# Metabolism of biosynthetic oligosaccharides by human-derived *Bifidobacterium breve* UCC2003 and *Bifidobacterium longum* NCIMB 8809

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**Abstract**

This work aimed to investigate the ability of two human-derived bifidobacterial strains, i.e. *Bifidobacterium breve* UCC2003 and *Bifidobacterium longum* NCIMB 8809, to utilize various oligosaccharides (i.e., 4-galactosyl-kojibiose, lactulosucrose, lactosyl-oligofructosides, raffinosyl-oligofructosides and lactulose-derived galacto-oligosaccharides) synthesized by means of microbial glycoside hydrolases. With the exception of raffinosyl-oligofructosides, these biosynthetic oligosaccharides were shown to support growth acting as a sole carbon and energy source of at least one of the two studied strains. Production of short-chain fatty acids (SCFAs) as detected by HPLC analysis corroborated the suitability of most of the studied novel oligosaccharides as fermentable growth substrates~~, since they were fermented, producing SCFAs,~~ for the two bifidobacterial strains, showing that ~~acetate~~ acetic acid is the main metabolic end product followed by lactic and formic acids. Transcriptomic and functional genomic approaches carried out for *B. breve* UCC2003 allowed the identification of key genes encoding glycoside hydrolases and ~~protein~~carbohydrate transport systems involved in the metabolism of 4-galactosyl-kojibiose and lactulosucrose. In particular, the role of β-galactosidases in the hydrolysis of these particular trisaccharides was demonstrated, highlighting their importance in oligosaccharide metabolism by human bifidobacterial strains.

**Keywords**

Prebiotic; Enzymatic synthesis; pure cultures; Short-chain fatty acid (SCFA); Structure–function relationship

## 1. INTRODUCTION

The gastrointestinal tract (GIT) of mammals is host to a complex and densely populated community of different microorganisms, known as the intestinal microbiota. Bacteria dominate the gut ecosystem, and *Bifidobacterium* is one of the main representative genera, being particularly abundant in healthy, breast-fed infants due to the ability of certain bifidobacteria to consume oligosaccharides found in human milk (Khonsari et al., 2016; Yatsunenko et al., 2012). In addition to human milk oligosaccharides, bifidobacteria can ferment a large variety of dietary oligosaccharides, although the ability to metabolize particular carbohydrates is species- and strain-dependent (de Vrese and Schrezenmeir, 2008).

Scientific evidence suggests that the composition and activity of the gut microbiota is responsive to diet. Thus, a substantial number of studies have demonstrated that dietary fibers, especially so-called non-digestible (i.e. not digested by the host) oligo-/polysaccharides, alter the number and/or activity of certain gut microbiota components, thereby leading to improvements in host health (Makki et al. 2018; Scott et al. 2008; So et al. 2018;Valdes et al. 2018). To date, several non-digestible carbohydrates, such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and inulin, have demonstrated such beneficial effects (Slavin 2013), and for this reason are referred to as prebiotics. In this context, bifidobacteria are often targeted for prebiotic intervention as they have been reported to confer various health benefits, including, but not limited to, immune-modulation (Hidalgo-Cantabrana et al. 2016; Sanchis-Chorda et al. 2018; Turroni et al. 2014), restriction of pathogenic bacteria through competitive exclusion and production of short chain fatty acids (SCFAs; examples are ~~butyrate~~ butyric and ~~propionate~~ propionic acids) (Ventura et al. 2012), as well as modulation of mucosal barrier function (Turroni et al. 2014).

Improving health and/or reducing the risk of (chronic) disease are some of the forces driving the development of functional foods for humans. There is both scientific and commercial interest in the concept of prebiotics which aim to beneficially modulate gut microbiota composition and associated bacterial metabolic activities. Significant scientific efforts are ongoing to identify novel and possibly improved compounds that possess such beneficial or prebiotic potential. In this sense, the (bio)synthesis of various oligosaccharides whose structural features make them promising candidates as new prebiotic ingredients has been described in recent years. Briefly, these oligosaccharides were produced by enzymatic synthesis using microbial glycoside hydrolases (GHs) acting on, in most cases, sucrose as donor and using different di- or trisaccharides as acceptors to produce 4-galactosyl-kojibiose (Diez-Municio et al. 2012a), lactulosucrose (Diez-Municio et al. 2012b), lactosyl-oligofructosides (Diez-Municio et al. 2015), raffinosyl-oligofructosides (Diez-Municio et al., 2016a) and lactulose-derived galacto-oligosaccharides (GOS-Lu) (Cardelle-Cobas et al., 2008; Martínez-Villaluenga et al., 2008). Following their synthesis, the ability of these particular novel carbohydrates to promote growth of specific probiotic strains has been investigated (Cardelle-Cobas et al., 2011; Garcia-Cayuela 2014), as well as their prebiotic potential using *in vitro* batch-culture fermentation systems inoculated with human faecal slurries (Cardelle-Cobas et al., 2009; Cardelle-Cobas et al., 2012; Diez-Municio et al. 2016b). Moreover, in the case of GOS-Lu, this prebiotic effect has been further corroborated by *in vivo* studies using different rat models (Fernández et al., 2018; Hernández-Hernández et al., 2012; Marín-Manzano et al., 2013). Remarkable findings were obtained regarding their strong bifidogenic effect by selectively promoting similar *Bifidobacterium* levels as compared to those produced by well-established prebiotics, such as lactulose and FOS. Nevertheless, further and precise information is needed regarding the affected bifidobacterial species, the associated enzymatic machinery required for the metabolism of these novel oligosaccharides and the corresponding metabolic end products.

Therefore, the main objective of this study was to gather knowledge on the utilization of a series of novel dietary oligosaccharides by specific beneficial human gut commensals represented by pure cultures of strains belonging to *Bifidobacterium breve* and *Bifidobacterium longum* (i.e. *B. breve* UCC2003 and *B. longum* NCIMB 8809), as well as to describe specific metabolic pathways required for their growth. Further insights were inferred from the relationship between structural features (i.e., monomer and glycosidic linkage type) and bifidogenic properties.

## 2. MATERIALS AND METHODS

### *2.1. Carbohydrates tested*

#### *2.1.12. Commercial carbohydrates*

Glucose (Glc), galactose (Gal), lactose (β-D-Gal-(1→4)-D-Glc), lactulose (β-D-Gal-(1→4)-D-Fru), maltose (α-D-Glc-(1→4)-D-Glc) and raffinose (α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and kojibiose (α-D-Glc-(1→2)-D-Glc) was purchased from Carbosynth (Berkshire, UK).

#### *2.1.12. Production of oligosaccharides*

The oligosaccharides employed in this study were synthesized by transglycosylation acceptor reactions catalyzed by microbial glycoside hydrolases and further structurally characterized by NMR as follows: 4-galactosyl-kojibiose (β-D-Gal-(1→4)-D-Glc-(2→1)-α-D-

Glc) was produced from lactose (or cheese whey) and sucrose using a *Leuconostoc mesenteroides* B-512F dextransucrase as described by Diez-Municio et al. (2012a), lactulosucrose (β-D-Gal-(1→4)-β-D-Fru-(2→1)-α-D-Glc) from lactulose and sucrose using a *Leuconostoc mesenteroides* B-512F dextransucrase as in Diez-Municio et al. (2012b), lactosyl-oligofructosides (LFOS) (β-D-Gal-(1→4)-α-D-Glc-[(1→2)-β-D-Fru]$_n$, n=2–4) from sucrose:lactosucrose or sucrose:lactose mixtures by transfructosylation reaction catalyzed by an inulosucrase from *Lactobacillus gasseri* DSM 20604 or by a levansucrase from *Bacillus subtilis* CECT 39 before the inulosucrase-catalyzed reaction as in Diez-Municio et al. (2015), raffinosyl-oligofructosides (RFOS) (α-D-Gal-(1→6)-α-D-Glc-[(1→2)-β-D-Fru]$_n$, n=2–5) from raffinose catalyzed by an inulosucrase from *Lactobacillus gasseri* DSM 20604 as in Diez-Municio et al. (2016b), and lactulose-derived galacto-oligosaccharides ~~derived from lactulose~~ (GOS-Lu) (comprising a complex mixture of oligosaccharides containing galactose units linked predominantly by β(1→6) bonds and a fructose residue at the reducing end) synthesized by transgalactosylation reaction using a β-galactosidase from *Aspergillus oryzae* as catalyst and lactulose as starting substrate as in Julio-Gonzalez et al. (2018).

