**Selective fermentation of potential prebiotic lactose-derived oligosaccharides by
probiotic bacteria**

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Abstract

The growth of potential probiotic strains from the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus* was evaluated with the novel lactose-derived trisaccharides 4'-galactosyl-kojibiose and lactulosucrose and the potential prebiotics lactosucrose and kojibiose. The novel oligosaccharides were synthesized from equimolar sucrose:lactose and sucrose:lactulose mixtures, respectively, by the use of a *Leuconostoc mesenteroides* dextranucrase and purified by liquid chromatography. The growth of the strains using the purified carbohydrates as the sole carbon source was evaluated by recording the culture optical density and calculating maximum growth rates and lag phase parameters. The results revealed an apparent bifidogenic effect of lactulosucrose, being also a moderate substrate for streptococci ~~and poorly~~ but badly utilized by lactobacilli. In addition, 4'-galactosyl-kojibiose was selectively fermented by *Bifidobacterium breve*, which was also the only tested bifidobacterial species able to ferment kojibiose. The described fermentation properties of the specific probiotic strains on the lactose-derived oligosaccharides would enable the design of prebiotics with a high degree of selectivity.

Keywords: Prebiotic; Lactose-derived oligosaccharides; Probiotic; *Bifidobacterium*; *Lactobacillus*; *Streptococcus*

1. Introduction

The human large intestine is densely colonized by a complex microbial ecosystem that plays a key role in human health and disease, being the subject of intensive studies for its characterization during last years (Aagaard et al. 2013; Qin et al. 2010). Intestinal bacteria provide a diverse range of biochemical and metabolic activities to complement host physiology and exert numerous protective effects by modulating mucosal and systemic immune responses (Clemente, Ursell, Parfrey, & Knight, 2012). Among the metabolic functions, the fermentation of dietary components, which are principally undigested fibre carbohydrates, and the impact on the composition and/or activity of the human gut microbiota are attracting considerable attention in food and nutrition research (Roberfroid et al. 2010).

Prebiotics are normally non-digestible dietary carbohydrates which are selectively fermented, resulting in specific changes in the composition and/or activity of a limited number of intestinal bacteria, thus conferring benefit(s) upon host health (Roberfroid et al. 2010). Currently, there are certain carbohydrates with well-known prebiotic status, and these include lactulose, inulin, fructo-oligosaccharides, galacto-oligosaccharides and resistant starch (Di Bartolomeo, Startek, & Van den Ende, 2013; Slavin, 2013). However, over the last decades there has been a growing search for new prebiotic carbohydrates which could be considered as emerging prebiotics, such as xylo-oligosaccharides, arabinoxylo-oligosaccharides, isomalto-oligosaccharides, lactosucrose and pectic-oligosaccharides, among others (Rastall & Gibson, 2002). Likewise, significant research efforts are currently focused on the search and/or production of novel prebiotic ingredients that have a series of desirable properties including: i) to be active at low dosage, ii) lack of side effects, iii) persistence through the colon, iv) fine control of microbiota modulation, v) good storage and processing stability and vi) to

possess additional biological activities, exerting beneficial effects on specific physiological functions and/or reducing the risk of disease, for example, through their effect on displacement of pathogens and/or regulation of the function of the immune system (Rastall & Hotchkiss, 2003). In this context, we have recently described the efficient dextranucrase-catalyzed synthesis of oligosaccharides such as lactulosucrose (Díez-Municio, Herrero, Jimeno, Olano, & Moreno, 2012a) and 2- α -glucosyl-lactose, also denominated 4'-galactosyl-kojibiose (Díez-Municio et al., 2012b), whose structural features makes them promising candidates for novel prebiotic ingredients. Nevertheless, to the best of our knowledge, no data on selective fermentation by bacteria have been reported for these novel oligosaccharides. In addition, high-yield and ~~high-high~~-purity kojibiose has been recently obtained from the complete ~~hydrolysis~~ *Kluyveromyces lactis* ~~β -galactosidase hydrolysis~~ of 4'-galactosyl-kojibiose ~~by~~ *Kluyveromyces lactis* ~~β -galactosidase~~ (Díez-Municio, Montilla, Moreno, & Herrero, 2014). Kojibiose is a natural disaccharide commercially available only in low amounts due to ~~different~~ drawbacks ~~in-related to~~ its isolation and/or synthesis. Nevertheless, ~~available~~ scarce data ~~already available~~ have pointed out kojibiose to have a promising prebiotic potential (Sanz, Gibson, & Rastall, 2005).

