**Hydrolysis of lactose and transglycosylation of selected sugar alcohols
by LacA β -galactosidase from *Lactobacillus plantarum* WCFS1**

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Abstract

The production, biochemical characterization and carbohydrate specificity of LacA β -galactosidase (locus *lp_3469*) belonging to the Glycoside Hydrolase family 42 from the probiotic organism *Lactobacillus plantarum* WCFS1 is addressed. The β -D-galactosidase activity was maximal in the pH range of 4.0-7.0 and 30-37 °C. High hydrolysis capacity towards the β (1 $\rightarrow$ 4) linkages between galactose and glucose (lactose) or fructose (lactulose) was found. High efficiency towards galactosyl-derivatives formation was observed when lactose and glycerol, xylitol or erythritol were used. Galactosyl-derivatives of xylitol were characterized for the first time as 3-O- β -D-galactopyranosyl-xylitol and 1-O- β -D-galactopyranosyl-xylitol displaying the great preference of LacA β -galactosidase for the transfer of galactosyl residues from lactose to the C1 or C3 hydroxyl group of xylitol. These results indicate the feasibility of using LacA β -galactosidase for the synthesis of different galactosyl-polyols, which could be promising candidates for beneficial and appealing functional and technological applications such as novel prebiotics or hypocaloric sweeteners.

Keywords: β -Galactosidase; *Lactobacillus plantarum* WCFS1; Prebiotics; Polyalcohols; Polyols

1. Introduction

β -D-Galactosidases (β -D-galactohydrolase, EC 3.2.1.23) are glycoside hydrolases (GHs) that catalyze the hydrolysis of lactose into galactose and glucose monomers but, interestingly, they can also catalyze transglycosylation reactions under some specific conditions to produce β -galactooligosaccharides (GOS)¹⁻³ whose chemical structure is based on galactose monomers linked by different β (1 $\rightarrow$ 1), β (1 $\rightarrow$ 2), β (1 $\rightarrow$ 3), β (1 $\rightarrow$ 4), and β (1 $\rightarrow$ 6) bonds and a terminal glucose^{4,5}. GOS are widely recognized as prebiotic oligosaccharides because of their resistance to gastric acidity and absorption in the small intestine, and their subsequent fermentation by the colonic microbiota⁶. In addition, some specific β -D-galactosidases can also transfer galactosyl residues from lactose to other nucleophile acceptors, such as fucose⁷, *N*-acetylglucosamine⁸, glucose, galactose and fructose yielding a variety of hetero-galactooligosaccharides⁹. In this context, it has been demonstrated that not only saccharides may act as the acceptors of galactosyl moieties. In fact, polyols (also known as sugar alcohols) could be, under specific conditions, better acceptors than carbohydrates favoring transglycosylation reaction over hydrolysis¹⁰, producing galactosyl derivatives of polyols (Gal-polyols)¹¹.

Polyols, a group of low-digestible carbohydrates, are ideal food sugar replacers delivering multiple functionalities in foodstuffs including sweetness, bulk and calorie reduction, as well as a number of physiological benefits, such as prevention of dental caries^{12,13}. Concretely, *in vivo* and *ex vivo* studies have confirmed that xylitol and erythritol have potential anti-diabetic and anti-obesogenic effects exerted through different mechanisms^{14,15}. Other studies have indicated that xylitol is capable of modifying the composition and activity of gut microbiota in mice¹⁶, as well as the fecal microbiota in rodents and humans¹⁷. By using different systems based on *in vitro* human

fecal cultures, the fermentation of xylitol promoted the bacterial production of short-chain fatty acids (SCFA), especially butyrate¹⁸ by affecting the metabolic activity of different bacteria, such as *Anaerostipes spp.*¹⁹ Lastly, the European Food Safety Authority (EFSA) has granted several health claims to both xylitol and erythritol²⁰⁻²².

However, intestinal absorption rates of xylitol and erythritol have been reported to be as high as 90-95% (depending on the dose level)²³⁻²⁵. Therefore, the interest of the synthesis of Gal-polyols relies on their use as a sucrose replacer that may efficiently pass through the small intestine and be available to the colonic microbiota²⁶. Interestingly, Klewicka and Klewicki²⁷ cultivated strains from *Lactobacillus casei*, *L. paracasei*, and *L. acidophilus* in the presence of galactosyl derivatives of erythritol, xylitol, and sorbitol, as well as with the polyols themselves. These authors found that none of the tested *Lactobacillus* sp. bacteria metabolised erythritol or xylitol alone, whereas these bacteria effectively utilized Gal-polyols, stimulating both their growth and acidifying activity. Therefore, these results clearly indicated that the galactosylation of polyols enhanced their fermentability by probiotic *Lactobacillus* sp. bacteria. Gal-polyols are considered GRAS (Generally Recognized As Safe) compounds²⁸ and they have been synthesized from reactions of lactose with xylitol, sorbitol or erythritol using β -galactosidase from different sources but all of them belonging to the fungi kingdom, such as *Kluyveromyces fragilis*^{29,30}, *Aspergillus oryzae*^{10,31} and *Kluyveromyces lactis*^{29,30}. However, there is a lack of knowledge about the synthesis of these Gal-polyols using lactic acid bacteria (LAB) such as *Lactobacillus ssp.* and *Bifidobacteria ssp.*³².

In this sense, *Lactobacillus plantarum* WCFS1 is the first *L. plantarum* strain whose genome was totally sequenced^{33,34} and is one of the best studied lactobacilli due to their high genetic variation in genes involved in hydrolysis, synthesis or

modifications of carbohydrates^{35,36}. Diverse glycosidase activities have been described in *Lactobacillus plantarum*, such as α -³⁷ and β -glucosidase^{38,39}, α -⁴⁰⁻⁴² and β -galactosidase⁶, β -fructofuranosidase⁴³ or α -ramnosidase⁴⁴.