Oligosaccharide purification was carried out by Size Exclusion Chromatography (SEC) following the procedure described by Martín-Ortiz et al. (2016). In brief, each analysis was run with 25 mL of the corresponding reaction mixture sample (20 % of total carbohydrates, w/v) injected into a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 cm × 5 cm) using degassed milli-Q water as the mobile phase at a flow of 1.5 mL/min and maintained at 4°C. The effluent was collected in 15 ml test tubes at the outlet of the column using a fraction collector (Frac-920; GE Healthcare Life Sciences, Mississauga, Canada). The degree of polymerization (DP) of collected fractions was determined by electrospray ionization-mass spectrometry (ESI-MS) on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector at positive polarity selecting the corresponding *m/z* values.

The electrospray ionization source was operated under positive polarity using 4 kV capillary voltage at 300 °C with a nitrogen drying gas flow of 12 L/min and a nebulizer (N2, 99.5 % purity) pressure of 276 kPa; the fragmentor voltage was varied from 80 to 110 V. Ions corresponding to $[M + Na]^+$ of the carbohydrates under analysis were monitored in SIM mode using default variable fragmentor voltages. Samples were diluted with 50:50 (v:v) acetonitrile (ACN):water and filtered through a 0.22 μm filter (Millipore, Madrid, Spain), and 5 μL was injected. HPChem Station software version 10.02 (Hewlett-Packard) was used for data processing. Fractions with DP ≥3 were pooled and freeze-dried.

The oligosaccharide profile of the purified samples was checked by LC-RID on an Agilent Technologies 1220 Infinity LC System-1260 RID (Boeblingen, Germany). The separation of carbohydrates was carried out with a Kromasil (100-$NH_2$) column (250 × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY) using acetonitrile:water (75:25, v:v) as the mobile phase and eluted in isocratic mode at a flow rate of 0.9 mL/min for 30-70 min, the injection volume being 50 μL. Data acquisition and processing were performed using the Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany).

*2.2. Growth assays. Bacterial strains and culture conditions*

Prior to executing growth experiments, bifidobacterial strains were routinely cultured in de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France) supplemented with 0.05 % cysteine-HCl~~, which makes the media more selective for bifidobacteria (but inhibitory to a wide range of non-bifidobacterial strains)~~.

Carbohydrate utilization by bifidobacterial strains was examined in modified de Man Rogosa and Sharpe (mMRS) medium prepared from first principles (de Man et al., 1960), without any carbohydrate source. The media was supplemented with the tested oligosaccharides, previously weighed and filter sterilised with 0.2 μm pore-size sterile filters, at a final concentration of 0.5 % (w/v). This medium was then inoculated (1 % inoculum) using an

overnight culture of a given bifidobacterial strain which had been grown on glucose. Negative controls were prepared using (i) inoculated basic medium without a carbon source, and (ii) basic uninoculated media containing a particular carbohydrate.

Bacterial growth was monitored in duplicate 30 ml Sterilin™ tubes (Thermo Scientific™, Newport, UK). The cultures were incubated under anaerobic conditions in a modular atmosphere-controlled system (Whitley Workstation A85, Davidson and Hardy, Belfast, Ireland) at 37 °C for 24h. Optical densities at 600 nm ($OD_{600nm}$) were recorded manually at 60 min intervals using a spectrophotometer (BioTek Instrument Inc., Winooski, VT, USA). Appropriate dilutions of cultures were carried out when needed to obtain optical density units in the linear relationship range (~0.3-0.7).

*2.3. Analysis of novel oligosaccharides consumption*

Consumption of the tested novel oligosaccharides by the two bifidobacterial strains was determined by Gas Chromatography with a flame ionization detector (GC-FID) on an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector, using nitrogen as a carrier gas at 1 mL/min. The trimethylsilyl oxime (TMSO) derivatives were prepared as previously described by Sanz et al. (2004) and separated using a HT5 carborane-methyl-polysiloxane capillary column (15 m × 0.32 mm internal diameter × 0.1 μm film thickness; J&W Scientific, Folsom, CA, USA). The initial oven temperature was 180 °C, increased at a rate of 3 °C/min to 250 °C and then at a rate of 3 °C/min to 380 °C. The injector and detector temperatures were 280 and 385 °C, respectively. Injections were made in the split mode (1:10). Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for carbohydrates were calculated from FID peak areas relative to phenyl-β-D-glucoside (internal standard).

For quantification of tested oligosaccharides before and after incubation with *B. breve* UCC2003 and *B. longum* NCIMB 8809 in growth medium samples, the reduction or increase in particular saccharide species was calculated as the relative percentage of the final concentration of those saccharides compared to the initial concentration (before incubation), respectively.

*2.4. Analysis of Short-Chain Fatty Acids (SCFA) and Lactic Acid*

Analysis of SCFAs (acetic, formic, propionic, and butyric acids) and lactic acid was carried out by high performance liquid chromatography (HPLC) with absorbance detector (UV) at 210 nm (Ruiz-Matute et al. 2011). Samples taken from pure culture tubes after 24h of incubation were centrifuged at 13,000 x *g* for 5 min to remove cells, and supernatants were then filtered through a 0.22 μm filter unit (Millipore), after which 30 μL of each sample was injected into the HPLC system (Hewlett-Packard HP1050 series) equipped with an absorbance detector and an automatic injector. The column was an ion-exclusion REZEX-ROA organic acid column (300 x 7.8 mm; Phenomenex, Chester, U.K.) maintained at 40 °C. The eluent was 0.005 N sulfuric acid in HPLC-grade water, and the flow was 0.5 mL/min. Quantification of the samples was carried out using external standard calibration curves for acetic, formic, propionic, butyric and lactic acids at concentrations ranging from 0.5 to 100 mM.

To evaluate the production of SCFAs and lactic acid, the original concentrations obtained after HPLC-UV analysis were corrected by subtracting the SCFA and lactic acid concentrations in the control sample from the respective SCFA and lactic acid concentration found in the supernatants of bifidobacterial culture samples. Likewise, a normalization of the final concentration of SCFAs and lactic acid against $OD_{600nm}$ density was performed in order to provide an adequate comparison between both strains.

*2.5. Analysis of global gene expression using B. breve UCC2003 DNA microarrays*

Global gene expression was determined during ~~log phase~~ the mid exponential phase of growth of *B. breve* UCC2003 in mMRS supplemented with 4-galactosyl-kojibiose, kojibiose, lactulose or lactulosucrose as the sole carbon/energy source ($OD_{600nm}$ of 0.5-0.6). The obtained transcriptome was compared to that determined for ~~log phase~~ *B. breve* UCC2003 cells when grown to the mid exponential phase in mMRS supplemented with ribose. DNA microarrays containing oligonucleotide primers representing each of the 1864 identified open reading frames on the genome of *B. breve* UCC2003 were designed and obtained from Agilent Technologies (Palo Alto, Ca., USA). A DNA microarray data analysis of two technical replicates per experiment was performed. Methods for cell disruption, RNA isolation, RNA quality control, complementary DNA synthesis and labelling were performed as described previously (Zomer et al. 2009). Labelled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-Colour Microarray-Based Gene Expression Analysis v4.0 manual (publication number G4140-90050). Following hybridization, microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A). Generated scans were converted to data files with Agilent's Feature Extraction software (Version 9.5). DNA-microarray data were processed as previously described (Garcia de la Nava et al. 2003). Differential expression tests were performed with the Cyber-T implementation of a variant of the t-test (Long et al. 2001).