In this work, the ability of kojibiose and novel lactose-derived oligosaccharides, i.e. 4'-galactosyl-kojibiose and lactulosucrose, to be metabolized by pure cultures of potential probiotic strains belonging to the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus* is evaluated and compared with well-established (lactulose) or emerging (lactosucrose) lactose-derived prebiotics. Considering that there is a strong link between the oligosaccharide chemical structure and its potential bioactivities, the results derived from this study could provide further insights into the influence of the monomer

composition and glycosidic linkage type on the selective fermentability of lactose-derived oligosaccharides by specific probiotic strains.

2. Materials and methods

2.1. Chemicals, reagents, standards and enzymes

All used chemicals and reagents were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO, USA), VWR (Barcelona, Spain), and Merck (Darmstadt, Germany). Ultra-pure water quality (18.2 MΩcm) with 1–5 ppb total organic carbon (TOC) and <0.001 EU mL⁻¹ pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA, USA).

Carbohydrates (fructose, glucose, galactose, sucrose, leucrose, lactulose and lactose) were all provided from Sigma-Aldrich (St. Louis, MO, USA), standard kojibiose was purchased from Carbosynth (Berkshire, UK) and lactosucrose from Wako Pure Chemical Industries (Osaka, Japan).

Dextranucrase from *Leuconostoc mesenteroides* B-512F was purchased from CRITT Bio-Industries (Toulouse, France). Specific activity was 0.4 U mg⁻¹, where 1 U is the amount of enzyme required to perform the transfer of 1 μmol of glucose per minute at a working temperature of 30 °C, a sucrose concentration of 100 g L⁻¹ at pH 5.2 in 20 mM sodium acetate buffer with 0.34 mM of CaCl₂. Soluble commercial preparation of β-galactosidase from *K. lactis* (Lactozym Pure 6500 L) was kindly supplied by Novozymes (Bagsvaerd, Denmark).

2.2. Synthesis, purification and characterization of the studied oligosaccharides

The trisaccharide lactulosucrose, β-D-galactopyranosyl-(1→4)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside, was enzymatically ~~synthetized~~synthesized from

sucrose:lactulose mixtures by the *L. mesenteroides* B-512F dextransucrase-catalyzed transfer of the glucosyl residue from sucrose to the C-2-hydroxyl group of the reducing unit of lactulose as described by Díez-Municio, Herrero, Jimeno, Olano, & Moreno (2012a). The enzymatic reaction was carried out with 30% (w/v) of sucrose and 30% (w/v) of lactulose and an enzyme charge of 2.4 U mL⁻¹ at 30 °C in 20 mM sodium acetate buffer with 0.34 mM CaCl₂ (pH 5.2) during 24 h of reaction time. Under these optimal conditions, lactulosucrose yield was around 30% in weight respect to the initial amount of lactulose.

The enzymatic synthesis of the trisaccharide 4'-galactosyl-kojibiose also termed as 2- α -D-glucopyranosyl-lactose, O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-O- α -D-glucopyranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranose, also termed as 2- α -D-glucopyranosyl-lactose was carried out in the presence of sucrose (donor) and lactose as acceptor (30:30, expressed in g/100 mL), in 20 mM sodium acetate buffer with 0.34 mM CaCl₂ (pH 5.2) at 30 °C using a *L. mesenteroides* B-512F dextransucrase (0.8 U mL⁻¹) as described by Díez-Municio et al. (2012b). The optimal synthesis conditions at 24 h of enzymatic reaction gave rise to yields around close to 50% (in weight respect to the initial amount of lactose).