In the present study we have heterologously produced and biochemically characterized the GH42 LacA β -galactosidase (Lp_3469) from *Lactobacillus plantarum* WCFS1. The LacA transgalactosylation properties to synthesize galactosyl derivatives of polyols using lactose (as galactosyl-donor) and xylitol or erythritol (as acceptors) mixtures were also studied and optimized. Finally, a comprehensive structural characterization of the synthesized galactosyl derivatives of xylitol was accomplished by NMR.

2. Materials and methods

2.1. Bacterial strains and plasmid

L. plantarum WCFS1 was kindly provided by Prof. M. Kleerebezem (Wageningen University & Research, The Netherlands). *E. coli* DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3-Cter vector, respectively⁴⁵. The *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 140 rpm. When required, ampicillin was added to the medium at a concentration of 100 mg L⁻¹.

2.2 Chemicals and reagents

Lactose monohydrate and acetonitrile (HPLC grade) were supplied by VWR (Barcelona, Spain). MOPS (3-(N-morpholino) propanesulfonic acid), xylitol, phenyl- β -glucoside, maltulose, maltose, cellobiose, mannitol, sorbitol, sucrose, and *p*NP- β -D-galactopyranoside were purchased from Sigma Aldrich (Stenheim, Germany).

Erythritol, isomaltulose, gentiobiose, isomaltose, lactulose and 2'-fucosyl-lactose, were purchased from Carbosynth (Berkshire, UK). KH_2PO_4 and K_2HPO_4 were supplied by Merck (Steinheim, Germany). All other chemicals were of analytical grade.

2.3 Production and purification of LacA β -galactosidase

PCR was used to amplify the *lacA* (*lp_3469*) gene from *L. plantarum* WCFS1 by using Advantage HD DNA polymerase (TaKaRa) and 1703 (5' *TAACTTTAAGAAGGAGATATACATatgtccaatccattaaagacaaca*) and 1704 (5' *GCTATTAATGATGATGATGATGATGttattcatcatatagacttgaac*) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lacA* gene sequence are written in lowercase letters) primers. The pURI3-Cter vector was used to insert the PCR product by a restriction enzyme- and ligation-free cloning strategy⁴⁵. The recombinant protein produced by this vector has a six-histidine affinity tag in their C-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were used to transform *E. coli* BL21 (DE3) competent cells for expression. *E. coli* BL21 (DE3) cells were transformed with the recombinant plasmid. *E. coli* cells harbouring pURI3-Cter-LacA plasmid were grown in LB medium containing 100 mg L⁻¹ ampicillin until an 0.4 optical density at 600 nm when cells were induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) at 0.4 mM final concentration. Induced cells were grown at 22 °C for 18 h, collected by centrifugation (8,000 g, 15 min, 4 °C), and resuspended in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl. A French Press was used to prepare protein extracts from the cell suspension. The insoluble fraction of the lysate was removed by centrifugation at 47,000 g for 40 min at 4 °C, and the supernatant was filtered through a 0.45 μm pore-size filter and then applied to a Talon Superflow resin (Clontech) equilibrated in phosphate buffer. The bound enzyme was eluted using 150 mM

imidazole in the same buffer. Fractions containing the His6-tagged LacA were pooled and dialyzed (50 mM sodium phosphate buffer, 300 mM NaCl, pH 7 at 4 °C) using 3.5 kDa pore diameter dialysis membranes. The purified protein was stored in 16% glycerol at -20°C. Glycerol was omitted when transgalactosylation reactions were assayed. The amino acid sequence of LacA (UniProtKB code: F9UUD6) can be found at https://www.ncbi.nlm.nih.gov/protein/WP_011102174.1.

2.4 LacA hydrolytic assays

The hydrolytic activity of LacA β -galactosidase from *L. plantarum* WCFS1 was determined by using *p*NP- β -D-galactopyranoside as substrate. The standard conditions chosen for the assay were LacA (4 μ g) in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl and 1mM of dithiothreitol (DTT) at 30 °C for 10 min in a 96-well Flat Bottom plate (Sarstedt). A reaction volume of sodium carbonate 1 M at pH 9.0 was used to stop the reaction. Hydrolysis of *p*NP- β -D-galactopyranoside was colorimetrically measured by liberation of *p*-nitrophenolate (*p*NP) at 420 nm using an extinction coefficient of $\epsilon = 9310 \text{ M}^{-1} \text{ cm}^{-1}$ in a spectrophotometer PowerWave HT (Bio-Tek, USA) by using Gene 5 version 2.06 software. Blanks without enzyme for spontaneous hydrolysis of the tested substrate were carried out and data were collected in triplicate and the average activities were quantified. One unit (U) of LacA activity was defined as the amount of enzyme required to release 1 μ mol of *p*NP per minute at saturated *p*NP- β -D-galactopyranoside concentration, at 30 °C in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl.

The hydrolytic activity of LacA β -galactosidase (1.16 U mL⁻¹) was also assayed by incubation at 30°C of different carbohydrates such as lactose, lactulose, cellobiose, gentiobiose, maltose, isomaltulose and sucrose at 200 g L⁻¹ with 300 μ L of enzyme in 50 mM of MOPS buffer supplemented with 20 mM of NaCl (pH=7) to a final volume

of 1 mL. In the case of 2-fucosyl-lactose, isomaltose, nigerose and maltulose the concentration was 0.5 g L⁻¹. Reactions were stopped at 24 hours by heating at 100°C for 5 min and samples were stored at -20°C for the subsequent analysis. Reactions were carried out in triplicate (*n* = 3). All these hydrolytic reactions were monitored by GC-FID.

2.5 Biochemical and kinetic properties of LacA

The biochemical properties of LacA β -galactosidase from *L. plantarum* WCFS1 were determined using *p*NP- β -D-galactopyranoside as substrate (1 mM final concentration) under the standard conditions previously described. The LacA optimal pH was determined by using citrate (pH 3), acetic acid-sodium acetate (pH 4-6), MOPS (pH 6.5 and 7) and Tris-HCl (pH 8) buffers (50 mM). The LacA optimal temperature was assayed by incubation at different temperatures (4, 22, 30, 37, 45, and 65 °C) in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl, 1mM DTT. For thermal stability assays, LacA β -galactosidase was incubated at 22, 30, 37, 55, and 65 °C for 30 min and 1, 2, 4, 6, and 20 h, in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl, and 1mM DTT. Aliquots were withdrawn at these incubation times to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%). The analyses were performed in triplicate.