*2.6. Biochemical and substrate ~~specificity~~ selectivity characterization*

*2.6.1. Plasmid construction for heterologous expression of lacZ genes.*

The strain *Lactococcus lactis* NZ9000-pNZ-*lacZ6* containing Bbr_1552 was used in previous studies (James, et al. 2016; O'Connell Motherway et al. 2010). The DNA manipulations to produce *L. lactis* NZ9000-pNZ-*lacZ7* were performed as described recently

(Ambrogi et al., 2019). Briefly, the Bbr_1833 gene was amplified from the genomic DNA of *B. breve* UCC2003 by PCR using primers bbr1833f (TGCATC *GATATC* ATG CAT CAC CAT CAC CAT CAC CAT CAC CAT CAC ATG ACA ACC GCA ACC AAC CGA G) and bbr1833r (TGCGCA *TCTAGA* TCA GGC GAC CTT GCA GTC G) containing an N-terminal His-tag-encoding sequence and the restriction sites EcoRV and XbaI indicated in italics. The PCR fragment (2127 nucleotides) was purified and cloned in digested pNZ8150. The resulting plasmid pNZ-LacZ7 was introduced into *L. lactis* NZ9000 by electroporation and selected using chloramphenicol.

*2.6.2~~1~~. Heterologous protein production and purification*

To investigate the role of the enzymes involved in the hydrolytic activity of specific substrates, His-tagged version of *lacZ6* and *lacZ7* (corresponding to the products of Bbr_1552 and Bbr_1833, see previous section, and termed here as *lacZ6*His and *lacZ7*His, respectively) were produced and purified as described previously (O'Connell Motherway et al. 2010; Pokusaeva et al. 2011). In brief, 400 ml of M17 broth supplemented with 0.5 % (w/v) glucose was inoculated with a 2 % inoculum of a particular *L. lactis* strain (specified in section 2.6.1), followed by incubation at 30 °C until an $OD_{600nm}$ of 0.5 was reached, at that point protein expression was induced by the addition of cell-free supernatant (800 µL) of a nisin-producing strain, followed by continued incubation for further 2 hours. Cells were harvested by centrifugation and the protein purification was performed as described previously (O'Connell Motherway et al. 2010) using~~. In brief,~~ a Ni-NTA affinity system (Qiagen GmBH, Hilden, Germany) ~~was used~~, after which the elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Rainbow pre-

stained low-molecular-weight protein markers (New England Bio-Labs, Hertfordshire, United Kingdom) were used to estimate the molecular weights of the purified proteins. Then, protein concentration were determined using the Bradford method (Bradford 1976).

*2.6.3~~2~~. Assay of β-galactosidase activities*

The hydrolytic activities specified by *lacZ6*His and *lacZ7*His were determined as described previously (O´Connell et al. 2013), using kojibiose, 4-galactosyl-kojibiose, lactulosucrose, lactulose or lactose as a substrate. ~~Briefly~~Specifically, ~~50 µL~~ 25 µg of purified proteins (*lacZ6* and *lacZ7*, respectively) were added to 500 µL of each of the carbohydrates ~~sugars~~ mentioned above dissolved in 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0) buffer (~~1~~2 mg/ml (w/v)). Then, MOPS buffer was added to a final reaction volume of 1mL, and this reaction mixture was then ~~followed by~~ incubated~~ion~~ for 24 hours at 37 °C. All samples were subjected to a final enzyme denaturation step at 85 °C for 15 minutes. Then, ~~they~~ samples were filtered by membrane filtration, using Spin-X centrifuge tube filters (pore size, 0.45 um; Costar; Corning Inc., NY), and were stored at -20°C prior to High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) analysis.

Carbohydrate fractions from the above-mentioned hydrolysis assays (25 µl aliquots) were analysed by HPAEC-PAD, as indicated previously (James et al., 2016). For this, a Dionex (Sunnyvale, CA) ICS-3000 system was used with a CarboPac PA1 analytical-exchange column (dimensions, 250 mm by 4 mm) and a CarboPac PA1 guard column (dimensions, 50 mm by 4 mm). Chromatographic profiles of standard carbohydrates were used for comparison of the results of their breakdown by the proteins tested. Chromeleon software (version 6.70; Dionex Corporation) was used for the integration and evaluation of the chromatograms obtained. A 1 mg/ml stock solution of each of the carbohydrates, as well as their putative breakdown products

used as reference standards was prepared by dissolving the particular carbohydrate ~~sugar~~ in Milli-Q water.

*2.7. Statistical analysis*

The comparisons of means using analysis of variance (ANOVA) were made using the statistical software package SPSS version 24 (SPSS Inc., IL, USA). Differences were considered significant when $P < 0.05$.

The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE132879.

## 3. RESULTS AND DISCUSSION

### *3.1. Chemical structure and degree of purity of the novel oligosaccharides.*

Synthesized oligosaccharides with a variety of structural features, such as monomer composition and type of glycosidic linkage, were employed in this study in order to determine their potential bifidogenic properties. The enzymes and starting substrates used for their production are specified in section 2.1.2. **Table 1** shows the chemical structures and degree of purity of the carbohydrates assessed in the present study. With the exception of GOS-Lu, the degree of purity of the novel oligosaccharides (considered as absence of mono- and disaccharides) was ≥99.0 % as determined by LC-RID. In the case of GOS-Lu, the starting substrate lactulose was not considered an impurity due to its prebiotic character (Corzo et al., 2015). Therefore, the GOS-Lu mixture comprised 8 % monosaccharides, 8 % disaccharides (mainly lactulose), 51 % of GOS-Lu trisaccharides and 33 % of GOS-Lu tetrasaccharides, leading to a degree of purity of 92 %.

### *3.2. Evaluation of Bifidobacterium breve UCC2003 and Bifidobacterium longum NCIMB 8809 growth on biosynthetic carbohydrates*

To determine the ability of bifidobacteria to grow on the purified oligosaccharides ~~to support bifidobacterial growth~~(based on final optical densities at 600 nm [$OD_{600nm}$]), *B. breve* UCC2003 (representative of an infant-associated bifidobacterial species) and *B. longum* NCIMB 8809 (representing a bifidobacterial species that is commonly isolated from adult and infant feces) were chosen based on their relevance. Concretely, they both have acted as model strains and, therefore, have been subject of genetic studies (Egan et al., 2014; James et al., 2016; O'Callaghan et al., 2015). Moreover, members of these two species are currently used as functional supplements in foods. These human-derived species were inoculated in a medium that included a particular carbohydrate, while also using a non-inoculated medium as a negative

control. A single carbon source (0.5 % (w/v)) was evaluated, considering either the proposed novel purified carbohydrates or other common and commercially available carbohydrates, such as lactose, lactulose, kojibiose or raffinose (selected because they represent core structures of many of the assessed oligosaccharides).

The results revealed that generally grow well (final OD600nm> 1.5) in several commercially available carbohydrates (Table 2). The final optical densities at 600 nm (all observed $OD_{600nm}$ were> 1.5) revealed that several commercially available carbohydrates (i.e., glucose, lactose, lactulose, maltose and raffinose) were remarkably metabolized by *B. breve* UCC2003 and *B. longum* NCIMB 8809 as a carbon source during 24h of cultivation (**Table 2**). In contrast, both bifidobacterial strains exhibited a very limited ability to ferment galactose, as previously described (Bottacini et al., 2014; Pokusaeva et al., 2011).