Kojibiose (2-O- α -D-glucopyranosyl- α -D-glucopyranose) was obtained from the hydrolysis of 4'-galactosyl-kojibiose by *K. lactis* β -galactosidase, after removal of residual monosaccharides by using a *Saccharomyces cerevisiae* treatment and further purification as described by Díez-Municio, Montilla, Moreno, & Herrero (2014).

Isolation of pure oligosaccharides was performed by liquid chromatography with refractive index detector (LC-RID) on an Agilent Technologies 1260 Infinity LC System (Boeblingen, Germany) using a Zorbax NH₂ PrepHT preparative column (250 $\times$ 21.2 mm, 7 μ m particle size) (Agilent Technologies, Madrid, Spain). Two mL of

reaction mixtures (~~150~~—200 mg of total carbohydrates) were eluted with acetonitrile:water as the mobile phase at a flow rate of 21.0 mL min⁻¹ for 30 min. The separated oligosaccharides were collected using an Agilent Technologies 1260 Infinity preparative-scale fraction collector (Boeblingen, Germany), and the fractions were pooled, evaporated in a rotatory evaporator R-200 (Büchi Labortechnik AG, Flawil, Switzerland) below 25 °C and freeze-dried. Purity grade was in all cases ≥ 99.0% as checked by LC-RID.

Lactulosucrose and 4'-galactosyl-kojibiose were then fully characterized by 1D and 2D [¹H, ¹H] and [¹H-¹³C] nuclear magnetic resonance (NMR) experiments (gCOSY, TOCSY, ROESY, multiplicity-edited gHSQC and gHMBC) (Díez-Municio, Herrero, Jimeno, Olano, & Moreno, 2012a; Díez-Municio et al., 2012b), whilst kojibiose was identified by LC-RID and gas chromatography with mass spectrometry detection (GC-MS) analyses by comparison with a commercial standard (Díez-Municio, Montilla, Moreno, & Herrero, 2014).

2.3. Bacterial strains and culture media

Streptococcus salivarius ZL50-7, *Lactobacillus reuteri* R13, *Lactobacillus delbrueckii* ZL95-27, *Bifidobacterium breve* 26M2 and *Bifidobacterium bifidum* HDD541 were isolated from milk of healthy mothers or infant feces and belong to the culture collection of the Department of Nutrition and Food and Science Technology, Universidad Complutense de Madrid (Madrid, Spain). *Streptococcus thermophilus* STY-31, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* LC-01 and *Bifidobacterium lactis* BB-12 were isolated from a commercial symbiotic product (Simbiotic Drink, Priégola, Madrid, Spain) as described by Tabasco, Paarup, Janer, Peláez, & Requena (2007). *Lactobacillus rhamnosus* GR-1 is a commercial probiotic

strain isolated from the human urogenital tract (Chan, Bruce, & Reid, 1984). Before being used in experiments, all strains were routinely cultured in MRS broth (Pronadisa, Madrid, Spain), except for *S. salivarius* ZL50-7 and *S. thermophilus* STY-31 that were grown in ESTY broth (Pronadisa) containing 20 g/L lactose (ESTY-L).

Basic media for experiments were MRS fermentation broth (Pronadisa), which does not contain either glucose or meat extract (De Man, Rogosa, & Sharpe, 1960), enriched with 0.2% Tween 80, 0.8% casein acid hydrolysate and 0.05% L-cysteine, or ESTY broth (Pronadisa), without any carbon source. These media were supplemented with the tested oligosaccharides at a final concentration of 0.3% (w/v). Each substrate was weighed and then added to the corresponding autoclaved basic media, and the mixtures were filter sterilized with 0.2- μ m-pore-size sterile filters. Glucose and lactulose were used as control of growth, being added at the same final concentration as the corresponding experimental oligosaccharides. Additional controls were made with basic medium and without carbon source and were incubated with each strain tested.

