
OsLu[b]	—	**21.00 (1.53)**

[a] GOS: ∑Allolactose, 6-galactobiose; 3′galactoyl-lactose (3′GaLa); 6′galactoyl-lactose (6′GaLa); unknown di-, tri- and tetrasaccharides.
[b] OsLu: ∑Allolactulose, 6-galactobiose; 6′galactoyl-lactulose (6′GaLu); unknown di-, tri- and tetrasaccharides.
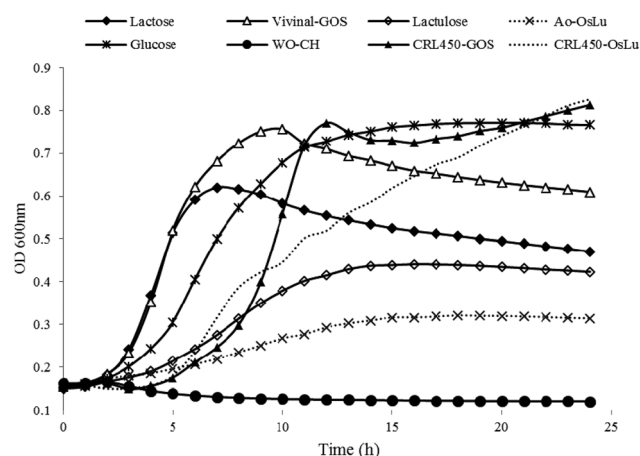

Fig. 3 Growth curves of *Lactobacillus bulgaricus* CRL 450 at expense of different carbohydrates at 0.5% (w/v) final concentration. Each curve is the average of two independent assays.

Table 3 Some relevant parameters related to growth of *L. bulgaricus* CRL450 at expense of different carbon sources. Data are means of two independent assays (±SD)

Growth parameters	Glucose	Lactose	Lactulose	Vivinal	*Ao*-OsLu	CRL450 GOS	CRL450 OsLu	No sugar
Max growth (h)	18	7	16	10	18	24	24	—
Lag (h)	2.83 ± 0.07^d	2.53 ± 0.02^d	3.36 ± 0.18^c	2.49 ± 0.02^d	$2.95 \pm 0.29^{c,d}$	7.32 ± 0.12^a	4.16 ± 0.21^b	—
OD_{max}	0.76 ± 0.02^a	$0.61 \pm 0.01^{a,b}$	$0.43 \pm 0.01^{b,c}$	0.75 ± 0.02^a	0.31 ± 0.00^c	0.80 ± 0.06^a	0.83 ± 0.25^a	0.16 ± 0.00^c
Log CFU mL^{-1}	8.13 ± 0.18^b	7.60 ± 0.03^b	6.51 ± 0.13^c	7.84 ± 0.02^b	6.50 ± 0.28^c	9.75 ± 0.22^a	10.05 ± 0.57^a	6.04 ± 0.06^c
Final pH^b	$4.17 \pm 0.11^{c,d}$	$4.43 \pm 0.10^{b,c,d}$	4.69 ± 0.08^b	4.05 ± 0.03^d	$4.51 \pm 0.15^{b,c}$	$4.30 \pm 0.03^{b,c,d}$	$4.34 \pm 0.04^{b,c,d}$	6.33 ± 0.02^a
μ (h^{-1})	0.079 ± 0.001^b	0.134 ± 0.014^a	$0.034 \pm 0.001^{b,c}$	0.145 ± 0.014^a	0.015 ± 0.000^c	0.151 ± 0.016^a	$0.056 \pm 0.040^{b,c}$	—
β-Gal (U mg^{-1})	0.89 ± 0.03^d	$2.06 \pm 0.03^{a,b}$	1.04 ± 0.21^d	$1.71 \pm 0.10^{b,c}$	$1.97 \pm 0.13^{a,b}$	1.51 ± 0.15^c	2.19 ± 0.02^a	0.90 ± 0.00^d
Lactic acid (mg mL^{-1})	0.43 ± 0.01^f	2.52 ± 0.03^b	1.98 ± 0.02^e	5.97 ± 0.04^a	2.25 ± 0.07^c	$2.10 \pm 0.03^{d,e}$	$2.11 \pm 0.00^{c,d}$	—

^{a} Initial Log CFU mL^{-1} 6.34 ± 0.06. ^{b} Initial pH was 6.50 ± 0.11. ^{a,b,c,d,e,f} Statistically significant differences between carbon source.