The effect of additives at 1 mM final concentration on β -galactosidase activity was studied by incubating LacA in the presence of different additives for 5 min at room temperature. Then, *p*NP- β -D-galactopyranoside was added, and the reaction mixture was incubated 10 min at 30 °C. After incubation, the LacA residual hydrolytic activity was measured. The additives used were KCl, CaCl₂, HgCl₂, ZnCl₂, CuCl₂, NiCl₂, FeCl₂, MnCl₂, Triton-X-100, Tween 20, Tween 80, SDS, urea, DMSO, cysteine, β -mercaptoethanol, PMSF, DEPC and EDTA. The hydrolytic activity measured in the

absence of any additive was taken as control (100%). Experiments were done in triplicate.

The kinetic parameters of LacA β -galactosidase were also studied by using *p*NP- β -D-galactopyranoside as substrate. K_m and V_{max} values were determined by nonlinear regression analysis fitting to Michaelis-Menten curves ($V_0 = V_{max}[S]/K_m + [S]$) of formation rates of *p*NP as a function of the concentration of substrate from 0 to 24 mM and with 1.5 μ g of enzyme and optimal conditions (50 mM MOPS buffer pH 5.0 containing 20 mM NaCl and 1mM $CaCl_2$) by using the software SigmaPlot version 13.0.

2.6 LacA transgalactosylation assays

Transgalactosylation assays catalyzed by LacA β -galactosidase (1.16 U mL⁻¹) were carried out at 37 °C in an orbital shaker at 900 rpm using 300 μ L of the enzyme, lactose (200 g L⁻¹) as the only substrate (acceptor and donor) and reaction mixtures containing lactose (200 g L⁻¹) / xylitol (200, 300 and 450 g L⁻¹) or erythritol (200, 300 and 400 g L⁻¹) prepared in 50 mM sodium phosphate buffer with 2 mM $MgCl_2$ (pH 6.5) to a final volume of 1 mL. Reactions were allowed to proceed up to 48 hours and samples were taken from the reaction mixture at different time intervals (0, 2, 8, 24, 32, 48 h), and monitored by GC-FID. The enzyme was inactivated by heating at 100°C for 5 min and samples were stored at -20°C for the subsequent analysis. Reactions were carried out in triplicate ($n = 3$). Synthesis yield is defined as a percentage (% w/w) of the total amount of galactosyl-derivatives with respect to the initial content of lactose (galactosyl donor). The content of galactosyl-derivatives is also given as percentage (w:w) of the total dry matter (estimated as the initial content of galactosyl donor and acceptor) of the reaction mixture.

2.7 Analysis of carbohydrates and galactosyl derivatives of polyols by gas chromatography (GC-FID)

The hydrolysis of lactose and transgalactosylation of polyols were followed by gas chromatography with a flame ionization detector (GC-FID) in an Agilent Technologies 7820A Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector (FID). The trimethyl silylated oximes (TMSO) were prepared⁴⁶ and the separation of the carbohydrates was carried out in a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane; 15 m × 0.32 mm i.d., 0.10 µm film thickness) (J&W Scientific, Folsom, California, USA). The oven temperature was programmed from 150 to 380° C at a rate of 3°C min⁻¹. The injector and detector temperatures were at 280°C and 385°C, respectively. Injections were carried out in split mode (1:20) using nitrogen at a flow rate of 1 mL min⁻¹. Data acquisition was performed using Agilent ChemStation software (Wilmington, DE, USA). Response factors were determined from duplicate analysis of standards solutions (glucose, galactose, fructose, lactose, raffinose and nystose) over the expected concentration range (0.005-2 mg) by internal standard calibration using phenyl-β-glucoside (0.5 mg mL⁻¹).

2.8 Purification of galactosyl derivatives of xylitol by hydrophilic interaction chromatography with refractive index detector (HILIC-RID)

A mixture of reaction of lactose and xylitol (20%:20%, w:w) after 48 h of incubation with LacA β-galactosidase from *Lactobacillus plantarum* WCFS1 was subjected to a fractionation procedure by HILIC in order to isolate and subsequently characterize the two main new peaks detected by GC-FID. A LC Agilent Technologies 1220 Infinity LC System 1260 equipped with a refractive index detector (RID) was used. The separation of main compounds present in the mixture was carried out with an

YMC-Pack Polyamine II column (250 x 4.6 mm, 5 μ m particle size) (Teknokroma, Barcelona, Spain) using acetonitrile: water 75:25 (v: v) as the mobile phase. The injection volume was 20 μ L and 1 mL of the reaction mixture were repeatedly injected and eluted in an isocratic mode at a flow rate of 1.0 mL min⁻¹ for 40 min. Two peaks labelled as fractions A and B (peaks 4 and 5, respectively, in **Figure S1 in Supporting Information**) were manually collected, pooled, evaporated in a rotary evaporator R-200 (Büchi Labortechnik AF, Flawil, Switzerland) and freeze-dried for subsequent characterization. Then, structure elucidation was carried out by NMR spectroscopy.