Among the novel tested oligosaccharides, 4-galactosyl-kojibiose was shown to support good growth for both strains, whereas lactulosucrose, lactosyl-oligofructosides (LFOS) and lactulose-derived galacto-oligosaccharides derived from lactulose (GOS-Lu) were differentially selectively fermented, showing varying growth abilities between the two studied strains (Figure S1). For example, *B. longum* NCIMB 8809 was shown to exhibited a higher growth ability on GOS-Lu as compared to the other assessed oligosaccharides. Furthermore, raffinosyl-oligofructosides (RFOS) were shown to represent a rather moderate growth substrate for *B. longum* NCIMB 8809, whilst *B. breve* UCC2003 exhibited very little growth on this substrate ($OD_{600nm}$< 0.5) (**Table 2**). The *B. longum* strain showed a higher growth ability when structures similar to FOS (i.e. LFOS and RFOS) were employed as the sole carbohydrate source in the growth medium when compared with that observed for *B. breve* UCC2003 (Figure S1).

*3.3. Carbohydrate fractions that are preferentially consumed by B. breve UCC2003 and B. longum NCIMB 8809.*

The levels of consumption of the tested oligosaccharides by the two studied bifidobacterial strains are shown in **Table 3**. In general terms, we found a positive correlation between growth ability of the strain (**Table 2**) and the level of carbohydrate consumption (**Table 3**). In the case of lactulosucrose, the difference found in growth data between *B. longum* NCIMB 8809 and *B. breve* UCC2003 was just 0.2 $OD_{600nm}$ units (that is, 0.8±0.01 and 1.0±0.04, respectively), consistent with a similar consumption level observed for both strains (**Table 3**). 4-Galactosyl-kojibiose was also consumed to a very similar extent by both strains, being in agreement with the observed growth abilities. Additionally, both trisaccharides were consumed in the narrow range of 24-28.5 % (**Table 3**). In contrast, GOS-Lu, LFOS and RFOS were metabolized to a significantly higher level by *B. longum* NCIMB 8809 when compared to *B. breve* UCC 2003, reaching total consumption levels of 79 %, 43 % and 16 %, respectively, by the former, being 1.4 to 1.9 times higher compared to that observed for *B. breve* UCC 2003 (**Table 3**).

Given that LFOS, RFOS and GOS-Lu are composed of an oligosaccharide mixture consisting of carbohydrates with a varying degree of polymerization (DPs), the impact of DP on the carbohydrate utilization preference was investigated. The obtained results revealed the existence of an inverse relationship between DP and consumption level for LFOS (i.e. the lower the DP of a given carbohydrate the higher the extent of utilization of this carbohydrate), whereas this effect was not observed in the case of GOS-Lu, likely due to the fact that the consumption rate was rather fast and, therefore, not a clear trend could be found following 24 h of growth. RFOS is dominated by DP4 (around 90 % of total content) and, consequently, no solid evidence could be inferred from the impact of the DP on RFOS utilization. Furthermore, β-glycosidic

linkages between galactose units, as in the case of GOS-Lu, appeared to be more prone to metabolism by either of the two bifidobacterial strains when compared to linkages involving fructose units (i.e., LFOS or RFOS) as based on growth (**Table 2**) and consumption (**Table 3**) data.

*3.4. SCFA analysis*

In order to obtain information about metabolite (such as SCFA and lactic acid) production following fermentation of the tested oligosaccharides, HPLC-UV analysis was employed to measure their concentrations in the supernatants of the two bifidobacterial strains. The obtained results showed that acetic acid, whose formation is consistent with the well-described bifidobacterial metabolic pathway (Palframan et al., 2003), was the predominant end metabolite formed in all cases, whereas lactic acid and formic acid were also found at different levels depending on the fermented oligosaccharide (**Table 4**). In this context, Macfarlane and Macfarlane (2003) have previously demonstrated that acetic acid and formic acid were the major bifidobacterial fermentation products formed during growth under carbohydrate limitation, whereas ~~acetate~~ acetic and ~~lactate~~ lactic acids were shown to be produced when carbohydrate is in excess. Similar outcomes were gathered from data collected from the *B. breve* species using a continuous culture set-up (Macfarlane and Gibson, 1995).

The highest acetic acid concentration (66-67 mM) was detected when lactose was used as a carbon source following 24h of cultivation. Concerning the various oligosaccharides assessed in the current study, GOS-Lu and lactulosucrose were metabolized to ~~acetate~~ acetic acid at levels ranging from 20 to 27 mM, or between 18 and 20 mM, or 11.5 and 19.7 mM when grown on 4-galactosyl-kojibiose or LFOS, respectively, whereas RFOS was the substrate with the lowest

produced levels of SCFAs, which is consistent with this substrate being a rather poor growth substrate for the bifidobacterial strains (**Table 2**). As expected, lactose was the substrate that generated the highest level of lactic acid for both bifidobacterial strains (29.7 and 37.1 mM), followed by raffinose (16.5 and 19.6 mM). In contrast, lactic acid levels found for the rest of oligosaccharides were below 6 mM. The lowest metabolite concentrations (values ranging from 0.5 to 3.1 mM) were found for ~~formate~~formic acid, whereas in some cases it was not detected at all. As expected, no ~~propionate~~propionic or ~~butyrate~~butyric acids were detected in any of the studied samples, as bifidobacterial strains do not have any metabolic abilities to produce these short chain fatty acids.

By considering the normalized values of SCFA and lactic acid production against $OD_{600nm}$ values obtained for both strains, the highest level of acetic acid was produced by the *B. longum* strain when grown on monosaccharides, whereas no ~~remarkable~~ differences were found between the two tested strains when they were grown on disaccharides with the exception of maltose which led to a very low ~~acetate~~ acetic acid level in the case of *B. longum* NCIMB 8809 when compared to *B. breve* UCC2003 (**Table 4**). Regarding the biosynthetic oligosaccharides, all of them presented normalized values for the production of acetic acid that were quite similar between the two strains for a given substrate with the exception of RFOS whose normalized production of acetic acid was ~2.5-fold higher for *B. breve* UCC2003 as compared to *B. longum* NCIMB 8809. A very similar trend as that described for acetic acid production following fermentation of the biosynthetic oligosaccharides was observed for normalized lactic acid values with the exception of LFOS which exhibited 1.7-fold higher levels in the case of *B. breve* UCC2003 (**Table 4**).

*3.5. Analysis of the 4-galactosyl-kojibiose and lactulosucrose-associated B. breve UCC2003 transcriptome*

To identify the specific enzymatic pathways that are required to metabolize the assessed oligosaccharides that supported growth, an exploratory global gene expression analysis was evaluated by microarray ~~analysis~~ during logarithmic growth of *B. breve* UCC2003 in mMRS medium supplemented with 4-galactosyl-kojibiose and lactulosucrose. These two oligosaccharides were selected based on their high purity and the fact that they are single compounds (**Table 1**), and because of their relatively high consumption by *B. breve* UCC2003 (**Tables 2** and **3**). Additionally, kojibiose and lactulose were studied since they are the core disaccharides of the aforementioned compounds. The obtained transcriptome data sets were compared with those obtained when the strain was grown in the same medium, yet supplemented with ribose as the sole carbohydrate source (thus to be used as a transcriptomic reference; its metabolic pathway and gene expression profile for growth of *B. breve* UCC2003 on this carbohydrate ~~sugar~~ is known (Bottacini et al., 2014; Egan et al., 2014; James et al., 2016; Lanigan et al., 2017; O'Connell et al., 2013; O'Connell et al., 2014; O'Connell Motherway et al., 2013; Pokusaeva et al. 2011).