24 h $OD_{600\,nm}$ of 0.83. The significantly highest ($p < 0.05$) μ_{max} were attained with CRL450-GOS and Vivinal used as carbon sources. On the contrary, lower growth rates and biomasses were obtained on lactulose and *Ao*-OsLu, reaching OD_{600} of 0.43 and 0.31 for each substrate, respectively, being OD_{600} values for *Ao*-OsLu significantly lower than in other conditions ($p<0.05$). Statistically significant differences were found between the studied substrates when the lag times, growth rates and maximum CFU, were also calculated (Table 3). In this case, when the growth of *L. bulgaricus* CRL 450 was evaluated at the expense of different carbon sources, final pH was correlated to Log CFU mL^{-1} and lactic acid production while OD_{600} and μ_{max} were most strongly correlated. In agreement with the absorbance data, no pH change was detected in the negative control, while a significant pH decrease of 1.57 to 2.62 units was observed in all of the other samples after 24 h. Lactic acid production was significantly higher in cultures grown on disaccharides and oligosaccharides than in a pure monosaccharide like glucose. Regarding β-gal, *L. bulgaricus* CRL450 displayed enzyme activity in the absence of carbon sources and the presence of glucose, suggesting a constitutive basal level of β-gal, but also the induction of synthesis by their specific substrates, showing the highest activity when CRL450 was grown at the expense of lactose and lactulose-derived oligosaccharides (Table 3).

However, it should be noted that these data correspond to the growth of *L. bulgaricus* CRL450 on mixtures of oligosaccharides that also contain monosaccharides; therefore, we considered it relevant to study the performance of the strain on purified oligosaccharides. At 24 h of incubation of CRL450 with the purified substrates, the increase on bacterial biomass was negligible (less than 0.1 at OD_{600}); however, the decrease of pH of around 1.5 units (from 6.5 to 5.1) and the appearance of lactic acid in the culture media (1.18 to 1.45 mg mL^{-1}) suggest metabolic activity of the microorganisms at the expense of these substrates (Table S1†). In relation to growth of *L. bulgaricus* CRL 450 with purified oligosaccharides, OD_{600} values for CRL450-GOS were significantly higher than those for Vivinal while CRL450-GOS and CRL450-OsLu showed significantly lower final pH values. No relevant differences were observed with regard to lactic acid production.

3.4. Determination of the prebiotic capacity of CRL450-GOS and CRL450-OsLu on the producer strain and probiotic cultures

As mentioned, prebiotic candidates should be able to stimulate the growth of intestinal bifidobacteria and lactobacilli.[41] A first step to test the prebiotic properties of the new GOS was to perform assays with pure cultures of recognized probiotics of intestinal origin. With this aim, *B. animalis* subsp. *lactis* BB-12 and *L. casei* CRL431 were inoculated in media containing glucose, or the not purified and purified Vivinal, *Ao*-OsLu, CRL450-GOS and CRL450-OsLu as the sole carbohydrate source. The growth of the strains was evaluated by bacterial biomass absorbance and pH determination at 24 h (Table 4). To have a prebiotic activity, the studied sugar should be metabolized by the test strain preferably better than glucose.

Both probiotics were able to develop on GOS and OsLu in spite of their microbial origin and purity, as evidenced by the increase in OD_{600} and the decrease in the pH of media (Table 4). Interestingly, the increase of biomass of both lactobacilli, CRL431 and CRL450 were strongly correlated. When the prebiotic index (PAS) was determined using as substrates the oligosaccharide mixtures without purification, a positive score was observed for all the strains and conditions tested (Fig. 4). This was due to the strong growth of LAB and bifidobacteria and the poor development of *E. coli* on all the oligosaccharides tested. *L. casei* CRL431 showed high growth rates on all oligosaccharides while *B. animalis* BB-12 and *L. bulgaricus* CRL450 grew significantly better on Vivinal-GOS than in the other substrates. The lowest growth rates were observed for *L. bulgaricus* CRL450 on *Ao*-OsLu and for *B. animalis* BB-12 on CRL450-GOS compared to the other studied strains (Fig. 4a).

When the prebiotic effect of the purified oligosaccharides was analyzed, it was observed that *L. casei* CRL431 and especially *L. bulgaricus* CRL450 showed poor growths (Table 4) and, therefore, their prebiotic scores were negative (Fig. 4b). However, *B. animalis* BB-12 still showed significantly good growths over purified *Ao*-OsLu, CRL450-GOS and CRL450-OsLu ($p < 0.5$).