2.9 Structural characterization of galactosyl derivatives of xylitol by nuclear magnetic resonance (NMR)

The chemical structures of synthesized galactosyl derivatives of xylitol were identified by an extensive ¹³C and ¹H NMR study. NMR spectra were recorded at 298 K, using D₂O as the solvent, on a Varian System 500 NMR spectrometer (¹H 500 MHz, ¹³C 125 MHz) equipped with a 5-mm HCN cold probe. Chemical shifts of ¹H (δ_H) and ¹³C (δ_C) in parts per million were determined relative to internal standards of sodium [2, 2, 3, 3-²H₄]-3-(trimethylsilyl)-propanoate in D₂O (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) in D₂O, respectively. One-dimensional (1D) NMR experiments (¹H, and ¹³C) were performed using standard Varian pulse sequences. Two-dimensional (2D) [¹H, ¹H] NMR experiments [gradient correlation spectroscopy (gCOSY), and total correlation spectroscopy (TOCSY), were carried out with the following parameters: delay time of 1 s, spectral width of 4310 Hz in both dimensions, 2048 complex points in t₂, 32 transients for each of 128 time increments, and linear prediction to 256. The data were zero-filled to 2048 $\times$ 2048 real points. 2D [¹H-¹³C] NMR experiments [gradient heteronuclear single-quantum coherence (gHSQC) and gradient heteronuclear multiple-bond correlation (gHMBC)] used the same ¹H spectral window, a ¹³C spectral window

of 8799 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to 2048×2048 real points. Typical numbers of transients per increment were 32 and 64, respectively.

3. Results and discussion

3.1 Biochemical characterization of LacA β -galactosidase from *Lactobacillus plantarum* WCFS1

Lactobacillus plantarum is a versatile lactic acid bacterial species, which possesses a complex carbohydrate metabolism due to the presence of a wide range of carbohydrate active enzymes. One of these enzymes, the LacA β -galactosidase from *L. plantarum* WCFS1 was recombinantly produced and characterized. The *lacA* (*lp_3469*) gene was cloned into the pURI3-Cter expression vector, and the obtained recombinant plasmid was transformed into *E. coli* BL21 (DE3). Cell extracts were used to detect, in a SDS-PAGE gel, the presence of the overproduced LacA β -galactosidase and whose estimated Mw, i.e. 78 kDa, fully matched with the theoretical Mw according to its amino acid sequence (**Figure 1**). Routinely, about 7.2 mg of purified protein from 1 L of culture was obtained.

The LacA protein exhibited β -D-galactosidase activity (E.C 3.2.1.23) on *p*-NP- β -D-galactopyranoside (14.33 U mg⁻¹ specific activity). LacA β -galactosidase showed its maximal activity from an acidic to a neutral broad pH range, that is from 4.0 to 7.0 (**Figure 2A**). Contrarily, β -galactosidases from the same species previously described have shown to exhibit a neutral pH for optimal activity, such as L.p FMNP01Gal-2 from *L. plantarum* FMNP01⁴⁷ (pH 7.0), LacLM from *L. plantarum* FUA3112 (pH 6.0-7.2), or LacLM from *L. plantarum* WCFS1 (pH 7.5)⁶. Similarly, β -galactosidases from other lactic acid bacteria also showed neutral pH for activity, LacA from *L. acidophilus*

(pH 6.5-7.2), LacLM from *Leuconostoc mesenteroides* subsp. *cremoris* (pH 6.8), and LacZ from *Streptococcus thermophilus* FUA3194 (pH 6.8), among others⁴⁸.

In relation to temperature, LacA β -galactosidase from *L. plantarum* WCFS1 presented an optimal temperature at 30-37 °C (**Figure 2B**). This optimal temperature range is similar to the value showed by LacLM β -galactosidase from *L. plantarum* FUA3112 (37 °C). However, β -galactosidases from lactic acid bacteria generally exhibit a higher optimal temperature, such as 45 °C for LacA from *L. acidophilus* FUA3191⁴⁸ or 55 °C for LacLM from *L. plantarum* WCFS1⁶ and for LacLM from *Lactobacillus helveticus* DSM 20075⁴⁹. It has been reported that the *L. plantarum* β -galactosidase L.p FMNP01Gal-2 showed a broad optimal temperature range, from 30 to 60 °C⁴⁷. This β -galactosidase also maintained 90% of its maximal activity during 4h incubation at 50 °C⁴⁷, being more thermostable than the LacA from *L. plantarum* WCFS1 characterized in this study that only maintained its activity up to 37 °C (**Figure 2C**).

The effect of several ions, surfactants, reductants, and inhibitors on *L. plantarum* LacA activity is shown in **Figure 3**. The results indicated that the activity of the protein LacA was not affected in the presence of most of the compounds analyzed. None of the additives assayed increased significantly LacA β -galactosidase activity. Among the 20 compounds tested, only three divalent cations, i.e. Ni²⁺, Cu²⁺ and Hg²⁺, affected LacA β -galactosidase activity by reducing the maximal activity 50, 90 and 98%, respectively. These cations also inhibited the activity of some β -galactosidases such as L.p FMNP01Gal-2⁴⁷ or LacLM from *L. helveticus* DSM 20075⁴⁹.

Kinetics studies performed in the conditions described previously, indicated that *L. plantarum* LacA for pNP- β -D-galactopyranoside showed a K_m 17.86 $\pm$ 3.55 mM, and V_{max} 14.33 $\pm$ 0.002 U mg⁻¹ values. From these data, the K_{cat} and catalytic efficacy (K_{cat}/K_m) were calculated, being the K_{cat} 1119.4 $\pm$ 0.0008 min⁻¹ and the catalytic efficacy

(K_{cat}/K_m) $62.68 \pm 12.46 \text{ mM}^{-1} \text{ min}^{-1}$. This catalytic efficacy is low as compared to the catalytic efficacy exhibited by a β -galactosidase from *Bacillus stearothermophilus*⁵⁰ with an $E_{cat} = 2.6 \times 10^5 \text{ mM}^{-1} \text{ min}^{-1}$. The specific activity showed by LacA β -galactosidase from *L. plantarum* WCFS1 (14.33 U mg^{-1} specific activity) is higher than the activity exhibited by other two *L. plantarum* β -galactosidases, L.p FMNP01Gal (0.96 U mg^{-1}) and L.p FMNP01Gal-2 (2.62 U mg^{-1})⁴⁷.