Employing this transcriptome approach, we identified genes exhibiting increased transcription when grown on 4-galactosyl-kojibiose, lactulosucrose, kojibiose and lactulose (as compared to growth on ribose). Unsurprisingly, many of these differentially transcribed genes were predicted to represent glycoside hydrolases (GHs) and carbohydrate~~sugar~~ transport systems. Lactulose and 4-galactosyl-kojibiose were the two substrates which caused a higher number of genes (i.e., 29 and 27, respectively) to exhibit increased transcription (at a cut-off of 2.5-fold increase in transcription, with an associated P value of 0.001) as compared to lactulosucrose (12 genes were shown to be transcriptionally upregulated) and kojibiose (6 upregulated genes).

Growth on 4-galactosyl-kojibiose corresponds to increased transcription of a particular gene cluster (Bbr_1551 to Bbr_1553), which represents genes that are required for growth on the human milk oligosaccharides lacto-N-tetraose and lacto-N-biose (James et al., 2016). Moreover, Bbr_1551, designated here as *lacS* which encodes a galactoside symporter, and *lacZ6* (corresponding to Bbr_1552), which encodes a β-galactosidase, have previously been shown to be involved in galacto-oligosaccharide metabolism in *B. breve* UCC2003 (O'Connell Motherway et al., 2010). The *lacZ6* gene was also significantly upregulated following growth of *B. breve* UCC2003 on lactulose and lactulosucrose (**Table 5**). Furthermore, the Bbr_0285 gene (designated here as *lacZ2*), which is predicted to encode another β-galactosidase, was highly upregulated when *B. breve* UCC2003 was cultivated on lactulose and lactulosucrose (**Table 5**). Two other gene clusters were significantly upregulated when UCC2003 was grown on 4-galactosyl-kojibiose. These induced clusters correspond to locus tags Bbr_1832 through to Bbr_1836, and Bbr_1878 through to Bbr_1880 (**Table 5**). The gene associated with locus tag Bbr_1833 (designated here as *lacZ7*) is predicted to encode a β-galactosidase, while the gene corresponding to locus tag Bbr_1836 encodes a predicted carbohydrate~~sugar~~-binding protein of ABC transporter system. The transcription of *lacZ7* was also significantly increased in the presence of mucin, probably because this enzyme is involved in the removal of galactose from mucin (Egan et al., 2014). The second transcriptionally upregulated cluster when UCC2003 is grown on 4-galactosyl-kojibiose contains a component of a phosphoenolpyruvate phosphotransferase (PEP-PTS) system and was also upregulated following incubation with lactulosucrose (**Table 5**).

In addition to data mentioned above, other gene clusters (corresponding to locus tags Bbr_0417 to Bbr_0422, Bbr_0526 to Bbr_0530 and Bbr_1865 to Bbr_1867) were upregulated on lactulose. These clusters have previously been shown to be involved in the metabolism of galacto-oligosaccharides and galactose-containing human milk oligosaccharides (James et al. 2016; O'Connell Motherway et al., 2013). Transcription of gene Bbr_1866 (encoding a putative transport system permease protein) was very highly upregulated (fold change 91.1) when grown on lactulose, suggesting its involvement in the metabolism of this disaccharide. The gene associated with locus tag Bbr_0529 (designated here as *lacZ5*, encoding a predicted β-galactosidase), which is located in one of the clusters and was previously reported to be involved in LNT and LNnT metabolism (James et al. 2016), was slightly upregulated when 4-galactosyl-kojibiose was used as substrate.

An interesting, if somewhat surprising, outcome of this study was the finding of an upregulated gene cluster (Bbr_0164 to Bbr_0169), involved in the metabolism of sialic acid and, therefore, not structurally related to the tested oligosaccharides, on lactulose and, especially, on 4-galactosyl-kojibiose (**Table 5**). This result may be explained by the fact that induction of certain gene clusters does not necessarily denote that their products are involved.

*3.6. Substrate ~~specificity~~ selectivity assays*

The suspected involvement of various identified β-galactosidases encoded by the carbohydrate~~sugar~~-induced genes (such as those corresponding to Bbr_1552 and Bbr_1833) in the metabolism of the novel oligosaccharides lactulosucrose and 4-galactosyl-kojibiose, as well as kojibiose, lactose and lactulose was further explored. To do this, the genes that encode these proteins were cloned and the corresponding proteins were overproduced in *L. lactis*, after which they were purified by affinity chromatography (see Materials and Methods). Analysis by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) revealed bands at

an apparent molecular weight of 118 kDa and 79.5 kDa for lacZ6His (corresponding to Bbr_1552) and lacZ7His (corresponding to Bbr_1833), respectively.

The ability of these two β-galactosidases to hydrolyse the oligosaccharides mentioned above was determined using *in vitro* enzymatic assays (by incubating the purified enzyme with its suspected substrate oligosaccharide for 24 h at 37 °C) and subsequent analysis of the reaction product by HPAEC-PAD. Based on the obtained HPAEC-PAD chromatograms (**Figure 1**), we found that lacZ6His did not hydrolyze the disaccharide kojibiose (dotted line in **Figure 1C**), apparently because the enzyme is unable to hydrolyze α-(1→2) glycosidic bonds. This finding is in agreement with the lack of upregulation observed for this particular gene when *B. breve* UCC2003 was grown on kojibiose (**Table 5**). In contrast, lacZ6His readily hydrolyzed lactose and lactulose (**Figures 1A** and **1B**, respectively), releasing galactose and glucose, or galactose and fructose, respectively. LacZ6His also hydrolyzed lactulosucrose, releasing sucrose and galactose (**Figure 1E**) and more efficiently 4-galactosyl-kojibiose ~~was efficiently hydrolyzed by lacZ6His, releasing~~ producing kojibiose and galactose (**Figure 1D**). This result revealed that lacZ6His has the ability to hydrolyze galactose units bonded by β(1-4) linkages either to fructose or glucose moieties. ~~Moreover, by comparing the obtained hydrolysis levels obtained with lacZ6His, 4-galactosyl-kojibiose was shown to be more completely hydrolyzed when compared to lactulosucrose. Similarly~~In addition, a significant higher upregulation of the *Bbr_1552* gene was observed for 4-galactosyl-kojibiose as compared to lactulosucrose in the *B. breve* UCC2003 transcriptome analysis (**Table 5**). In contrast, it was observed that lacZ7His (dashed line) did not exert any hydrolytic activity on any of the substrates employed (kojibiose, 4-galactosyl-kojibiose, lactose, lactulose and lactulosucrose). This lack of activity on these carbohydrates ~~sugars~~may be due to the tested conditions, which were optimal for lacZ6His (as specified in James et al., 2016), or to a misfolding of the protein. Of course, it is possible that this enzyme possesses a substrate ~~specificity~~selectivity different from the substrates used.

Bearing in mind the possible limitations of the current study (such as the additional information that could provide the testing of these carbohydrates in a wider variety of bifidobacteria species, as well as the performance of additional microarray experiments and larger quantitative growth assays), the results of this work constitutes an ~~remarkable~~ advance in the knowledge on the utilization of novel dietary oligosaccharides by two relevant beneficial human gut bacteria, through the elucidation of specific metabolic pathways and structural features (i.e., monomer and glycosidic linkage type) required for their growth.