2.4. Evaluation of bacterial growth on synthesized carbohydrates

The bacterial strains were cultured overnight at 1% in MRS or ESTY-L broth, depending on the strain. The cells were harvested by centrifugation ($10,000 \times g$ for 10 min at 4 °C), washed twice with and resuspended in sterile saline solution (0.85%), and inoculated (1%) into the basic media plus the tested carbohydrate. Bacterial growth was monitored in duplicate 300 μ L-wells of sterile 96-well microplates with lid (Sarstedt, La Roca del Vallès, Spain). All strains were grown in aerobic conditions at 37 °C for 48 h, with the exception of the *Bifidobacterium* strains. The optical densities at 600 nm (OD_{600}) of the strains growing aerobically were recorded at 60 min intervals with an automated microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham,

MA, USA), at duplicate experiments. Maximum growth rates ($\mu_{\max}$) and lag parameter (lag) of strains growing under aerobic conditions were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.2.1 (Barayni & Roberts, 1994) (available at <http://www.ifr.ac.uk/safety/DMfit/default.html>). In the case of the anaerobic strains, *B. breve* 26M2, *B. lactis* BB-12 and *B. bifidum* HDD541, microplates were kept at an anaerobic incubator at 37 °C for 48 h (Bactron Anaerobic Chamber; Sheldon Manufacturing, Cornelius, OR, USA). The OD₆₀₀ was recorded at the microplate reader at 0 h, 24 h and 48 h.

3. Results

The structures of the tested oligosaccharides, including ~~the~~ commercially available lactulose and lactosucrose are depicted in Fig. 1. ~~Kojibiose, 4' Ggalactosyl kojibiose and lactulosucrose were synthesized from equimolar sucrose:lactose and sucrose:lactulose mixtures, respectively, by using a *L. mesenteroides* B 512F dextranucrase which catalyzed the transfer of the glucosyl residue from sucrose to the 2-hydroxyl group of the reducing unit of lactose and lactulose, respectively (Díez-Municio, Herrero, Jimeno, Olano, & Moreno, 2012a; Díez-Municio et al., 2012b). Additionally, the synthesis of kojibiose from 4' galactosyl kojibiose required additional stages such as monosaccharide removal and β -galactosidase hydrolysis before chromatographic purification (Díez-Municio, Montilla, Moreno, & Herrero, 2014).~~

Maximum optical densities at 600 nm (OD_{max}), maximum growth rates ($\mu_{\max}$) and lag times (h) during growth of the strains under aerobic conditions on the assayed oligosaccharides are shown in Table 1. As expected, all strains grew well on glucose, reaching *L. rhamnosus* GR-1 the highest OD_{max} and *L. reuteri* R13 the highest $\mu_{\max}$.

values. Overall, the registered bacterial growth parameters ~~registered~~ were lower with lactulose than with glucose, showing both *L. delbrueckii* ZL95-27 and *L. acidophilus* LA-5 no growth on lactulose. Among the synthesized carbohydrates, none of the strains tested under aerobic conditions ~~did grow~~ grew with 4'-galactosyl-kojibiose as carbon source and only the *Streptococcus* strains grew on lactulosucrose (Table 1). Kojibiose and lactosucrose promoted the growth of five and four bacterial strains, respectively, out of the seven tested strains (Fig. 2). Main differences between strains when growing on lactosucrose were observed for lag times, with the shortest time registered for *L. casei* (5.75 h) and longer lag times (31.82 h and 40.20 h) for *L. reuteri* R13 and *L. acidophilus* LA-5. On the other hand, except for *L. acidophilus* LA-5, lag times of the strains growing on kojibiose were similar and shorter than 5 h, with the highest OD_{max} value recorded for *L. rhamnosus* GR-1. This strain was characterized by growing on the assayed disaccharides lactulose and kojibiose but not utilizing the tested trisaccharides as carbon sources (Table 1).

