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Human milk and mucosal lacto- and galacto-*N*-biose synthesis by transgalactosylation and their prebiotic potential in *Lactobacillus* species

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Abstract Lacto-*N*-biose (LNB) and galacto-*N*-biose (GNB) are major building blocks of free oligosaccharides and glycan moieties of glyco-complexes present in human milk and gastrointestinal mucosa. We have previously characterized the phospho-β-galactosidase GnbG from *Lactobacillus casei* BL23 that is involved in the metabolism of LNB and GNB. GnbG has been used here in transglycosylation reactions, and it showed the production of LNB and GNB with *N*-acetylglucosamine and *N*-acetylgalactosamine as acceptors, respectively. The reaction kinetics demonstrated that GnbG can convert 69 ± 4 and 71 ± 1 % of *o*-nitrophenyl-β-D-galactopyranoside into LNB and GNB, respectively. Those reactions were performed in a semi-preparative scale, and the synthesized disaccharides were purified. The maximum yield obtained for LNB was 10.7 ± 0.2 g/l and for GNB was 10.8 ± 0.3 g/l. NMR spectroscopy confirmed the molecular structures of both carbohydrates and the absence of reaction byproducts, which also supports that GnbG is specific for β1,3-glycosidic linkages. The purified sugars were subsequently tested for their potential prebiotic properties using *Lactobacillus* species. The results showed that LNB and GNB were fermented by the tested strains of *L. casei*, *Lactobacillus rhamnosus* (except *L. rhamnosus* strain ATCC 53103), *Lactobacillus zeae*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*. DNA hybridization experiments suggested that the metabolism of those disaccharides in 9 out of 10 *L. casei* strains, all *L. rhamnosus* strains and all *L. zeae* strains tested relies upon a phospho-β-galactosidase homologous to GnbG. The results presented here support the putative role of human milk oligosaccharides for selective enrichment of beneficial intestinal microbiota in breast-fed infants.

Keywords Lacto-*N*-biose · Galacto-*N*-biose · Human milk oligosaccharides · Transglycosylation · *Lactobacillus* · Prebiotic

Introduction

The biological role of the high amount of oligosaccharides present at human milk (human milk oligosaccharides, HMOs) is largely unknown (Bode 2012; Garrido et al. 2013; Thurl et al. 2010). HMOs are not digested, and therefore, they reach the infant colon (Engfer et al. 2000). In this environment, it has been suggested that those oligosaccharides are metabolized by the resident microbiota, and that they can act as prebiotic substrates. Lacto-*N*-biose I (LNB; Gal-β1–3GlcNAc) is the main building block of type-1 HMOs, and it has also been found as a free sugar in human milk (Balogh et al. 2015). This disaccharide is fermented in vitro by all the tested strains of *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *infantis, B. longum* subsp. *longum*, and *Bifidobacterium breve*, and by some strains of *Bifidobacterium pseudocatenulatum*, *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium pseudolongum* (Xiao et al. 2010). Additionally, fermentation of LNB using stool samples from infants showed a significantly promoted

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growth of *B. bifidum* (Satoh et al. 2013). Other important disaccharide is galacto-*N*-biose (GNB; Gal-β1–3GalNAc), which is commonly found in glyco-complexes at the human mucosal surfaces, and it also constitutes the functional structure of the T-antigen (Liu and Newburg 2013; Moran et al. 2011; Roy and Baek 2002). Regarding the metabolism of LNB and GNB by *Bifidobacterium* species, it has been shown that *B. bifidum* cleaves lacto-*N*-tetraose (LNT) in the culture supernatant by the action of a lacto-*N*-biosidase. The LNB generated, as well as GNB, are transported inside the cells and then metabolize by a phosphorylase enzyme (Wada et al. 2008). By contrast, *B. longum* subsp. *infantis* transports LNT inside the cells and hydrolyzes this tetrasaccharide into galactose and lacto-*N*-triose by a specific β-1,3-galactosidase (Yoshida et al. 2012).

In addition to *Bibidobacterium*, the other genus with many recognized probiotic strains is *Lactobacillus* (Hill et al. 2014; Turroni et al. 2014). Species of this group are common inhabitants of the human gastrointestinal tract, and they are already found at early stages of life. Regarding this, strains of *Lactobacillus casei*, *Lactobacillus gasseri*, and *Lactobacillus rhamnosus* are frequently isolated from breast-fed infant feces (Albesharat et al. 2011; Martin et al. 2007; Rubio et al. 2014). Positive probiotic effects of *Lactobacillus* species have been shown on immune system stimulation, atopic disease, and several types of diarrhea in children among others (Ashraf and Shah 2014; Kalliomaki et al. 2003; Pedone et al. 2000; Rodriguez-Diaz and Monedero 2013; Szajewska et al. 2013). We have previously demonstrated that fucosyl-*N*-acetylglucosamine disaccharides, that form part of HMOs and also part of glyco-complexes from the mucosa, were fermented by strains of *L. casei*, *L. rhamnosus*, *B. breve*, and *B. pseudocatenulatum* (Becerra et al. 2015), suggesting a potential prebiotic effect for those carbohydrates. Additionally, we have characterized in *L. casei* strain BL23 the *gnb* operon (*gnbREFGBCDA*) that is involved in the transport and catabolism of LNB and GNB (Bidart et al. 2014). Both disaccharides are transported and phosphorylated by a specific phosphoenolpyruvate: sugar phosphotransferase system, and then they are hydrolyzed into galactose-6P and the corresponding *N*-acetylhexosamine by the phospho-β-galactosidase encoded by *gnbG* gene. These results and the above described metabolism of LNB and GNB by *Bifidobacterium* species support the utilization of both disaccharides as potential prebiotics. To completely determine the biological function of LNB and GNB as prebiotics, further investigation is needed, and consequently, synthesis of sufficient amounts of them would be necessary. Chemical synthesis of LNB, GNB, and LNB-containing oligosaccharides such as Lewis a and b blood group antigens has long been performed. However, several protection and de-protection stages of the carbonyl groups are necessary in order to get the proper selectivity, and hence, the chemical synthesis became a time-consuming and expensive process (Lemieux and Driguez 1975; Wilstermann and Magnusson 1995). As an alternative, the use of enzymes to synthesize HMOs has the convenience of catabolizing specific glycosidic bonds in one step and independently of other carbonyl groups. LNB and GNB have been previously synthetized by the transglycosylation activity of β-galactosidases (Fujimoto et al. 1998; Vetere et al. 2000) and by multi-enzymatic systems (Nishimoto and Kitaoka 2007b; Nishimoto and Kitaoka 2009; Yu et al. 2010). In the present work, we have utilized the phospho-β-galactosidase GnbG from *L. casei* BL23 (Bidart et al. 2014) in transgalactosylation reactions with *N*-acetylglucosamine and *N*-acetylgalactosamine as acceptors and showed that it very efficiently synthesized LNB and GNB, respectively. Afterward, the reactions were performed in a semi-preparative scale and the produced disaccharides were purified and analyzed for their in vitro fermentation by *Lactobacillus* species.