## 4. CONCLUSIONS

The data assembled in this study provide information on the abilities of *B. breve* UCC2003 and *B. longum* NCIMB 8809 to grow on a number of novel oligosaccharides. Overall, the tested biosynthetic oligosaccharides supported reasonable to good growth of, at least, one of the two studied strains with the exception of RFOS which appears to represent a poor substrate for either of the strains. The identification of key genes encoding for ~~protein~~carbohydrate transport systems and glycoside hydrolases, particularly β-galactosidases, revealed the importance of the presence of galactose connected by β-glycosidic linkages in the metabolism of these oligosaccharides by human-derived bifidobacterial strains.

The described fermentation properties of the novel oligosaccharides and the relationship to their chemical structure are highly relevant to the potential design of prebiotics with bifidogenic attributes. ~~a high degree of selectivity.~~

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**Figure caption**

Figure 1. HPAEC-PAD profiles of (A) lactose, (B) lactulose, (C) kojibiose, (D) 4-galactosyl-kojibiose, (E) lactulosucrose, when incubated in MOPS buffer (pH 7) and the tested β-galactosidases (lacZ6His: dotted line, lacZ7His: dashed line).

**Table 1.** Chemical structure, hexose units and purity of all tested carbohydrates.

| Oligosaccharide tested | Chemical structure | Hexose units | Purity |
|---|---|---|---|
| Glucose | | 1 | ≥99.5% |
| Galactose | | 1 | ≥99% |
| Lactose | β-D-Gal-(1→4)-D-Glc | 2 | 98% |
| Lactulose | β-D-Gal-(1→4)-D-Fru | 2 | 98% |
| Maltose | α-D-Glc-(1→4)-D-Glc | 2 | ≥99% |
| Kojibiose | α-D-Glc-(1→2)-D-Glc | 2 | >99% |
| Raffinose | α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru | 3 | ≥98% |
| 4-Galactosyl-kojibiose | β-D-Gal-(1→4)-D-Glc-(2→1)-α-D-Glc | 3 | ≥99% |
| Lactulosucrose | β-D-Gal-(1→4)-β-D-Fru-(2→1)-α-D-Glc | 3 | ≥99% |
| Lactosyl-oligofructosides (LFOS) | β-D-Gal-(1→4)-α-D-Glc-[(1→2)-β-D-Fru]$_n$, n=2-4 | 3-6 | ≥99% |
| Raffinosyl-oligofructosides (RFOS) | α-D-Gal-(1→6)-α-D-Glc-[(1→2)-β-D-Fru]$_n$, n=2-5 | 4-7 | ≥99% |
| Lactulose-derived galacto-oligosaccharides (GOS-Lu) | General structure: complex mixture of (Gal)$_n$-Fru, (Gal)$_n$-Gal and (Gal)$_n$-Fru-Gal, involving β(1→1), β(1→4) and β(1→6) linkages. | 2-4 | 92% |

**Table 2.** Carbohydrate utilization (0.5 %, w/v) by the tested strains of *Bifidobacterium* on mMRS medium (24 h growth).

| Strain | Glucose | Galactose | Lactose | Lactulose | Maltose | Raffinose | Kojibiose | 4-Galactosyl-kojibiose | Lactulosucrose | LFOS | RFOS | GOS-Lu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***B. breve* UCC 2003** | ++++ | + | ++++ | +++ | ++++ | ++++ | ++ | ++ | ++ | + | - | ++ |
| ***B. longum* NCIMB 8809** | ++++ | + | ++++ | ++++ | ++++ | ++++ | + | ++ | + | ++ | + | +++ |

A minus sign (-) indicates that final $OD_{600nm}$ <0.5; + indicates final OD600nm = 0.5–1; ++ indicates final OD600nm =1–1.5; and +++ indicates final OD600nm >1.5–2; and ++++ final OD600nm >2.

| Strain | Glucose | Galactose | Lactose | Lactulose | Maltose | Raffinose | Kojibiose | 4-Galactosyl-kojibiose | Lactulosucrose | LFOS | RFOS | GOS-Lu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. breve* UCC 2003 | 3.00± 0.31* | 1.37± 0.09 | 3.27± 0.05 | 1.67± 0.19 | 2.23± 0.13 | 2.51± 0.01 | 1.11± 0.10 | 1.23 ± 0.07 | 1.00 ± 0.04 | 0.62± 0.01 | 0.27± 0.06 | 1.38± 0.03 |
| *B. longum* NCIMB 8809 | 3.44± 0.06 | 0.87± 0.07 | 3.86± 0.16 | 2.16± 0.32 | 2.62± 0.41 | 3.09± 0.11 | 0.60± 0.01 | 1.07 ± 0.02 | 0.80 ± 0.01 | 1.14± 0.03 | 0.66± 0.02 | 1.82± 0.05 |

* Results shown as mean (*n*=3) with the corresponding standard deviation (±) between parentheses.

**Table 3.** Quantification of tested oligosaccharides before (Pre) and after (Post) incubation with *B. breve* UCC2003 (*B.b*) and *B. longum* NCIMB 8809 (*B. lo*) in samples having different saccharide fractions (DP*1, DP2, DP3, DP4, DP5 and DP6).

| | | Concentration (mg/L) | | | | | | % Total consumed *** |
|---|---|---|---|---|---|---|---|---|
| | | DP*1 | DP2 | DP3 | DP4 | DP5 | DP6 | |
| LFOS | Pre | - | - | 0.93±(0.01)** [a] | 1.28±(0.07) [a] | 0.06±(0.01) [a,b] | - | |
| | *B.b* | 0.047±(0.002) [a] | 0.060±(0.003) [a] | 0.65±(0.02) [b] | 0.96±(0.05) [b] | 0.05±(0.002) [b] | - | 22.7 [a] |
| | *B. lo* | 0.01±(0.001) [b] | 0.01±(0.001) [b] | 0.19±(0.05) [c] | 1.02±(0.09) [b] | 0.06±(0.01) [a] | - | 43.2 [b] |
| RFOS | Pre | 0 [a] | 0 [a] | 0.06±(0.02) [a] | 2.09±(0.06) [a] | 0.10±(0.02) [a] | 0.10±(0.003) [a] | |
| | *B.b* | 0.07±(0.001) [b] | 0.03±(0.002) [b] | 0.02±(0.003) [b] | 1.80±(0.09) [a] | 0.09±(0.002) [a] | 0.08±(0.01) [a] | 10.9 [a] |
| | *B. lo* | 0.07±(0.01) [b] | 0.03±(0.002) [b] | 0.09±(0.02) [a] | 1.76±(0.5) [a] | 0.11±(0.02) [a] | 0.07±(0.03) [a] | 16.0 [b] |
| GOS-Lu | Pre | 0.33±(0.003) [a] | 0.23±(0.01) [a] | 1.56±(0.02) [a] | 0.61±(0.06) [a] | - | - | |
| | *B.b* | 0.13±(0.01) [b] | 0.16±(0.01) [b] | 0.62±(0.05) [b] | 0.309±(0.001) [b] | - | - | 55.2 [a] |
| | *B. lo* | 0.08±(0.002) [c] | 0.05±(0.01) [c] | 0.38±(0.02) [c] | 0.065±(0.02) [c] | - | - | 78.6 [b] |
| 4-Galactosyl-kojibiose | Pre | - | 0 [a] | 2.58±(0.06) [a] | - | - | - | |
| | *B.b* | - | 0.02±(0.01) [b] | 1.85±(0.08) [b] | - | - | - | 28.4 [a] |
| | *B. lo* | - | 0.06±(0.01) [c] | 1.90±(0.01) [b] | - | - | - | 26.5 [a] |
| Lactulosucrose | Pre | - | 0 [a] | 2.66±(0.25) [a] | - | - | - | |
| | *B.b* | - | 0.03±(0.01) [b] | 2.03±(0.1) [b] | - | - | - | 23.8 [a] |
| | *B. lo* | - | 0.02±(0.01) [b] | 1.99±(0.09) [b] | - | - | - | 25.1 [a] |