Regarding ~~the~~ *Bifidobacterium* strains, OD₆₀₀ could not be recorded continuously at 60 min intervals due to the required anaerobic growth conditions of the species. The OD₆₀₀ values after 24 h and 48 h growth of *B. lactis* BB-12, *B. breve* 26M2 and *B. bifidum* HDD541 with the six carbohydrates used as carbon sources are shown in Table 2. Lactulosucrose was fermented by the three bifidobacterial strains, which reached at 24h higher cell densities than when growing with lactosucrose or lactulose, ~~and~~ being also the only oligosaccharide fermented by *B. bifidum* HDD541. Remarkably, *B. breve* 26M2 was the only strain among the bacteria assayed that was able to growth with all the tested carbohydrates and particularly with kojibiose and 4'-galactosyl-kojibiose. In the case of *B. lactis* BB-12, it exhibited growth on lactulose, lactosucrose

and lactulosucrose, although no growth was observed on kojibiose and 4'-galactosyl-
kojibiose.

4. Discussion

This article reports important differences in the ability of the studied lactose-derived oligosaccharides to promote the growth of representative probiotic strains from three different genera (*Lactobacillus*, *Bifidobacterium* and *Streptococcus*). These results highlight the key role played by the monomer composition, molecular weight and glycosidic linkage on their fermentable properties, as well as reinforce the fact that the ability to metabolize particular carbohydrates is species- and strain-dependent (de Vrese & Schrezenmeir, 2008).

Among the tested carbohydrates, lactulose and lactosucrose are commercially available prebiotics, and kojibiose has also been described as a potential prebiotic (Sanz, Gibson, & Rastall, 2005), ~~although~~ ~~However~~, the low availability and high cost of kojibiose impairs its use and explains the lack of studies on its bioactive properties (Díez-Municio, Montilla, Moreno, & Herrero, 2014). Regarding lactulosucrose and 4'-galactosyl-kojibiose, to the best of our knowledge, no data on their prebiotic properties had been described so far in the literature. In this study, lactulosucrose had an apparent bifidogenic effect, being also a moderate substrate for streptococci ~~and poorly~~ but badly utilized by lactobacilli. In contrast, lactosucrose allowed the growth of some strains of lactobacilli and bifidobacteria (Tables 1 and 2). By comparing both structures (Fig. 1), i.e. β -D-Gal-(1 $\rightarrow$ 4)- β -D-Fru-(2 $\rightarrow$ 1)- α -D-Glc vs β -D-Gal-(1 $\rightarrow$ 4)- α -D-Glc-(1 $\rightarrow$ 2)- β -D-Fru, it can be inferred that monomer position might play an important role on the fermentation properties of oligosaccharides. Lactosucrose has proven to be scarcely hydrolyzed by human digestive enzymes (Fujita et al., 1991) and selectively utilized by

intestinal *Bifidobacterium* (Fujita, Ito, & Kishino, 2009; Ohkusa, Ozaki, Sato, Mikuni, & Ikeda, 1995; Takei, Akakura, Ueda, Mikami, & Ito, 2006), warranting its use as a prebiotic ingredient (Mu, Chen, Wang, Zhang, & Jiang, 2013). Although more information and further experiments are needed, considering the results reported in this work, lactulosucrose might also present a great potential as prebiotic, specially targeted to the growth of bifidobacteria (Table 2). Moreover, having lactulose as a core structure, lactulosucrose might possess the beneficial properties attributed to lactulose, e.g. prebiotic activity, enhancement of mineral absorption, blood glucose lowering effects and reduction in intestinal transit time, among others (Schuman, 2002). Lastly, since longer carbohydrate chains are normally fermented at slower rates (Perrin, Fournies, Grill, Jacobs, & Schneider, 2002; Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004), lactulosucrose might also exhibit lower fermentation rates as compared to lactulose, which would increase the interest as a novel prebiotic by increasing promoting its capacity to reach the distal parts of the colon.