Materials and methods

Transgalactosylation activity assay

GnbG phospho-β-galactosidase (GH35 family) was expressed with a His-tag and purified as previously described (Bidart et al. 2014). Transgalactosilation activity of GnbG was analyzed in a solution containing 100 mM Tris-HCl buffer, pH 7.5, *o*-nitrophenyl β-D-galactopyranoside (*o*NP-gal) 40 mM (donor) and *N*-acetylglucosamine (GlcNAc) 200 mM or *N*-acetylgalactosamine (GalNAc) 200 mM (acceptors). After the addition of GnbG to 0.17 U/ml (one unit was defined as the amount of enzyme able to release 1 μmol of *o*NP in 1 min at 42 °C, pH 7.5 and with *o*NP-gal 5 mM), the reactions were incubated at 42 °C and samples were withdrawn at different times and immediately heated at 100 °C for 3 min to stop the reaction.

Transgalactosylation reactions aimed at the purification of the produced disaccharides were performed in the conditions described above for 3 h, and they were heat-treated to stop the reaction.

Analytical and semi-preparative HPLC analysis

The transgalactosylation reaction products were analyzed and purified by HPLC (Jasco PU2080Plus system) using analytical and preparative Rezex RCM-Monosaccharide columns (Phenomenex, Torrance, CA, USA), respectively, as described previously (Becerra et al. 2015). Di- and monosaccharides were confirmed by comparison of their retention times with those of standards (LNB, GNB, galactose, GlcNAc, and GalNAc). LNB and GNB were obtained from Carbosynth Ltd. (Compton, Berkshire, UK).

Nuclear magnetic resonance spectroscopy

Lyophilized samples were dissolved in 550 μl of deuterated water and analyzed immediately. Nuclear magnetic resonance (NMR) spectra were recorded at 27 °C using a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm TCI cryoprobe. One-dimensional (1D) ^{1}H spectra with presaturation and a noesy mixing period for water suppression and improved baseline were acquired with 256 transients. ^{1}H- ^{13}C heteronuclear single quantum coherence (HSQC) experiments were acquired with 200 transients over a spectral width of 3.5 (for ^{1}H) and 30 ppm (for ^{13}C) and 128 points in the indirect dimension. Total correlation spectroscopy (TOCSY) experiments were acquired with 32 transients over a spectral width of 3.5 ppm in both dimensions and 128 points in the indirect dimension. NMR spectra were processed using the program Topspin3.2 (Bruker Biospin).

Microorganisms and culture media

The *Lactobacillus* strains used in this study are listed in Table 1. They were routinely grown at 37 °C under static conditions in MRS medium (Difco, Sparks, MD, USA). The recombinant *Escherichia coli* strain PE159 that expresses the phospho-β-galactosidase GnbG (Bidart et al. 2014) was grown in Luria-Bertani (LB) medium (Pronadisa, Madrid, Spain) with ampicillin (100 μg/ml) at 37 °C under vigorous shaking. The corresponding solid media were prepared by adding 1.8 % agar.

Culture of *Lactobacillus* strains with lacto-*N*-biose and galacto-*N*-biose

The *Lactobacillus* strains were grown at 37 °C under static conditions in MRS basal medium as previously described (Becerra et al. 2015). Lacto-*N*-biose (LNB) and galacto-*N*-biose (GNB) utilization was determined by measuring growth in 96-well microtiter plates in a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany) at 37 °C under static conditions. Each well contained 100 μl of basal MRS plus 4 mM of purified LNB or GNB. At least three independent replicates for each condition were obtained. The carbohydrates utilization at the end of the fermentation was analyzed by HPLC as described above in filtered culture supernatants.

Southern blot analysis

Total DNA was isolated from *Lactobacillus* strains with the REALPURE "SSS" genomic DNA extraction kit (Durviz, Paterna, Spain). A *gnbG* digoxigenin-labelled probe was obtained by PCR with oligonucleotides LNB5Fow (5′-TCG ATC GAT CAT GAG TTT ATG-3′) and LNB5Rev (5′-TGC CTC ATC GCT GGC TTG-3′) and the PCR Digoxigenin-labeling Mix (Roche) using Taq DNA polymerase and *L. casei* BL23 DNA as template. Chromosomal DNA from the *Lactobacillus* strains was digested with *Eco*RV and separated in a 0.8 % agarose gel. The digested DNA was transferred to Hybond-N+ membranes (GE Healthcare) as previously described (Sambrook et al. 1989). The membranes were hybridized