These results showed a low growth of *L. bulgaricus* CRL450 on the oligosaccharides produced by the strain, contrary to

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 not purified and purified

	Lactobacillus casei CRL431		Bifidobacterium animalis subsp. lactis BB-12		Lactobacillus bulgaricus CRL450	
	Increase of biomass[a]	Final pH[b]	Increase of biomass[a]	Final pH[b]	Increase of biomass[a]	Final pH[a]
Glucose	0.60 ± 0.02^{c}	4.04 ± 0.01^{c}	$0.75 \pm 0.06^{a,b}$	5.02 ± 0.01^{d}	$0.44 \pm 0.01^{a,b}$	4.12 ± 0.11^{c}
Vivinal GOS	0.77 ± 0.07^{b}	3.80 ± 0.06^{d}	0.81 ± 0.04^{a}	$5.76 \pm 0.08^{a,b}$	0.55 ± 0.03^{a}	5.61 ± 0.07^{a}
Ao-OsLu	0.84 ± 0.05^{b}	3.82 ± 0.03^{d}	$0.61 \pm 0.02^{b,c}$	5.82 ± 0.08^{a}	$0.21 \pm 0.00^{b,c}$	5.51 ± 0.03^{a}
CRL450-GOS	1.03 ± 0.03^{a}	4.10 ± 0.03^{c}	0.50 ± 0.11^{c}	4.51 ± 0.16^{e}	0.66 ± 0.05^{a}	4.30 ± 0.03^{c}
CRL450-OsLu	1.12 ± 0.05^{a}	3.84 ± 0.06^{d}	$0.72 \pm 0.04^{a,b}$	5.04 ± 0.09^{d}	0.53 ± 0.02^{a}	4.34 ± 0.04^{c}
Purified Vivinal GOS	0.20 ± 0.02^{f}	4.78 ± 0.01^{a}	0.27 ± 0.04^{d}	$5.69 \pm 0.10^{a,b,c}$	0.04 ± 0.00^{c}	5.61 ± 0.07^{a}
Purified Ao-OsLu	$0.25 \pm 0.01^{e,f}$	4.57 ± 0.01^{b}	$0.64 \pm 0.03^{b,c}$	$5.26 \pm 0.05^{c,d}$	0.03 ± 0.01^{c}	5.51 ± 0.03^{a}
Purified CRL450-GOS	$0.32 \pm 0.05^{d,e}$	4.52 ± 0.00^{b}	$0.63 \pm 0.05^{b,c}$	5.07 ± 0.10^{d}	0.02 ± 0.02^{c}	5.03 ± 0.02^{b}
Purified CRL450-OsLu	0.41 ± 0.02^{d}	4.28 ± 0.08^{c}	$0.63 \pm 0.04^{b,c}$	$5.32 \pm 0.19^{b,c,d}$	0.02 ± 0.01^{c}	5.19 ± 0.03^{b}

[a] Determination of biomass in pure cultures was done by turbidimetry and reported as OD_{600}. [b] 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,c,d,e,f] Statistically significant differences between groups.
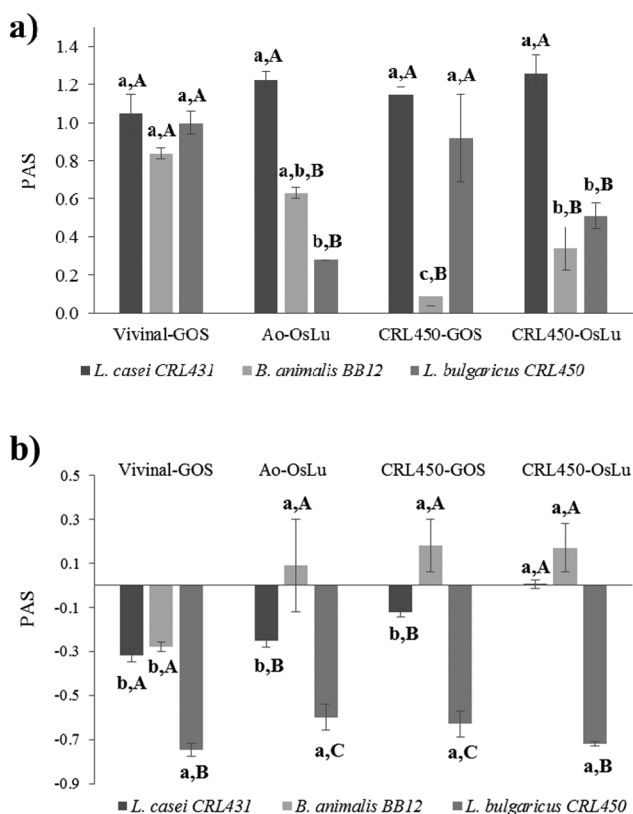