* DP: Degree of polymerization; ** ~~Standard deviation in parenthesis (n=3);~~ Results shown as mean (*n*=3) with the corresponding standard deviation (±) ~~between parentheses~~ *** Reduction or increase in the saccharide species was calculated as the relative percentage of the final concentration of those saccharides compared to the initial concentration (before incubation), respectively. Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

**Table 4.** SCFA and lactic acid concentrations (mM) from supernatants of bacterial cultures (24 h of *in vitro* incubation) determined by HPLC.

| | | Concentrations (mM) | | | Normalized values** | | |
|---|---|---|---|---|---|---|---|
| | | Lactic | Formic | Acetic | Lactic | Formic | Acetic |
| **Glucose** | *B. breve* UCC2003 | 3.6±(0.4)*$^{abcdefg}$ | 1.8±(0.3)$^{a}$ | 32.7±(6.0)$^{ab}$ | 1.12 | 0.56 | 10.16 |
| | *B. longum* NCIMB 8809 | 22.7±(3.9)$^{h}$ | 1.2±(0.3)$^{b}$ | 49.9±(8.7)$^{c}$ | 6.52 | 0.34 | 14.32 |
| **Galactose** | *B. breve* UCC2003 | 8.6±(0.3)$^{i}$ | 2.6±(0.2)$^{d}$ | 26.5±(1.2)$^{adef}$ | 6.01 | 1.82 | 18.51 |
| | *B. longum* NCIMB 8809 | 7.5±(0.05)$^{ij}$ | n.d. | 34.0±(0.1)$^{b}$ | 8.15 | 0 | 36.96 |
| **Lactose** | *B. breve* UCC2003 | 29.7±(1.8)$^{k}$ | 1.6±(0.1)$^{c}$ | 66.1±(2.0)$^{g}$ | 8.97 | 0.48 | 19.97 |
| | *B. longum* NCIMB 8809 | 37.1±(1.8)$^{l}$ | 0.5±(0.1)$^{b}$ | 67.6±(2.7)$^{g}$ | 9.34 | 0.13 | 17.02 |
| **Lactulose** | *B. breve* UCC2003 | 4.6±(0.01)$^{cdefgj}$ | 2.4±(0.2)$^{d}$ | 27.3±(1.3)$^{abf}$ | 2.54 | 1.33 | 15.10 |
| | *B. longum* NCIMB 8809 | 3.1±(0.4)$^{abcdef}$ | n.d. | 24.0±(1.7)$^{defh}$ | 1.30 | 0 | 10.07 |
| **Maltose** | *B. breve* UCC2003 | 24.3±(0.1)$^{h}$ | 3.0±(0.04)$^{e}$ | 50.3±(2.7)$^{c}$ | 11.36 | 1.40 | 23.50 |
| | *B. longum* NCIMB 8809 | 0.7±(0.04)$^{a}$ | n.d. | 2.1±(0.2)$^{i}$ | 0.24 | 0 | 0.72 |
| **Raffinose** | *B. breve* UCC2003 | 16.5±(0.7)$^{m}$ | 3.1±(0.1)$^{e}$ | 47.9±(0.2)$^{c}$ | 6.60 | 1.24 | 19.16 |
| | *B. longum* NCIMB 8809 | 19.6±(0.5)$^{n}$ | n.d. | 52.7±(0.1)$^{c}$ | 6.19 | 0 | 16.66 |
| **Kojibiose** | *B. breve* UCC2003 | 2.8±(0.2)$^{abcde}$ | n.d. | 6.8±(0.3)$^{ij}$ | 2.38 | 0 | 5.78 |
| | *B. longum* NCIMB 8809 | 2.3±(0.1)$^{abcd}$ | n.d. | 4.8±(0.4)$^{ij}$ | 3.81 | 0 | 7.95 |
| **4-Galactosyl-kojibiose** | *B. breve* UCC2003 | 2.6±(0.1)$^{abcde}$ | n.d. | 17.7±(0.3)$^{hk}$ | 2.02 | 0 | 13.79 |
| | *B. longum* NCIMB 8809 | 3.4±(0.2)$^{abcdefg}$ | n.d. | 19.5±(0.1)$^{dh}$ | 3.14 | 0 | 17.99 |
| **Lactulosucrose** | *B. breve* UCC2003 | 6.4±(0.4)$^{gji}$ | n.d. | 24.1±(0.9)$^{defh}$ | 6.27 | 0 | 23.63 |
| | *B. longum* NCIMB 8809 | 5.9±(0.3)$^{fgji}$ | n.d. | 20.3±(0.2)$^{defh}$ | 7.41 | 0 | 25.5 |
| **LFOS** | *B. breve* UCC2003 | 4.9±(0.1)$^{defgj}$ | n.d. | 11.5±(1.2)$^{jk}$ | 7.92 | 0 | 18.58 |
| | *B. longum* NCIMB 8809 | 5.4±(0.04)$^{efgj}$ | n.d. | 19.7±(0.5)$^{deh}$ | 4.66 | 0 | 16.98 |
| **RFOS** | *B. breve* UCC2003 | 1.6±(0.1)$^{abc}$ | n.d. | 5.6±(0.4)$^{ij}$ | 7.02 | 0 | 24.56 |
| | *B. longum* NCIMB 8809 | 1.9±(0.3)$^{abcd}$ | n.d. | 6.3±(0.1)$^{ij}$ | 2.83 | 0 | 9.38 |
| **GOS-Lu** | *B. breve* UCC2003 | 4.1±(0.4)$^{bcdefg}$ | n.d. | 24.3±(1.5)$^{defh}$ | 2.91 | 0 | 17.23 |
| | *B. longum* NCIMB 8809 | 4.3±(0.6)$^{bcdefg}$ | n.d. | 27.0±(0.1)$^{abef}$ | 2.32 | 0 | 14.59 |

n.d.: non detected

* Results shown as mean ($n$=3) with the corresponding standard deviation (±) ~~between parentheses~~. Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level. ** Normalized values: Data regarding normalization of the SCFA and lactic acid concentration values against $OD_{600nm}$ density.

**Table 5.** Carbohydrate-dependent transcriptional upregulation of specific genes during growth of *Bifidobacterium breve* UCC2003 in mMRS medium supplemented with selected substrates[a]