Table 1 List of *Lactobacillus* strains used in this study

Species	Strain[a]	Culture collection[b]
Lactobacillus rhamnosus	BL1	CECT 278
Lactobacillus rhamnosus	BL2	CECT 276
Lactobacillus rhamnosus	BL3	CECT 275
Lactobacillus plantarum	BL8	CECT 748
Lactobacillus curvatus	BL14	CECT 904
Lactobacillus acidophilus	BL17	CECT 903
Lactobacillus casei	BL23	CECT 5275
Lactobacillus casei	BL28	64 H
Lactobacillus casei	BL32	CECT 4040
Lactobacillus delbruckii subsp. *bulgaricus*	BL33	CECT 4005
Lactobacillus pentosus	BL35	CECT 4023
Lactobacillus casei	BL82	CECT 277
Lactobacillus casei	BL83	CECT 4043
Lactobacillus casei	BL86	CECT 4045
Lactobacillus casei	BL87	ATCC 11578
Lactobacillus casei	BL91	CECT 4646
Lactobacillus casei	BL93	61 BG
Lactobacillus casei	BL94	ATCC 393
Lactobacillus zeae	BL95	ATCC 15820
Lactobacillus salivarius	BL158	CECT 4063
Lactobacillus johnsonii	BL261	AD99
Lactobacillus plantarum	BL166	NCIMB 8826
Lactobacillus zeae	BL257	IFI-CA
Lactobacillus gasseri	BL277	ATCC33323
Lactobacillus acidophilus	BL280	CECT 4179
Lactobacillus johnsonii	BL281	CECT 289
Lactobacillus johnsonii	BL287	DSM 10533
Lactobacillus intestinalis	BL288	DSM 6629
Lactobacillus rhamnosus	BL327	CECT 288
Lactobacillus taiwanensis	BL340	DSM 21041
Lactobacillus rhamnosus	BL358	HN001
Lactobacillus rhamnosus	BL377	ATCC 53103

[a] BL, Culture Collection from our laboratory

[b] CECT, Spanish Type Culture Collection; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCIMB, National Collection of Industrial, Food and Marine Bacteria, UK; *L. casei* strains 64 H y 61 BG are described in (Gasser and Mandel 1968); *L. rhamnosus* HN001, also known as DR20, is described in (Prasad et al. 1998); *L. zeae* BL257 was isolated from biological aging wine (Moreno-Arribas and Polo 2008). *L. johnsonii* BL261 was isolated from rat intestinal content (Sarmiento-Rubiano et al. 2007)

with *gnbG*, washed, and visualized with CDP-Star chemiluminescent reagent as recommended by the supplier (Roche).

Results

Transglycosylation activity of the phospho-β-galactosidase GnbG

The phospho-β-galactosidase GnbG from *L. casei* BL23 has been previously characterized showing that this enzyme is able to hydrolyze lacto-*N*-biose (LNB) and galacto-*N*-biose (GNB) (Bidart et al. 2014). These substrate specificities conducted us in this work to examine the feasibility of using GnbG to synthesize those disaccharides. We tested the transgalactosylation activity of the 6×(His)GnbG with *N*-acetyl-glucosamine (GlcNAc) or *N*-acetyl-galactosamine (GalNAc) as acceptor and *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) as donor. HPLC analyses suggested that oligosaccharides were being synthesized in each transglycosylation reaction whose retention time coincided with that of LNB and GNB standards, respectively (Fig. S1). To analyze the transglycosylation kinetics of GnbG, a time course reaction was performed and the synthesis of LNB and GNB with GlcNAc and GalNAc as acceptor, respectively, were determined (Fig. 1). The maximum transient yields of 69 ± 4 % for LNB and 71 ± 1 % for GNB with respect to the added *o*-NPG were obtained after 3 h, respectively.

Preparative scale synthesis, purification and characterization of lacto-*N*-biose and galacto-*N*-biose

The transglycosylation reactions described above for GnbG were scaled from microliters to milliliters in the conditions described in the material and methods section. Both reactions using GlcNAc and GalNAc as acceptor, respectively, were independently loaded in a semi-preparative scale ion-exclusion chromatography and the fractions containing LNB and GNB were collected (Fig. S1). The maximum yields obtained of LNB and GNB were 10.7 ± 0.2 and 10.8 ± 0.3 g/l, respectively. The purified LNB and GNB were characterized by NMR spectroscopy and compared with the commercial disaccharides. Analysis of the corresponding 1D ^{1}H and the 2D ^{1}H-^{13}C HSQC spectra (Figs. 2 and 3) revealed identical ^{1}H and ^{13}C chemical shift values for purified and commercial LNB and GNB. In addition, the acquisition of 2D TOCSY experiments (Fig. S2) showed exactly the same spin coupling patterns for the synthetized compounds and the standard samples, and it also showed the lack of any additional signals in the spectra of the purified compounds. These results demonstrated the absence of reaction byproducts in the purified samples and confirmed the glycosidic linkage specificity for both disaccharides. This further indicates that the phospho-β-galactosidase GnbG is specific for β1,3-linkages production.

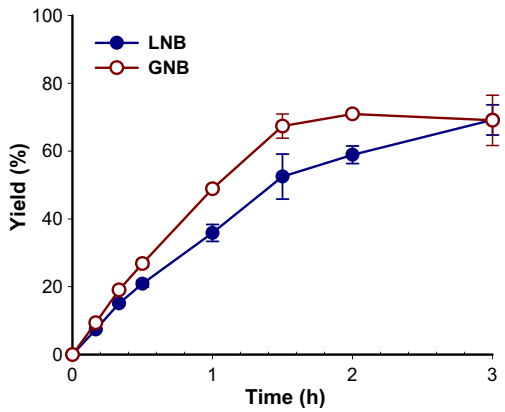


Fig. 1 Time-dependent synthesis of lacto-*N*-biose (*close symbols*) and galacto-*N*-biose (*open symbols*) by transglycosylation catalyzed by the phospho-β-galactosidase GnbG from *Lactobacillus casei*. Yield is expressed as a percentage of the donor concentration (*o*-nitrophenyl-β-D-galactopyranoside 40 mM)