Fig. 4 Prebiotic activity scores (PAS) of oligosaccharides not purified (panel a) and purified (panel b) on selected probiotics and L. bulgaricus CRL 450. [a, b, c] Statistically significant differences among carbon sources. [A, B, C] Statistically significant differences between strains.

expectations, since it is assumed that oligosaccharides produced by probiotics may exert better prebiotic properties.[31] To determine the extent of the metabolic activity of bacteria when they grow at the expense of the different purified oligosaccharides, carbohydrate consumption was analyzed after 24 h of fermentation. Table 5 shows the use of the substrates by the probiotics and L. bulgaricus CRL450. Consumption of total carbohydrates was evidenced in all cases, ranging from 79% for L. casei CRL431 with CRL450-GOS and 38% to B. animalis BB12 with the same substrate. In this respect, L. casei CRL 431 was the most metabolically active microorganism with the highest consumption of GOS and OsLu from L. bulgaricus CRL450, while B. animalis BB-12 was the least active.

As expected, in general, monosaccharides were the most efficiently metabolized, so when the oligosaccharides from lactulose were the substrates, the fructose consumption was total (with the exception of CRL431 on Ao-OsLu), whereas more than 55% of glucose was consumed when GOS were the carbon sources. However, galactose was metabolized differently according to the bacteria tested, since the consumption was high for recognized probiotics, especially B. animalis BB-12, but significantly less efficient for L. bulgaricus CRL450, leaving a large amount of this monosaccharide. It is well known that in the presence of excess lactose, many L. bulgaricus strains metabolize the glucose moiety of lactose and release galactose into the growth medium. This feature has been associated with the accumulation of galactose in cheeses and with maturation defects.[46] Regarding disaccharides, lactose was highly metabolized by LAB, leading to a complete consumption for L. bulgaricus CRL450 grown on Vivinal-GOS, and 79% for L. casei CRL431 grown on CRL450-GOS, whereas lactulose was consumed between 95% for L. casei CRL431 grown on Ao-OsLu and 51% for L. bulgaricus CRL450 on CRL450-OsLu. With respect to B. animalis BB-12, low lactose and lactulose consumption was observed, ranging from 32 to 52%. Finally, GOS were more efficiently metabolized, between 32 and 76%, than OsLu, between 9 and 48%, regardless of their microbial origin. However, large amount of oligosaccharides from L. bulgaricus CRL450 remained unmetabolized.

As mentioned, it is known that the chemical structure of the oligosaccharides is related to their bioactivities.[35] Other

Table 5 Carbohydrate content (% of total carbohydrates in the sample) found in the culture media supplemented with 5 mg mL⁻¹ of purified GOS or OsLu that were inoculated with LAB and bifidobacteria and incubated for 24 and 48 h, respectively