3.2. Hydrolytic activity of LacA protein (lp_3469) on different carbohydrates

Hydrolytic activity of LacA protein lp_3469 (in 16% glycerol, w:w) was tested on ten disaccharides and one trisaccharide, being lactose (Gal- β (1 $\rightarrow$ 4)-Glc) and lactulose (Gal- β (1 $\rightarrow$ 4)-Fru) the only disaccharides that were hydrolyzed (**Figure 4**). Lactulose (**Figure 4A**) was hydrolyzed releasing fructose (peak 1) and galactose (peak 2). However, the ability of LacA to hydrolyze lactulose was not as high as compared to lactose (**Figure 4B**), which was efficiently hydrolyzed into galactose (peak 2) and glucose (peak 3). Therefore, our results confirmed the LacA gene's annotation as a gene encoding a β -galactosidase (EC 3.2.1.23) involved in the hydrolysis of lactose. In addition, when lactose is linked to L-fucose, from the galactose unit through an α (1 $\rightarrow$ 2) bond to comprise the human milk oligosaccharide 2'-fucosyllactose, LacA was not capable of hydrolyzing the β (1 $\rightarrow$ 4) glycosidic linkage. This behavior was expectable based on the fact that GH42 β -galactosidases are classified as exoglycosidase enzymes and, therefore, a substitution of the target galactose residue at the O-2 position blocked LacA activity.

Unknown compounds labelled as peaks 4 and 6 (**Figure 4**) were detected in both reactions using lactose and lactulose, and were tentatively identified as 2-O- β -D-galactopyranosyl-glycerol and 1-O- β -D-galactopyranosyl-glycerol, respectively, by comparison of their relative retention times with those compounds previously

synthesized and identified in another study using two commercial enzyme preparations (Pectinex Ultra SP-L and Lactozym 3000 L HP G) which contained glycerol as stabilizer⁵¹. In our particular case, LacA preparation was also provided as a first step with 16 % glycerol as stabilizer; in consequence, galactosyl derivatives of glycerol could be formed from hydrolysis of lactose or lactulose and transgalactosylation of glycerol catalyzed by the β -galactosidase activity of LacA. Indeed, the formation of galactosyl-glycerols was higher than the formation of the most abundant GOS (**Figure 4B**), with a concentration of 0.43 and 2.80 g L⁻¹ of 2-O- β -D-galactopyranosyl-glycerol and 1-O- β -D-galactopyranosyl-glycerol, respectively. To the best of our knowledge, this is the first time that galactosyl-polyol formation has been described for LacA from *Lactobacillus plantarum* WCFS1. This fact could suggest that this protein may have a transgalactosylation preference for alternative acceptors, such as polyols, rather than lactose.

In addition, GOS formation from hydrolysis and transgalactosylation of lactose was also detected (**Figure 4B**) corresponding to di- (multiple peaks labelled as 9) and trisaccharides (multiple peaks labelled as 10); although the efficiency of their synthesis was lower than that observed for galactosyl-glycerol formation. Our finding is in agreement with the low transglycosylation activity ascribed to β -galactosidases from GH 42 family to synthesize GOS⁵². Despite some exceptions for GH 42 β -galactosidases from *Bifidobacterium* subsp have been reported⁵³⁻⁵⁴, site-directed mutagenesis has had to be applied to enhance the GOS production through biosynthesis catalyzed by GH 42 β -galactosidases.⁵⁵⁻⁵⁶

3.3 Transgalactosylation activity of LacA protein on reaction mixtures of lactose and selected polyols

Firstly, hydrolysis and transgalactosylation reactions of lactose (acting both as acceptor and donor) were carried out using LacA β -galactosidase preparation without glycerol. Hydrolysis of lactose was around 64% at 48 hours of reaction; however, low levels of di- and trisaccharides GOS were produced (10.6 g L⁻¹ and 3.4 g L⁻¹, respectively). Therefore, LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 displayed high hydrolytic activity but low capability of transgalactosylation when lactose is used as a single substrate acting both as donor and acceptor.

Secondly, different assays using reaction mixtures containing lactose and diverse polyols (sorbitol, mannitol, xylitol and erythritol) were performed. In mixtures of lactose:mannitol or lactose:sorbitol (20%:20%, w:w) no formation of galactosyl-polyols derivatives was detected. In contrast to these results, other studies reported galactosyl-sorbitol formation using β -galactosidases from fungal origin (*Aspergillus oryzae*, *Kluyveromyces lactis* and *Kluyveromyces fragilis*)^{10,29,30}. Besides, a study revealed the synthesis of galactosyl-mannitols using a β -galactosidase from *Kluyveromyces lactis*¹¹. Previous studies revealed that molecules of polyol of small size have a greater chance to diffuse to the active sites of the enzyme and synthesize galactosyl-polyols³⁰. Thus, LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 could not favor the synthesis of galactosyl-derivatives when polyols having six carbons in their structure, as the case of the isomers mannitol and sorbitol, are acting as acceptors, whereas glycerol, erythritol and xylitol containing three, four and five carbons, respectively, were efficiently transgalactosylated.

3.3.1 Formation of galactosyl derivatives of erythritol

Transgalactosylation reactions using lactose and erythritol (20%:20%, w:w) catalyzed by LacA β -galactosidase from *L. plantarum* WCFS1 (1.16 U mL⁻¹) were carried out, and representative GC-FID profiles at 0 and 48 h of reaction are shown in

Figure 5A. As expected, the presence of major compounds corresponding to erythritol (peak 1) and lactose (peak 10) were detected and galactose (peak 3) and glucose (peak 4) were also found as a consequence of hydrolysis of lactose. Di- (multiple peaks labelled as 11) and trisaccharide GOS (multiple peaks labelled as 12) were also detected, although at low levels, as a result of transglycosylation of lactose by LacA β galactosidase activity. Interestingly, a series of unknown and newly detected peaks (6 and 7) eluting near to the disaccharide region could correspond to galactosyl derivatives of erythritol as a result of the transfer of the galactosyl residue released from the hydrolysis of lactose to erythritol. They could be tentatively assigned as (2R, 3S)- and (2S and 3R)-4-O- β -galactopyranosyl-erythritol based on structural data of compounds previously reported following the transgalactosylation of erythritol using immobilized β -galactosidase from *Aspergillus oryzae*¹⁰.