In vitro fermentation of lacto-*N*-biose and galacto-*N*-biose by *Lactobacillus* strains

The utilization of LNB and GNB by *Bifidobacterium* species has been previously reported (Nishimoto and Kitaoka 2007a; Satoh et al. 2013; Xiao et al. 2010). However, the fermentation of these disaccharides by *Lactobacillus*, a genus with also many probiotics strains, has not been tested. Therefore, in order to analyze the capacity of several species of this genus (Table 1) to metabolize LNB and GNB, the growth pattern in MRS basal medium supplemented with these disaccharides synthesized here was determined. No growth was observed in the presence of LNB and GNB for the tested strains of *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Lactobacillus acidophilus*, *Lactobacillus delbruckii* subsp. *Bulgaricus*, *Lactobacillus pentosus*, *Lactobacillus salivarius*, *Lactobacillus intestinalis*, and *Lactobacillus taiwanensis* (data not shown). In contrast, utilization of LNB and GNB was evidenced in strains of *L. casei*, *L. rhamnosus*, *Lactobacillus zeae*, *L. gasseri*, and *Lactobacillus johnsonii*. All the tested strains of *L. casei* were able to grow on LNB and GNB as carbon source (Fig. 4), including strain BL23, whose capacity to ferment both sugars has been recently demonstrated in our lab and it was used here as a positive control (Bidart et al. 2014). Utilization of LNB and GNB was also evidenced in 5 out of 6 strains of *L. rhamnosus* (Fig. 5). No growth difference was observed for *L.rhamnosus* strain BL377 (the widely marketed *L. rhamnosus* GG strain) in the presence of these disaccharides with respect to the culture control without carbohydrate source. Sugar content analysis of the culture supernatants detected LNB and GNB, respectively, in the

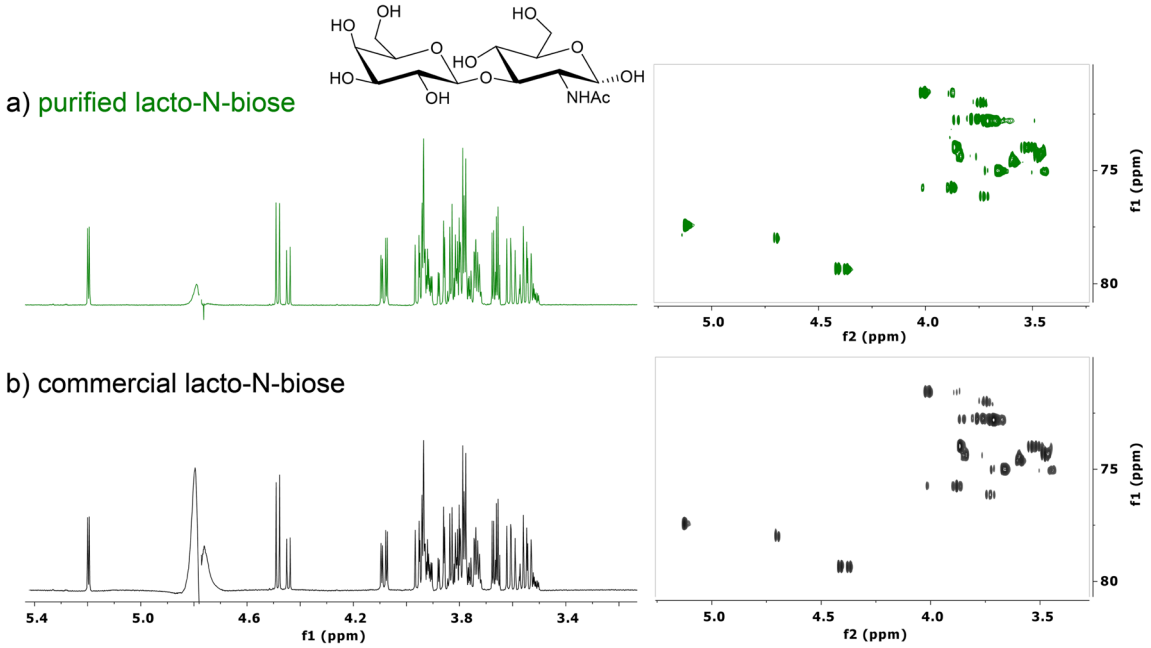


Fig. 2 ^{1}H 1D and 2D heteronuclear single quantum coherence (HSQC) NMR spectra acquired at 600 MHz and 27 °C ^{1}H- ^{13}C of **a** purified lacto-*N*-biose and **b** commercial lacto-*N*-biose

supernatants from strain BL377, while they were consumed by the rest of the strains tested from *L. casei* and *L. rhamnosus* species.

The *L. zeae* (strains BL95 and BL257), *L. gasseri* (strain BL277), and *L. johnsonii* (strains BL261, BL281, and BL287) tested here were also able to grow with LNB and GNB (Fig. 6). Sugar content analysis of the culture supernatants of these strains showed that LNB and GNB were consumed. Curiously, *L. gasseri* and *L. johnsonii* species, which are phylogenetically close (Zhang et al. 2011), presented similar growth patterns, showing an autolytic phenotype. The cell lysis might be due to induction of the already characterized prophages in *L. gasseri* (Baugher et al. 2014; Ismail et al. 2009) and *L. johnsonii* (Denou et al. 2008; Ventura et al.