	Fru	Gal	Glu	Lac	Lu	Di-GOS	Tri-GOS	GOS	Totals
Vivinal-GOS (mg mL⁻¹) T₀ [a]	—	0.10 (0.00)	0.18 (0.01)	0.55 (0.08)	—	1.19 (0.25)	2.84 (0.46)	5.42 (0.74)	6.25 (0.84)
CRL431 T₂₄ (%)	—	27.23[d] (3.53)	8.25[c] (0.13)	8.15[d] (0.60)	—	14.65[d] (0.95)	57.91[c] (2.56)	40.48[c] (1.73)	36.49[c,d] (1.39)
BB12 T₄₈	—	39.80[d] (1.45)	29.50[b] (3.53)	57.50[b] (0.35)	—	40.85[b] (0.05)	56.67[c] (3.26)	54.02[c] (4.44)	53.38[a,b] (3.96)
CRL450 T₂₄	—	441.14[a] (8.96)	22.95[b] (18.40)	0.00[f]	—	42.98[b,c] (16.51)	83.02[b] (2.53)	59.07[b,c] (4.63)	59.09[a] (3.62)
Ao-OsLu (mg mL⁻¹) T₀	0.42 (0.01)	0.37 (0.02)	—	—	1.67 (0.25)	0.73 (0.16)	2.24 (0.25)	3.45 (0.60)	5.91 (0.88)
CRL431 T₂₄	35.61[a] (1.97)	19.40[d] (2.39)	—	—	5.47[e] (0.06)	12.48[d] (10.06)	79.05[b] (4.67)	53.99[c] (5.15)	36.83[b,c,d] (3.28)
BB12 T₄₈	0.00[b]	0.00[f]	—	—	48.35[b] (5.05)	113.67[a] (0.18)	73.59[b] (9.64)	71.74[b] (6.30)	55.51[a] (5.10)
CRL450 T₂₄	0.00[b]	126.99[c] (46.58)	—	—	32.56[b] (2.62)	8.74[d] (0.57)	76.85[b] (4.54)	51.78[c] (3.07)	47.32[a,b,c] (1.85)
CRL450-GOS (mg mL⁻¹) T₀	—	0.24 (0.03)	0.391 (0.03)	2.44 (0.25)	—	0.77 (0.14)	2.98 (0.16)	3.75 (0.02)	6.82 (0.23)
CRL431 T₂₄	—	20.69[d] (2.69)	2.86[c] (0.05)	21.21[c] (4.31)	—	20.64[c] (1.75)	24.30[d] (1.11)	23.55[d] (0.53)	21.42[d] (1.74)
BB12 T₄₈	—	6.99[e] (1.51)	4.13[c] (0.10)	66.74[a] (1.97)	—	89.48[a] (21.43)	62.87[b,c] (8.03)	68.33[b] (10.78)	61.89[a] (6.58)
CRL450 T₂₄	—	426.91[a] (29.00)	45.28[a] (24.93)	2.60[e] (0.54)	—	8.56[a] (3.75)	76.08[a] (12.71)	62.23[b] (9.33)	52.93[a,b,c] (4.54)
CRL450-OsLu (mg mL⁻¹) T₀	0.31 (0.01)	0.19 (0.00)	—	—	3.88 (0.29)	0.25 (0.00)	0.77 (0.12)	1.03 (0.12)	5.40 (0.19)
CRL431 T₂₄	0.00[b]	27.61[d] (0.26)	—	—	12.04[d] (0.47)	40.93[b] (4.48)	107.38[a] (5.01)	90.80[d] (4.87)	26.88[d] (1.27)
BB12 T₄₈	0.00[b]	9.73[e] (0.29)	—	—	67.88[a] (5.20)	0.00[g]	79.31[b] (15.91)	59.69[b,c] (11.97)	60.46[a] (6.00)
CRL450 T₂₄	0.00[b]	327.24[b] (49.17)	—	—	48.60[b] (6.79)	2.19[f] (3.10)	87.96[b] (1.53)	66.74[b] (1.91)	58.91[b] (6.21)

[a] Mean (standard deviation). [b] % remaining percentage respect to initial content. [a,b,c,d,e,f,g] Statistically significant differences between strains.

studies have evaluated the prebiotic effect of lactose and lactulose derived oligosaccharides on pure cultures of potential probiotic strains by assessing growth and/or metabolite production.[31,35,40,47,48] However, substrate consumption has not been assessed. Our results show that, although no strong growth was obtained with purified substrates, the microorganisms were able to metabolize to different extents the oligosaccharides derived from lactose and lactulose, in agreement with previous studies using this type of bacteria and oligosaccharides.[35,47] In addition, the present study revealed the differential consumption of carbohydrates.

4. Conclusions

In recent years, the production of tailor-made oligosaccharides synthesized by probiotic-β-galactosidases has gained attention due to their potential use as specific functional ingredients for improving the composition and activity of gut microbiota. Given the interest of potentially bioactive carbohydrates, novel glycosidases were screened to produce oligosaccharides efficiently. Our results demonstrated that, although a significant number of LAB produced enzymes with transgalactosidase activity, few of them effectively synthesize oligosaccharides. This is the case of *L. bulgaricus* CRL450, achieving high yields of prebiotic oligosaccharides from lactose (GOS) and lactulose (OsLu), the latter synthetized for the first time with a β-galactosidase from *Lactobacillus* sp. These oligosaccharides were mainly composed by β(1 → 6), and β(1 → 3) linked trisaccharides. The CRL450-derived oligosaccharide mixtures were consumed and supported growth of the producer and recognized probiotic strains, showing bifidogenic effect. Furthermore, these bacteria were able to metabolize different GOS and OsLu. Since these new oligosaccharides represent a promising alternative for the development of new prebiotic food additives, further studies dealing with the optimization of transgalactosidase conditions as well as the behavior of oligosaccharides formed during *in vitro* and *in vivo* fermentation by the complex intestinal microbiota are on course at present.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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