The kinetic behaviour of erythritol:lactose mixture (20%:20%, w:w) throughout the transgalactosylation reaction is shown in **Figure 6 (A-B)**. Lactose was efficiently hydrolyzed (only 36.5% of this disaccharide remained intact after 48 hours of reaction) and the highest hydrolysis rate took place mainly from the eighth hour of reaction, releasing, concomitantly, galactose and glucose (**Figure 6A**). In the case of the sugar alcohol, a moderate decrease of 9% was quantified by GC-FID. GOS di- and trisaccharides were formed in similar amounts but at very low levels, whereas galactosyl derivatives of erythritol were more predominant, especially that labelled as 2 (maximum levels of 20.7 g L⁻¹, **Figure 6B**), which corresponded to peak 7 (**Figure 5A**), as compared to that labelled as 1 (9.7 g L⁻¹) and corresponding to peak 6 in **Figure 5A**. The maximum formation rate of the major galactosyl-erythritol compound took place in the time period from 8 to 24 hours to, then, reached a plateau until the end of the

reaction, whereas the minor galactosyl-erythritol compound exhibited a moderate but progressive synthesis throughout the enzymatic reaction (**Figure 6B**).

In order to study the effect of initial concentration of erythritol in the formation of their galactosyl derivatives, two additional sets of experiments with mixtures of erythritol:lactose 30%:20% (w:w) and 40%:20% (w:w) were carried out and results are shown in **Figure 6 (C-F)**. Lactose progressively decreased in the two studied enzymatic mixtures, whilst galactosyl derivatives of erythritol formed due to the transfer of the galactosyl residue of lactose to erythritol increased progressively when higher starting concentrations of erythritol were used. Thus, in reactions using 30 and 40% of erythritol, galactosyl-erythritol 2 showed a constant increase until 48 hours (28.8 g L⁻¹ and 30.1 g L⁻¹, respectively) (**Figure 6D-F**). This increase was in line with a more pronounced decrease percentage in the concentration of erythritol as compared to enzymatic reactions performed with initial concentration of erythritol:lactose of 20%:20% (w:w). Likewise, no decrease in the content of the galactosyl-erythritols compounds was observed in all reactions tested, indicating that either they were not hydrolyzed by LacA or their synthesis prevailed over their potential hydrolysis.

The increase in the initial concentration of erythritol acting as acceptor promoted significantly the synthesis of galactosyl derivatives of erythritol, reaching a maximum yield (expressed as a percentage (w:w) of the initial amount of lactose) of 17.8% (40%:20%) and 14.4% (30%:20%) at 48 hours of reaction, as compared to 10.7% in reactions using 20%:20% (w:w) mixtures. These values were also converted to percentage (w:w) of the total dry matter of the reaction mixture, which ranged from 7.4 to 8.0%, to ensure an appropriate comparison to those reported by Klewicki et al ³⁰ who showed a maximum content of galactosyl derivatives of erythritol of 17.3%, 18.6% and 15.3% with β -galactosidases from *Kluyveromyces lactis*, *Kluyveromyces fragilis* and

Aspergillus oryzae, respectively, using concentrations of 40%:20% of erythritol:lactose (w:w). In consequence, both the source of β -galactosidase and, specially, the initial concentration of polyol could affect the production of galactosyl derivatives of erythritol.

Finally, the percentage of galactosyl derivatives of erythritol with respect to all transfer products formed in reaction at 48 h was similar in reactions using 30% or 40% as starting concentration of erythritol (77 and 78%, respectively) as compared to reactions with 20% (73%). Likewise, the content of GOS found in all assays was similar in all reactions because the same concentration of lactose was used in all cases and, therefore, a potential inhibitory effect of erythritol concentration in the GOS synthesis could be ruled out.

3.3.2 Formation of galactosyl derivatives of xylitol

Figure 5B shows a chromatographic profile of a reaction mixture of xylitol:lactose (20%:20%, w:w) and LacA β -galactosidase from *L. plantarum* WCFS1 (1.16 U mL⁻¹) at 0 and 48 h of reaction. In addition to the starting substrates xylitol (peak 2) and lactose (peak 10), galactose (peak 3) and glucose (peak 4) were also found as a consequence of the hydrolysis of lactose. Di- (peak 11) and trisaccharides (peak 12) GOS were also detected as a result of transglycosylation of lactose at much lower abundances than several unknown compounds labelled as 8 (composed of three chromatographic peaks) and 9 (including two peaks) which could correspond to galactosyl derivatives of xylitol formed by transglycosylation catalyzed by LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 using galactosyl residues derived from lactose hydrolysis.

Figure 7 (A-B) shows the evolution of lactose and xylitol content, released monosaccharides, and synthesized galactosyl-polyols throughout course reaction (48 h) on xylitol:lactose mixtures (20%:20%,w:w) with LacA β galactosidase from *Lactobacillus plantarum* WCFS1. Remarkably, this β galactosidase displayed high hydrolytic activity towards lactose, considering a hydrolysis degree of 75% after 48 hours of reaction and the subsequent release of monosaccharides. By considering the ability of xylitol as the acceptor of galactose units released from lactose, new compounds, labelled as Gal-xylitol 1 and 2 in **Figure 7B** and corresponding to GC peaks 8 and 9, respectively, (**Figure 5B**) were detected from the second hour of reaction, reaching the maximum content (9.4 and 42.1 g L⁻¹) at the end of reaction. However, the production of GOS di- and trisaccharides was observed at very low quantities.