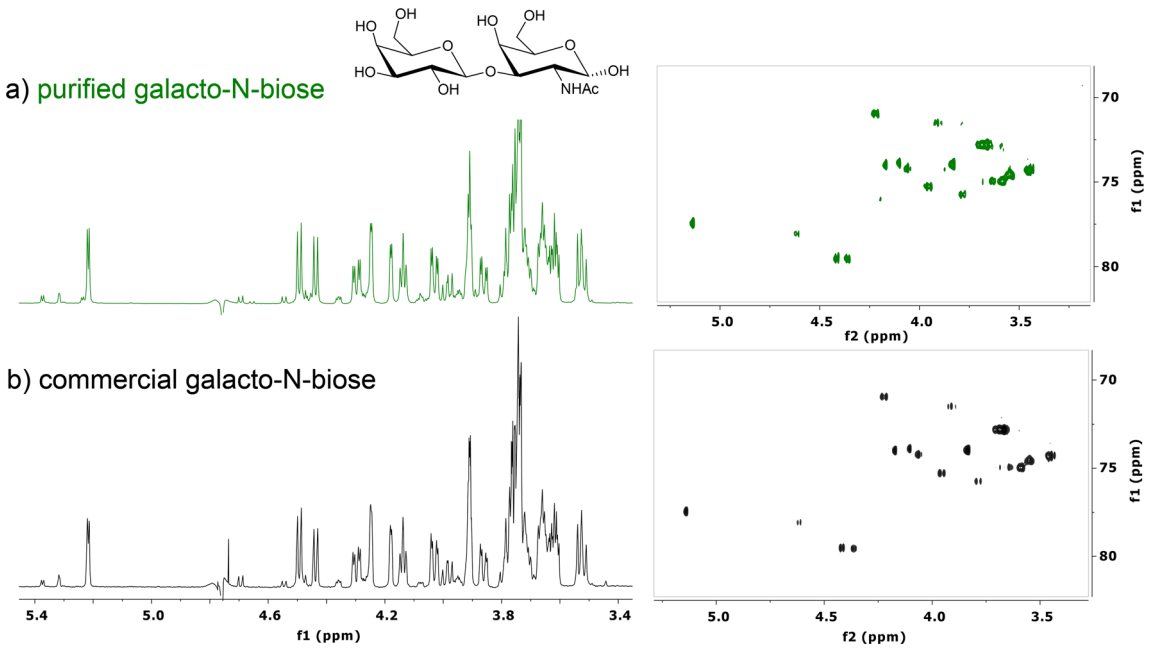
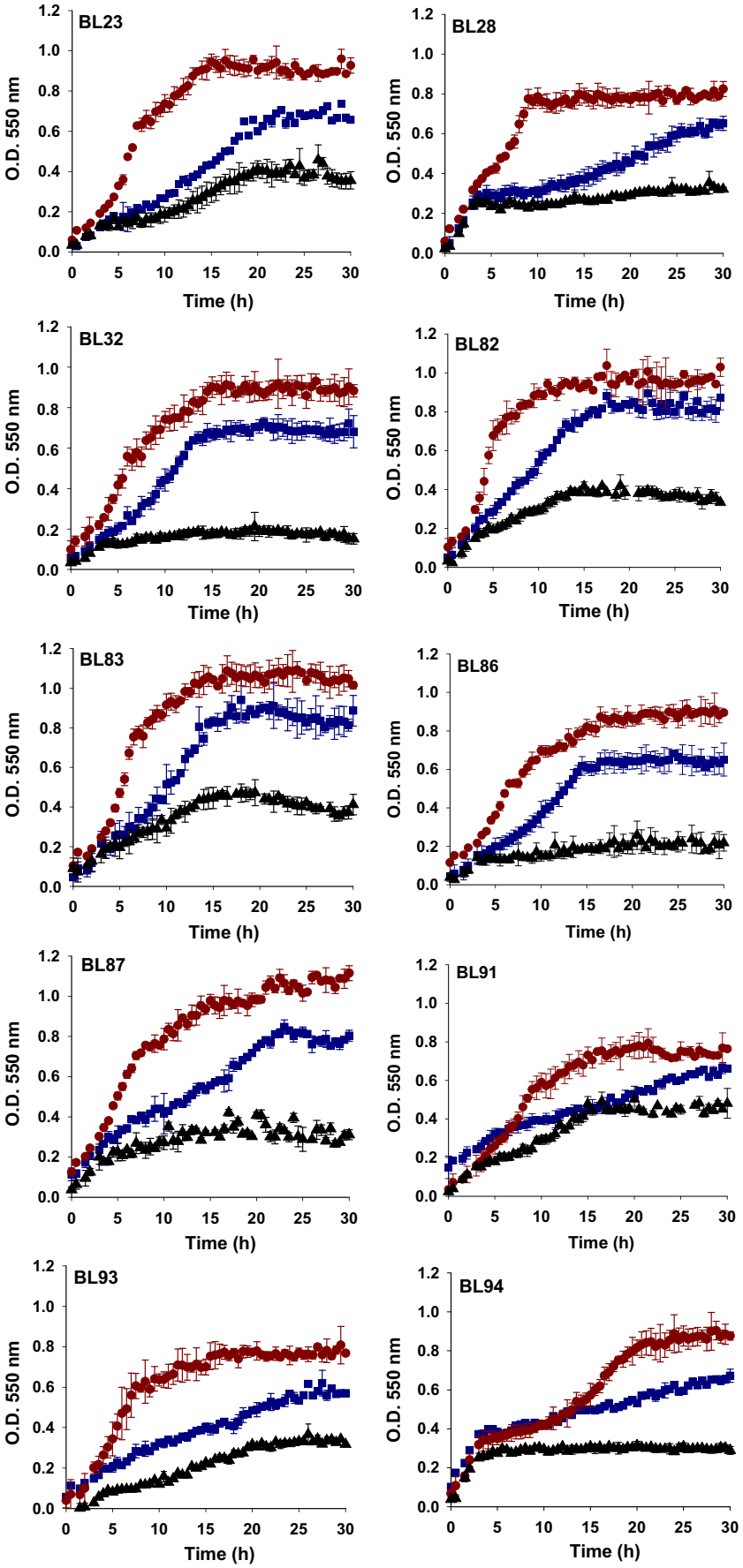