A valuable result obtained from this study is the selective fermentation of 4'-galactosyl-kojibiose by *B. breve* 26M2 (Tables 1 and 2). Nakada, Nishimoto, Chaen, & Fukuda, (2003) evaluated the utilization by several intestinal bacteria of two koji-oligosaccharides (degree of polymerization 3 and 4) linked with only α -1,2 glucosidic linkages and, in good agreement with data reported for 4'-galactosyl-kojibiose, they did not observe any growth on four *Lactobacillus* strains (*L. acidophilus*, *L. gasseri*, *L. reuteri* and *L. salivarius*), although both koji-oligosaccharides strongly enhanced growth of *B. breve*. In addition, *B. breve* 26M2 selectively utilized kojibiose, which was also a good substrate for lactobacilli (Tables 1 and 2), confirming the prebiotic potential of the disaccharide stated by Sanz, Gibson, & Rastall, et al. (2005). Similarly, Nakada, Nishimoto, Chaen, & Fukuda, (2003) observed a good utilization of kojibiose by *B.*

breve but not by *B. bifidum*. O'Connel et al. (2013) have recently demonstrated the presence in *B. breve* of a distinctive α -glucosidase (MelD) with α -(1→2)-glycosyl hydrolase activity that could be involved in the hydrolysis of the disaccharide. A considerable variability in the carbohydrate metabolic abilities has been described between bifidobacterial strains (Hopkins, Cummings, & Macfarlane, 1998; Pokusaeva, Fitzgerald, & van Sinderen, 2011). In fact, the increasing access to bifidobacteria genome sequences has revealed that these organisms contain several oligosaccharide uptake systems and carbohydrate-hydrolyzing enzymes, sugar ABC (ATP Binding Cassette) transporters or PEP-PTS (phosphoenolpyruvate-phosphotransferase system) components, that vary between species (Macfarlane, Blackett, & Macfarlane, 2010; Pokusaeva, Fitzgerald, & van Sinderen, 2011).

5. Conclusions

In conclusion, findings reported in this work point out the selective fermentation properties of a series of emerging or novel lactose-derived oligosaccharides on specific probiotic strains. Although pure culture models do not reflect bacterial interactions in the host (Roberfroid, 2007; Watson et al., 2012), this type of assays is adequate for screening studies aimed to the initial selection of non-digestible carbohydrates that would be able to selectively enrich probiotic strains within the gut microbiota diversity, and to assist in the design of prebiotics with a high degree of selectivity. In addition, these results provide new or additional evidences for further investigation of the potential effects of these oligosaccharides on the diversity and/or activity of the gut microbiota, as well as on the metabolic consequences for the host.

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Table 1. Maximum optical density at 600 nm (OD_{max}), maximum growth rate ($\mu_{\max}$, h⁻¹) and lag (h) parameters of bacteria growing under aerobic conditions on glucose, lactulose, lactosucrose and synthesized oligosaccharides as carbon sources.