Lactose was also efficiently hydrolyzed (85%) by LacA β galactosidase when 30 or 45% (w:w) of starting xylitol was used (**Figure 7C and E**). Regarding transfer products, the increase in the content of xylitol (30% or 45%, w:w) promoted the synthesis of xylitol-derivatives, being around 3-4 folds superior to the formation of galactooligosaccharides (**Figure 7D and F**). The concentration of the major galactosyl-xylitol compound increased progressively to 53.3 and 57.7 g L⁻¹ (using 30 and 45% of starting xylitol, respectively) whereas the other detected galactosyl-xylitol compound experimented an increase up to 18.2 and 20.8 g L⁻¹ when 30 and 45% of xylitol was used at the beginning of the enzymatic reaction. Following the conversion of the concentration values to percentage (w:w) of the total dry matter of the reaction mixture, the maximum content of galactosyl derivatives of xylitol were within the range from 12.3 to 13.4%.

In a similar way to the synthesis of Gal-erythritol, the increase in the initial concentration of xylitol promoted significantly the synthesis of their galactosylated derivatives, reaching a maximum yield (expressed as a percentage (w:w) of the initial amount of lactose) of 24.4% (45%:20%, xylitol:lactose) and 23.7% (30%:20%, xylitol:lactose) at 48 hours of reaction, as compared to 18.0% in reactions using 20%:20% (w:w) mixtures. In consequence, our maximum reported synthesis yields were similar to those obtained from another study using β -galactosidases from *Kluyveromyces lactis*, *Kluyveromyces fragilis* and *Aspergillus oryzae*, whose maximum yields varied from 12 to 21% (expressed as a percentage of the initial amount of ONPG as a galactosyl donor)³⁰. Therefore, these results confirmed that LacA from *Lactobacillus plantarum* WCFS1 displayed high transgalactosylation activity, being capable of synthesizing galactosylated derivatives of xylitol at a similar level as selected enzymes from molds and yeasts.

Finally, the percentage of galactosyl derivatives of xylitol with respect to all transfer products formed was barely similar in reactions using 20% or 30% of xylitol (76 and 77%, respectively), reaching 82% when the initial concentration of xylitol was 45%.

Therefore, LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 displayed preference for acceptors such as glycerol (**Figure 4**), erythritol (**Figure 6**) or xylitol (**Figure 7**) over lactose when mixtures of lactose/polyol were used.

3.3.3 NMR characterization of galactosyl derivatives of xylitol

Two fractions (i.e., **A** and **B**) containing galactosyl xylitol **1** and **2** respectively were isolated by HPLC (**Figure S1**) and fully characterized by an extensive NMR study. Complete structural elucidation of these two fractions was carried out by the combined use of 1D and 2D [¹H-¹H] and [¹H-¹³C] NMR experiments (gCOSY, TOCSY,

multiplicity-edited gHSQC, TOCSY-HSQC and gHMBC). ^{13}C NMR assignments for identified compounds are given in **Table S1**. A full set of spectra are collected in the Supporting Information (**Figures S2-S15**).

For fraction **A**, the ^1H NMR and ^{13}C spectra showed the existence of a complex mixture. So in the ^{13}C spectrum a major compound and seven minor compounds with different populations were identified. By comparison of ^{13}C spectrum with data from the published literature^{57,58}, the minor compounds were β - and α - D-lactose, β - and α - D-Gal, β - and α - D-Glc, and xylitol. The major compound was an unknown compound (**1**). ^{13}C NMR spectrum of **1** displayed 11 signals. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances, indicating the presence of three methylene and eight methine carbons. From these results, we determined the existence of an anomeric proton at δH 4.49. gCOSY, TOCSY experiments revealed the presence of a sugar moiety, which was determined to be galactose with a β configuration [$J(\text{H1}, \text{H2}) = 7.7 \text{ Hz}$]. In addition, the existence of a xylitol moiety was detected. gHSQC-TOCSY experiment was used for assigning all carbons in the molecule. The linkage between the xylitol unit and the sugar was established from gHMBC correlations between the anomeric proton at δH 4.49 and the xylitol methyne central carbon at δC 79.61 and between the xylitol proton at δH 3.90 and the anomeric carbon at δC 103.84 (**Figure 8**). Consequently, the structure **3-O- β -D-galactopyranosyl-xylitol** was deduced for the unknown compound **1**.

Following the same procedure, the ^1H NMR and ^{13}C spectra of fraction **B** showed the existence of two new similar compounds (**2a** and **2b**) in a 70:30 ratio. In addition, small quantities of β - and α - D-lactose were observed. For both compounds, ^1H and ^{13}C NMR spectra were similar to that of compound **1**. gCOSY, TOCSY and gHSQC spectra were consistent with the presence of a β -galactosyl configuration [J

(H1_{2a}, H2_{2a}) = 7.8 Hz, J (H1_{2b}, H2_{2b}) = 7.8 Hz] and a xylitol moiety for each isomer. For both isomers, the linkage between xylitol and galactose moieties was established from gHMBC correlations (**Figure 8**) So, relevant correlation peaks between the galactosyl anomeric proton (δ H1_{2a} 4.426 and δ H1_{2b} 4.428) and the methylene xylitol moiety carbon (δ C1'_{2a} 70.93 and δ C1'_{2b} 71.28) and between the corresponding methylene protons (δ H1'_{2a} 4.01, 3.84 and δ H1'_{2b} 4.07, 3.75) and the galactosyl anomeric carbon (δ C1_{2a} 103.12 and δ C1_{2b} 103.42). Taking into account that glycosylation would make the xylitol residue asymmetric, which would thus lead to the formation of a mixture of two diastereomeric galactosyl-xylitol molecules. Consequently, the structure **(2R)-**and **(2S)-1-O- β -D-galactopyranosyl-xylitol** was deduced for the unknown compound **2** (a and b).