Fig. 3 ^{1}H 1D and 2D heteronuclear single quantum coherence (HSQC) NMR spectra acquired at 600 MHz and 27 °C ^{1}H- ^{13}C of **a** purified galacto-*N*-biose and **b** commercial galacto-N-biose


BL23
BL28
BL32
BL82
BL83
BL86
BL87
BL91
BL93
BL94
O.D. 550 nm
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
20
25
30

◀ **Fig. 4** Growth curves of *Lactobacillus casei* strains BL23, BL28, BL32, BL82, BL83, BL86, BL87, BL91, BL93, and BL94 in MRS basal medium without added carbon source (*triangles*), with lacto-*N*-biose (*squares*), or galacto-*N*-biose (*circles*) as the only carbon source. Data presented are mean values based on at least three replicates. *Error bars* indicate standard deviations

2003). As occurs in the majority of the tested lactobacilli, the *L. johnsonii* strains grew better in GNB than in LNB. In this later sugar, the cells began exponential growth only after 20 (strain BL287) or 40 h (strains BL261 and BL281), suggesting a low induction of the metabolic genes by LNB or the involvement of different metabolic routes for LNB and GNB.

Presence of genes homologous to *gnbG* in *Lactobacillus* strains

We have previously shown that the ability of *L. casei* strain BL23 to grow in the presence of LNB and GNB is dependent upon the activity of the phospho-β-galactosidase GnbG encoded by *gnbG* gene (Bidart et al. 2014). To find out if the above *Lactobacillus* strains that ferment LNB and GNB also contain a homologous *gnbG* gene, Southern blot analysis was performed (Fig. 7). The results demonstrated that the *L. casei* strains (BL28, BL32, BL82, BL83, BL86, BL87, BL91, and BL93), *L. rhamnosus* strains (BL1, BL2, BL3, BL327, and BL358), and *L. zeae* strains (BL95 and BL257) that grow with LNB and GNB as carbon sources presented hybridization signals, suggesting that, in these strains, the metabolism of those disaccharides also relies on a phospho-β-galactosidase homologous to GnbG. The only exception would be *L. rhamnosus* strain BL377 that as mentioned below may carry a non-functional *gnb* operon. *L. casei* strain BL94 (ATCC 393) (Fig. 4), *L. gasseri*, and *L. johnsonii* species (Fig. 6) can be cultured in the presence of LNB or GNB, but in agreement with the lack of *gnbG* homologues in their genomes as described below, no hybridization bands were present (Fig. 7), indicating that the hydrolysis of these carbohydrates must depend on a different glycosidase.

Discussion

Many studies suggest that HMOs play important roles in infant health (Bode 2012); however, the low availability of these oligosaccharides limits the research about their functionality and their eventual application. In the present work, we have utilized the phospho-β-galactosidase GnbG from *L. casei* BL23 (Bidart et al. 2014) in transgalactosylation reactions directed to synthesize LNB and GNB. These are major building blocks of oligosaccharides present in human milk and gastrointestinal mucosa. The transgalactosylation efficiency of GnbG is remarkably high compared with the efficiency of other glycosidases, which usually is moderately ranged from 20 to 40 % (Schmaltz et al. 2011; Trincone 2015). Indeed, LNB and GNB have been previously

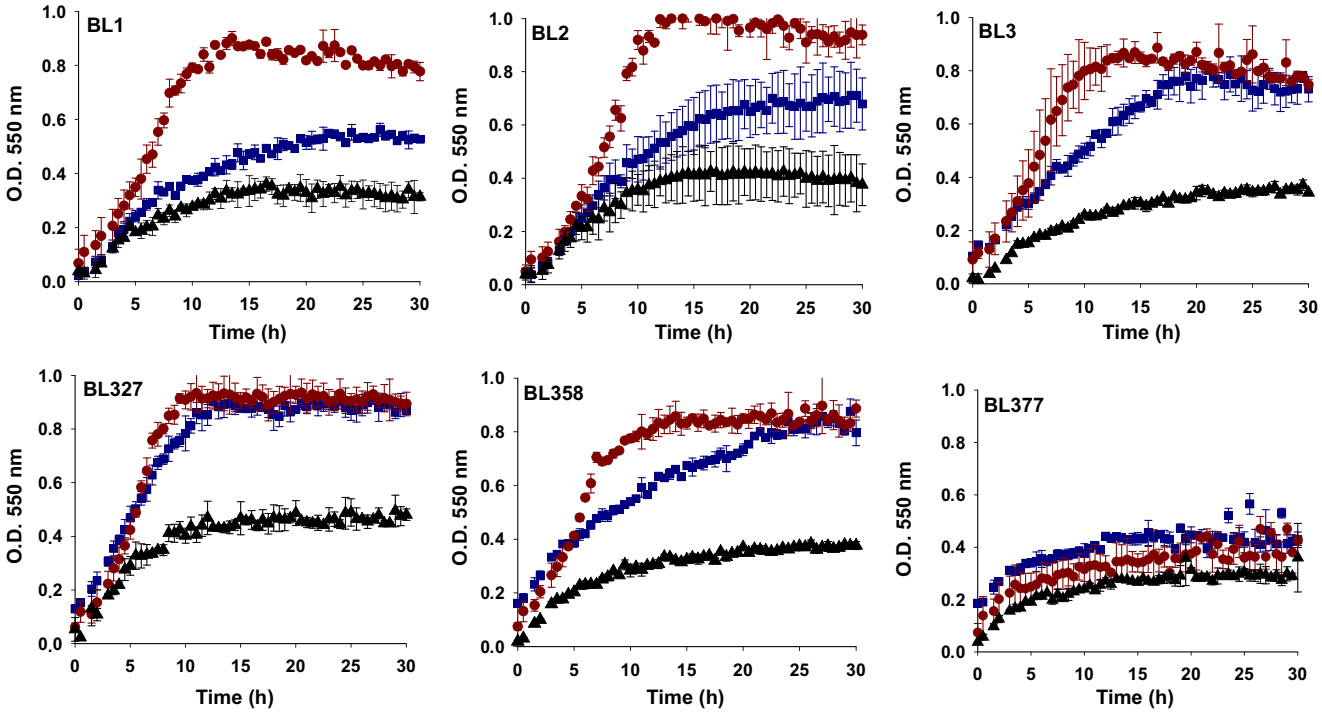


Fig. 5 Growth curves of *Lactobacillus rhamnosus* strains BL1, BL2, BL3, BL327, BL358, and BL377 in MRS basal medium without added carbon source (*triangles*), with lacto-*N*-biose (*squares*) or galacto-*N*-biose (*circles*) as the only carbon source. Data presented are mean values based on at least three replicates. *Error bars* indicate standard deviations