Species		Glucose	Lactulose	Lactosucrose	Kojibiose	Lactulosucrose	4'-galactosyl- kojibiose
<i>S. thermophilus</i> STY-31	OD _{max}	1.09 ± 0.03	0.73 ± 0.01	NG	NG	0.47 ± 0.03	NG
	$\mu_{\max}$	0.29 ± 0.02	0.08 ± 0.01			0.01 ± 0.00	
	lag	1.74 ± 0.01	2.50 ± 0.06			11.52 ± 3.82	
<i>S. salivarius</i> ZL50-7	OD _{max}	1.12 ± 0.01	1.09 ± 0.01	0.93 ± 0.01	0.84 ± 0.02	0.48 ± 0.06	NG
	$\mu_{\max}$	0.49 ± 0.01	0.42 ± 0.01	0.13 ± 0.01	0.07 ± 0.01	0.01 ± 0.00	
	lag	5.75 ± 0.04	7.46 ± 0.02	21.95 ± 0.11	4.62 ± 0.10	15.11 ± 1.85	
<i>L. reuteri</i> R13	OD _{max}	1.09 ± 0.01	0.62 ± 0.01	0.51 ± 0.01	0.73 ± 0.01	NG	NG
	$\mu_{\max}$	0.54 ± 0.01	0.09 ± 0.00	0.05 ± 0.01	0.12 ± 0.01		
	lag	1.69 ± 0.02	0.96 ± 0.01	31.82 ± 1.22	0.84 ± 0.08		
<i>L. delbrueckii</i> ZL95-27	OD _{max}	0.67 ± 0.01	NG	NG	NG	NG	NG
	$\mu_{\max}$	0.14 ± 0.00					
	lag	5.92 ± 0.10					
<i>L. acidophilus</i> LA-5	OD _{max}	0.72 ± 0.01	NG	0.75 ± 0.02	0.48 ± 0.01	NG	NG
	$\mu_{\max}$	0.11 ± 0.01		0.07 ± 0.00	0.02 ± 0.00		
	lag	7.92 ± 0.09		40.20 ± 0.50	20.23 ± 0.56		
<i>L. rhamnosus</i> GR-1	OD _{max}	1.29 ± 0.06	0.82 ± 0.02	NG	1.03 ± 0.01	NG	NG
	$\mu_{\max}$	0.23 ± 0.01	0.09 ± 0.01		0.09 ± 0.00		
	lag	2.07 ± 0.16	4.65 ± 0.41		2.11 ± 0.01		
<i>L. casei</i> LC-01	OD _{max}	0.90 ± 0.03	0.51 ± 0.03	0.79 ± 0.01	0.48 ± 0.03	NG	NG
	$\mu_{\max}$	0.12 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.04 ± 0.00		
	lag	3.50 ± 0.04	3.15 ± 0.11	5.75 ± 0.02	1.18 ± 0.04		

Data represent means +/- SD of at least 2 replicates. NG: no growth.

433 **Table 2.** Growth (OD₆₀₀) of *Bifidobacterium* strains with glucose, lactulose, lactosucrose and synthesized oligosaccharides as carbon sources.

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	<i>B. lactis</i> BB-12		<i>B. breve</i> 26M2		<i>B. bifidum</i> HDD541	
	24 h	48 h	24 h	48 h	24 h	48 h
Glucose	1.11 ± 0.01	1.10 ± 0.12	0.79 ± 0.02	0.75 ± 0.01	0.76 ± 0.03	0.66 ± 0.02
Lactulose	0.69 ± 0.02	1.08 ± 0.01	0.69 ± 0.03	0.89 ± 0.07	NG	NG
Lactosucrose	0.55 ± 0.01	0.73 ± 0.02	1.03 ± 0.01	1.04 ± 0.04	NG	NG
Kojibiose	NG	NG	1.20 ± 0.02	0.91 ± 0.01	NG	NG
Lactulosucrose	0.76 ± 0.05	0.70 ± 0.01	1.21 ± 0.02	0.84 ± 0.01	1.08 ± 0.06	0.49 ± 0.02
4'-galactosyl-kojibiose	NG	NG	0.72 ± 0.03	0.55 ± 0.01	NG	NG

435 Data represent means +/- SD of at least 2 replicates. NG: no growth.

436 **Figure legends.**

437 **Figure 1.** Chemical structures of the studied oligosaccharides. A) lactulose; B)
438 lactosucrose; C) kojibiose; D) lactulosucrose; E) 4'-galactosyl-kojibiose.

439 **Figure 2.** Growth curves of the bacterial strains on A) kojibiose and B) lactosucrose.

440

441 **Figure 1.**

442

443 **Figure 2.**

444