In conclusion, this work describes an efficient synthesis of galactosyl polyols through hydrolysis of lactose and transgalactosylation of xylitol and erythritol using a biochemically characterized LacA β -D-galactosidase from *Lactobacillus plantarum* WCFS1. Remarkably, LacA exhibited a great preference towards both polyols acting as acceptors as compared to lactose leading to a much higher production of galactosyl polyols than conventional GOS. Xylitol and erythritol are widely used in a variety of food and oral care products as sugar replacers but their usefulness can be boosted further by gathering scientific evidence that highlights a number of health benefits, including hypoglycemic, anti-hyperglycemic, anti-diabetic and anti-obesogenic effects¹⁵. In this context, the efficient galactosylation of monomer polyols, as that demonstrated in our work, could reduce their absorption in the small intestine and, consequently, enhance their fermentative properties by making them readily available for the colonic microbiota. The knowledge of the detailed structure of the newly

synthesized galactosyl-polyols is of crucial importance to better elucidate their possible functional applications, such as novel prebiotics, sweeteners or bulking agents, as well as anticariogenic or hypocaloric compounds. Further studies are warranted to confirm the low digestibility of these galactosyl-derivatives, as well as the suitable immobilization of LacA β -galactosidase from *Lactobacillus plantarum* WCFS1. In addition, different approaches leading to the potential enhancement of LacA transgalactosylation activity, such as nucleophile mutagenesis or the addition of several ions, could be also carried out. These data would add value for maximizing the production of these novel compounds and also for supporting their potential incorporation into functional and/or conventional food products.

4. Abbreviations

GOS, galactooligosaccharides; Gal-polyols, galactosyl derivatives of polyols; LB, Luria-Bertani; IPTG, isopropyl- β -D-thiogalactopyranoside; *p*NP, *p*-nitrophenolate; DTT, dithiothreitol; MOPS, 3-(N-morpholino) propanesulfonic acid.

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7. Supporting Information

HPLC-RID chromatographic profile of transgalactosylation reaction based on lactose:xylitol at 20%:20% (**Figure S1**); ^{13}C NMR assignments (**Table S1**) and a full set of spectra for identified compounds (**Figures S2-S15**) (PDF).

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FIGURE CAPTIONS

Figure 1. Purification of LacA β -galactosidase from *L. plantarum* WCFS1. SDS-PAGE analysis LacA protein eluted after affinity resin. The gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE Standards, Bio-Rad).

Figure 2. Biochemical properties of LacA β -galactosidase from *L. plantarum* WCFS1. (A) LacA pH activity profile. (B) LacA temperature activity profile. (C) LacA thermal stability after incubation at 22 °C (◆), 30 °C (■), 37 °C (▲), 45 °C (x) and 65 °C (●) in MOPS buffer (50 mM, 20 mM NaCl, 1 mM DTT, pH 7) at indicated times. The mean value and standard error are shown (n=3). The percentage of residual activity was calculated by comparing to the non-incubated enzyme.

Figure 3. Effect of additives at 1mM on *L. plantarum* LacA β -galactosidase relative activity. The activity of LacA in the absence of additives was defined as 100%. The mean value and standard error are shown (n=3).

Figure 4. GC-FID profiles resulting from the hydrolysis of lactulose (a) and lactose (b) at an initial concentration of 200 g L⁻¹ and at 0 (dashed line) and 24 hours (dark line) of reaction catalyzed by LacA protein (lp_3469) from *Lactobacillus plantarum* WCFS1 (1.16 U mL⁻¹). Peaks identification: 1, fructose; 2, galactose; 3, glucose; 4, 2-O- β -D-galactosyl-glycerol; 5, internal standard; 6, 1-O- β -D-galactosyl-glycerol; 7, lactulose; 8, lactose; 9, GOS (disaccharides); 10, GOS (trisaccharides).

Figure 5. GC-FID profiles of hydrolysates of mixtures of (a) erythritol: lactose 20%:20% and (b) xylitol: lactose 20%:20% (200 g L⁻¹ each) after 0 (dashed line) and 48 hours (dark line) of reaction using Lac A β -galactosidase from *Lactobacillus plantarum* WCFS1 (1.16 U mL⁻¹) at 37°C. Peak identification: 1, erythritol; 2, xylitol; 3, galactose; 4, glucose; 5, internal standard; 6, galactosyl-erythritol 1; 7, galactosyl-erythritol 2; 8,

galactosyl-xylitol 1; 9, galactosyl-xylitol 2; 10, lactose; 11, GOS (disaccharides); 12, GOS (trisaccharides).

Figure 6. Concentration of monosaccharides, erythritol, lactose, and formed GOS (galactooligosaccharides) and galactosyl-erythritol derivatives upon transgalactosylation reactions catalyzed by LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 (1.16 U mL⁻¹) at 37°C using as starting substrate mixtures of erythritol:lactose 20%:20% (**a and b**), 30%:20% (**c and d**) and 40%:20% (w:w) (**e and f**). Gal-erythritol 1 and Gal-erythritol 2 are the peaks labelled as 6 and 7, respectively, in GC-chromatograms (Figure 5a). Vertical errors represented standard deviations (n=3).

Figure 7. Concentration of monosaccharides, xylitol, lactose, formed GOS (galactooligosaccharides) and galactosyl-xylitol derivatives upon transgalactosylation reaction catalyzed by LacA β -galactosidase from *Lactobacillus plantarum* WCFS1 (1.16 U mL⁻¹) at 37°C using as starting substrate mixtures of xylitol:lactose 20%:20% (**a and b**), 30%:20% (**c and d**) and 45%:20% (w:w) (**e and f**), as starting substrate. Gal-xylitol 1 and Gal-xylitol 2 are the peaks labelled as 8 and 9, respectively, in GC-chromatograms (Figure 5b), Vertical errors represented standard deviations (n=3).

Figure 8. Structure of compounds **1-2**. Relevant interglycosidic gHMBC NMR correlations are indicated by arrows. Compound **1**: 3-O- β -D-galactopyranosyl-xylitol; Compound **2**: (2R)- and (2S)-1-O- β -D-galactopyranosyl-xylitol.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure

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Figure 6.

Figure

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Figure 8.

9. For Table of Contents only