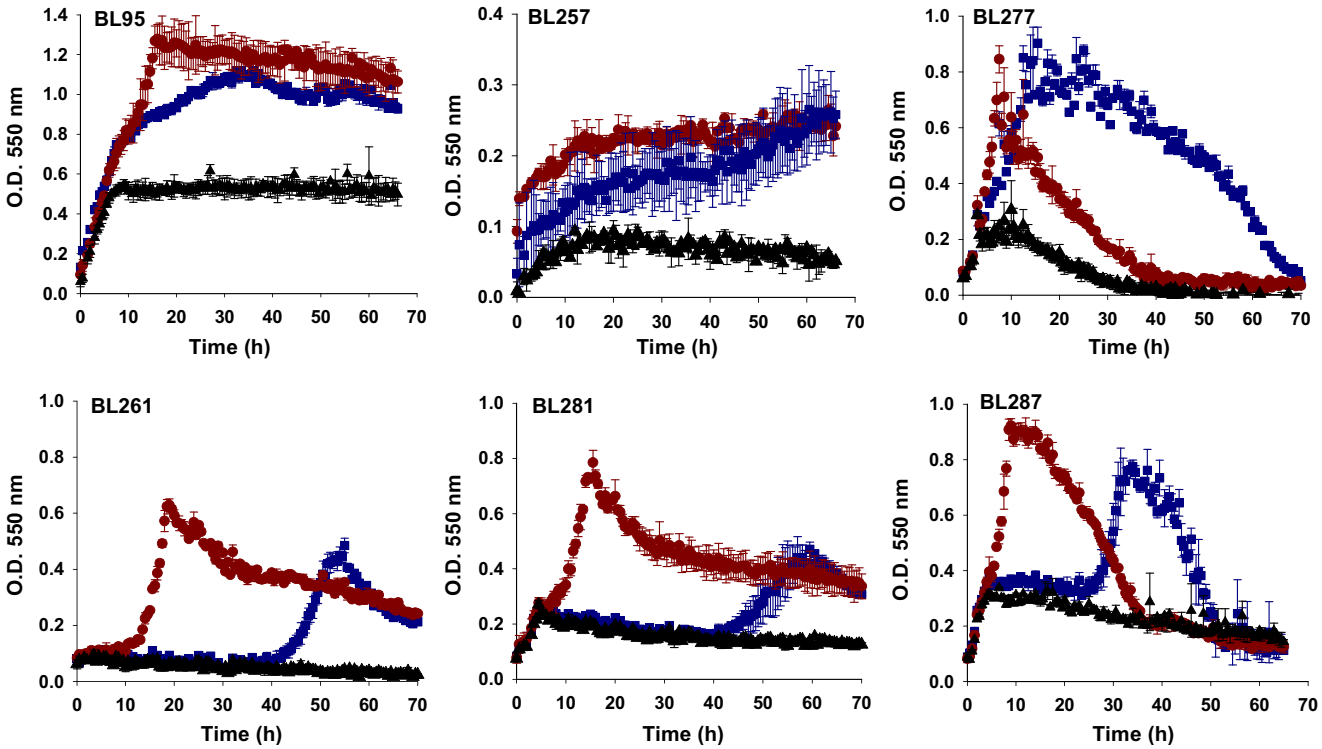


Fig. 6 Growth curves of *Lactobacillus zeae* strains BL95 and BL257, *Lactobacillus gasseri* strain BL277, and *Lactobacillus johnsonii* strains BL261, BL281, and BL287 in MRS basal medium without added carbon source (*triangles*), with lacto-*N*-biose (*squares*), or galacto-*N*-biose (*circles*) as the only carbon source. Data presented are mean values based on at least three replicates. *Error bars* indicate standard deviations

synthesized in transglycosylation reactions with the β-galactosidase BgaC from *Bacillus circulans*, but with low yields (12.2 and 10.1 % for LNB and GNB, respectively) (Fujimoto et al. 1998). Although both enzymes, GnbG and BgaC, belong to the glycoside hydrolase family 35, they only showed a 41 % sequence identity, which might explain the strong difference in the transgalactosylation efficiency between both enzymes. LNB has also been produced by the transglycosylation activity of a β-galactosidase isolated from the plant pathogen *Xanthomonas manihotis* (Vetere et al. 2000). This enzyme was shown to produce LNB at a maximum yield of 55 % after 20 h reaction. Therefore, GnbG displayed the highest transglycosylation rate for the synthesis of LNB and GNB reported to date. Both disaccharides have also been synthesized using a mix of four (sucrose phosphorylase, lacto-*N*-biose phosphorylase, UDP-hexose 1-P, and UDP-glucose 4-epimerase) (Nishimoto and Kitaoka 2007b; Nishimoto and Kitaoka 2009) or two enzymes (galactose-1P kinase and lacto-*N*-biose phosphorylase) (Yu et al. 2010) resulting in a high efficiency of synthesis.