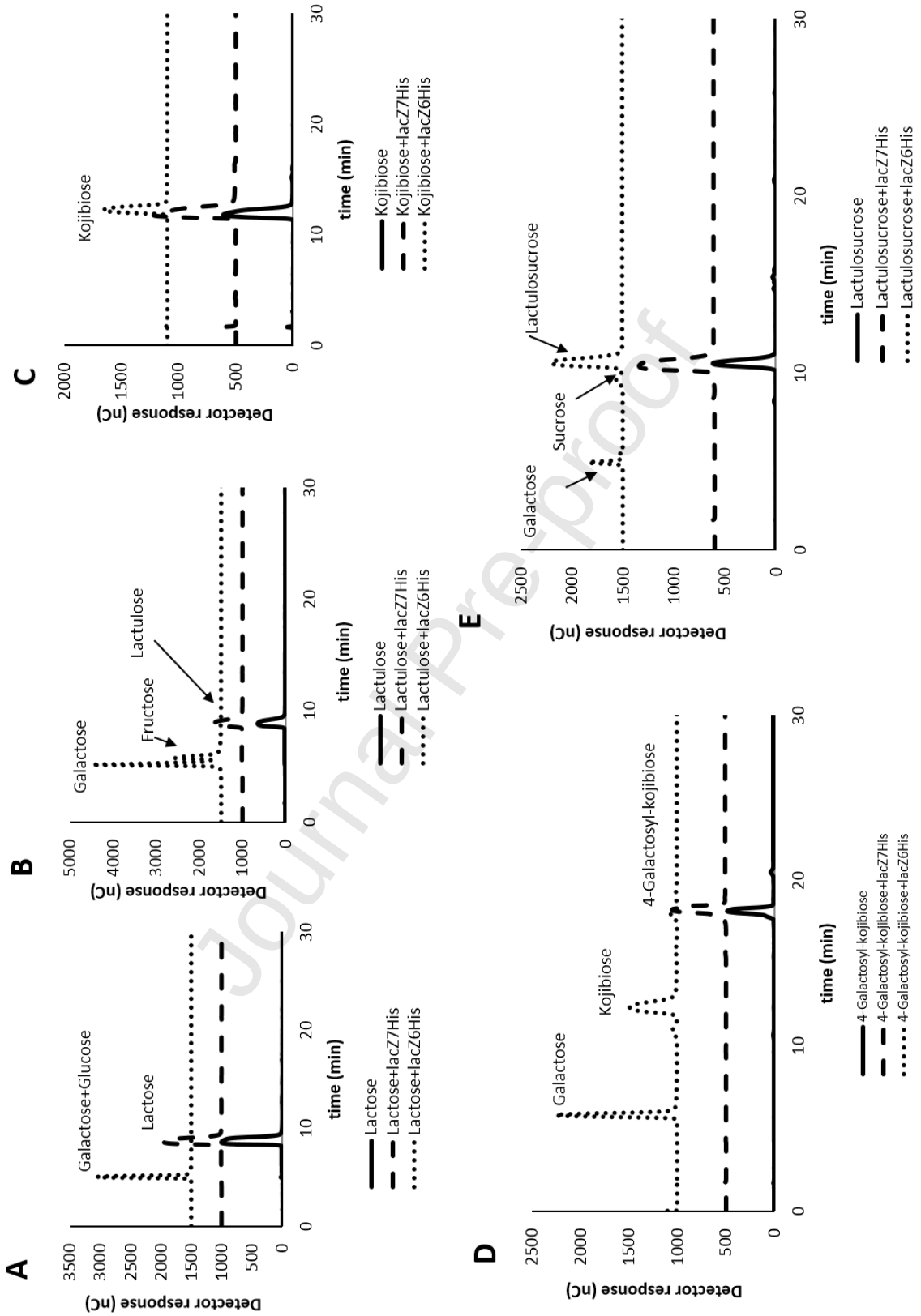
| | | Fold upregulation[b] during growth on: | | | |
|---|---|---|---|---|---|
| Gene ID | Gene name and function | 4-Galactosyl kojibiose | Kojibiose | Lactulose | Lactulosucrose |
| Bbr_0164 | oppA1 sialic acid-binding protein | 21.7 | — | 9.4 | — |
| Bbr_0165 | oppB1 sialic acid transport system permease protein | 15.5 | — | 21.1 | — |
| Bbr_0166 | oppD1 sialic acid transport ATP-binding protein | 20.0 | — | 11.2 | — |
| Bbr_0167 | oppF1 sialic acid transport ATP-binding protein | 14.4 | — | 5.3 | — |
| Bbr_0168 | sialidase | 14.2 | — | 2.8 | — |
| Bbr_0169 | nagB1 Glucosamine-6-phosphate isomerase | 13.6 | — | 3.1 | 3.6 |
| Bbr_0284 | Bbr_0284 Transport protein | 2.9 | — | 17.8 | 11.5 |
| Bbr_0285 | lacZ2 Beta-galactosidase | 3.7 | — | 39.7 | 21.1 |
| Bbr_0417 | Bbr_0417 Solute-binding protein of ABC transporter system for galacto-oligosaccharides | — | — | 4.0 | — |
| Bbr_0418 | Bbr_0418 Permease protein of ABC transporter system for galacto-oligosaccharides | — | — | 2.5 | — |
| Bbr_0419 | Bbr_0419 Permease protein of ABC transporter system for galacto-oligosaccharides | — | — | 3.5 | — |
| Bbr_0422 | galA Glycosyl hydrolases family 53, Endogalactanase | — | — | 6.7 | — |
| Bbr_0526 | Bbr_0526 Transcriptional regulator, LacI family | — | — | 4.3 | — |
| Bbr_0527 | Bbr_0527 Permease protein of ABC transporter system for sugars | — | — | 2.7 | — |
| Bbr_0528 | Bbr_0528 Permease protein of ABC transporter system for sugars | — | — | 3.6 | — |
| Bbr_0529 | lacZ5 Beta-galactosidase (involved in LN9n)T metabolism | 2.7 | — | 3.8 | — |
| Bbr_0530 | Bbr_0530 Solute-binding protein of ABC transporter system for sugars | 6.3 | 3.0 | 6.4 | 4.1 |
| Bbr_1551 | lacS Galactoside symporter | 14.2 | — | 20.3 | 3.1 |
| Bbr_1552 | LacZ6 Beta-galactosidase | 45.1 | — | 35.9 | 11.7 |
| Bbr_1553 | Bbr_1553 Transcriptional regulator, LacI family | 2.8 | — | 2.9 | — |
| Bbr_1566 | Bbr_1566 Universal stress protein family | 5.3 | 2.9 | 3.9 | 5.9 |
| Bbr_1831 | Bbr_1831 Transcriptional regulator, LacI family | 4.6 | — | — | — |

| | | | | | |
|---|---|---|---|---|---|
| Bbr_1832 | Bbr_1832 Hypothetical protein | 34.1 | — | — | — |
| Bbr_1833 | lacZ7 Beta-galactosidase | 56.4 | — | 3.6 | 3.2 |
| Bbr_1834 | ABC sugar uptake system, permease component | 47.9 | — | — | — |
| Bbr_1835 | ABC sugar uptake system, ATP-binding protein | 45.1 | — | — | — |
| Bbr_1836 | ABC sugar uptake system, solute binding protein. | 96.6 | — | — | — |
| Bbr_1865 | Bbr_1865 Raffinose transport system permease protein | 8.5 | — | 18.9 | 2.7 |
| Bbr_1866 | Bbr_1866 Raffinose transport system permease protein | 10.6 | — | 91.1 | — |
| Bbr_1867 | Bbr_1867 Raffinose-binding protein | 6.2 | — | 39.8 | — |
| Bbr_1878 | Bbr_1878 Hypothetical protein | 11.3 | 2.5 | 6.5 | 7.1 |
| Bbr_1879 | Bbr_1879 PTS system, IIABC component | 12.5 | 4.2 | 12.0 | 10.9 |
| Bbr_1880 | Bbr_1880 PTS system, IIBC component | 18.7 | 4.7 | 18.2 | 13.1 |
| Bbr_1890 | Bbr_1890 ATP-binding protein of ABC transporter system for sugars | 5.4 | 2.6 | 2.6 | — |

[a] Microarray data were obtained using *B. breve* UCC2003 grown on 0.5% of the corresponding carbohydrate~~sugar~~ and were compared with array data obtained when *B. breve* UCC2003 was grown on ribose as a reference.

[b] The cut-off point is 2.5-fold, with a *P* value of 0.001. —, value below the cut-off.

**Figure 1.**



**Highlights**

1.- Utilization of biosynthetic oligosaccharides by two bifidobacterial strains was tested
2.- Differences in utilization are linked to carbohydrate molecular weight and structure
3.- Transcriptome analysis was ~~performed~~ done for *B. breve* UCC2003 when grown on ~~some of them~~ two trisaccharides.
4.- Upregulated genes represented glycosyl hydrolases and sugar transport systems
5.- The role of β-galactosidases in hydrolysis of particular trisaccharides was demonstrated