Knowledge about the fermentation of LNB and GNB is almost limited to *Bifidobacterium* species (Satoh et al. 2013;

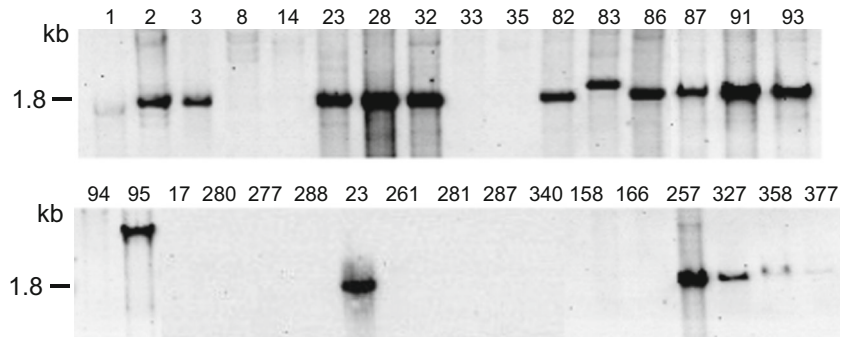


Fig. 7 Southern blot analysis of the *Eco*RV-digested genomic DNA from *Lactobacillus rhamnosus* strains (BL1, BL2, BL3, BL327, BL358, BL377), *Lactobacillus casei* strains (BL23, BL28, BL32, BL82, BL83, BL86, BL87, BL91, BL93, BL94), *Lactobacillus zeae* strains (BL95 and BL257), *Lactobacillus gasseri* strain (BL277), and *Lactobacillus johnsonii* strains (BL261, BL281 and BL287). A DNA fragment corresponding to *gnbG* from *Lactobacillus casei* strain BL23 was used as a probe. Genomic DNA from this strain was used as positive control. The *numbers on the left* indicate the size in kb of the DNA fragments from BL23 strain

Xiao et al. 2010). Here, we have shown that all *L. casei* strains and all but one *L. rhamnosus* strains tested were able to grow in the presence of both disaccharides. These results are in agreement with the fact that more than 80 % of the available genomes of the *L. casei/paracasei/rhamnosus* group contain the *gnb* gene cluster involved in the transport and catabolism of both sugars (Bidart et al. 2014). *L. rhamnosus* strain BL377 has a complete *gnb* operon (GenBank genome accession number FM179322); however, it did not ferment LNB and GNB (Fig. 5). An analysis of the *gnb* cluster in this strain showed an extra open reading frame (ORF), encoding a putative protein of 82 amino acids that overlaps with the 3′ end of the *gnbG* gene. The transcription and/or translation of that ORF might affect the proper transcription and/or translation of the downstream *gnb* genes, which would result in the impairment of strain BL377 to transport and hydrolyze LNB and GNB. Two strains of *L. zeae*, a species which is phylogenetically close to the *L. casei/paracasei/rhamnosus* group (Vasquez et al. 2005), were also able to grow in the presence of LNB and GNB (Fig. 6). Analysis of the available genome sequence of *L. zeae* strain BL95 (GenBank accession number BACQ00000000) showed the presence of a complete *gnb* operon, encoding proteins which deduced amino acid sequences ranging from 86 to 93 % identity respect to the *L. casei* BL23 *gnb* gene products (Bidart et al. 2014). Contrarily, *L. casei* strain BL94 (ATCC 393) can be cultured in the presence of LNB or GNB (Fig. 4) but lacks *gnbG* homologues in its genome (GenBank accession number AZCO00000000). Similarly, the *L. gasseri* and *L. johnsonii* strains tested here were able to ferment both disaccharides, but do not contain a *gnb* cluster. BLAST search using the amino acid sequence of phospho-β-galactosidase GnbG from *L. casei* BL23 (Bidart et al. 2014) against the translated genomes of *L. gasseri* BL277 (ATCC 33323) (Azcarate-Peril et al. 2008) and *L. johnsonii* BL287 (DSM 10533) (Sun et al. 2015) did not show relevant homology with any protein, suggesting the absence of an enzyme homologous to GnbG in those strains. This is in agreement with the DNA hybridization analysis showed above. Therefore, the hydrolysis of both disaccharides must rely upon other glycosidases. Indeed, the *L. gasseri* BL277 genome encodes 20 putative glycosidases (Azcarate-Peril et al. 2008), including several phospho-β-glycosidases (Honda et al. 2012; Nagaoka et al. 2008). As well, the *L. johnsonii* BL287 genome also encodes more than 20 putative glycosidases (Sun et al. 2015). However, the specificity of these enzymes is not known. Growth patterns, genome inspection, and hybridization with the *gnbG* probe demonstrated that LNB and GNB utilization is a widespread feature in *L. casei/paracasei/rhamnosus/zeae*. This is a group of species linked to the gastrointestinal habitat for which genes for the utilization of other important human milk and mucosal oligosaccharides have been described (Rodriguez-Diaz et al. 2012), highlighting the role of the utilization of this substrates in the development in this particular niche. Whether *L. gasseri*, *L. johnsonii*, and the *L. casei* ATCC393 strain rely on systems similar to the bifidobacteria for LNB and GNB catabolism, or they express new glycosidases with LNB and GNB specificities remains to be investigated.

In this work, we have shown that the phospho-β-galactosidase GnbG isolated from *L. casei* BL23 can be used in transglycosylation reactions directed to synthesize LNB and GNB, which are important disaccharides present in human milk, either free or forming part of the structure of oligosaccharides and glycoproteins. The reactions were efficiently scaled and the produced LNB and GNB easily purified. The prebiotic potential of these carbohydrates for enhancement of specific *Bifidobacterium* species, as shown previously, and *Lactobacillus* species, as shown here, support the putative role of human milk oligosaccharides for selective stimulation of the growth of beneficial gastrointestinal microbiota in breast-fed infants. Furthermore, GnbG might be used in transglycosylation reactions to produce derivative compounds, such as glycosides of LNB and GNB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors

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