Journal of
Applied Microbiology

© 2012 University College Cork Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Selective carbohydrate utilization by lactobacilli and bifidobacteria

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Keywords
Bifidobacterium, fructo-oligosaccharides, galacto-oligosaccharides, inulin, *Lactobacillus*, lactulose, microbiota, polydextrose, prebiotic, probiotic.

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2013/1421: received 7 August 2012, revised 10 December 2012 and accepted 10 December 2012

doi:10.1111/jam.12105

Abstract

Aim: To evaluate the ability of specific carbohydrates, including commercially available products, to support the growth of representatives of two well-known groups of gut commensals, namely lactobacilli and bifidobacteria.

Methods and Results: Sixty-eight bacterial strains, representing 29 human-derived lactobacilli and 39 bifidobacteria (both human- and animal-derived), were tested for their ability to metabolize 10 different carbohydrates. Analysis of growth and metabolic activity was performed using a combination of diagnostic parameters, such as final OD_{600}, final pH, fermentation end products and growth rate.

Conclusions: The data assembled in this study provide significant complementary and comparative information on the growth-promoting properties of a range of carbohydrates, while also investigating interspecies differences between lactobacilli and/or bifidobacteria with regard to their carbohydrate utilization abilities. Galacto-oligosaccharides (GOS) and lactulose were shown to support the most favourable growth characteristics, whereas relatively poor growth of lactobacilli and bifidobacteria was observed on inulin, maltodextrin and polydextrose. GOS/inulin (9 : 1) and fructo-oligosaccharides (FOS)/inulin mixtures supported mostly similar growth abilities to those obtained for GOS and FOS, respectively. Microbial consumption of GOS, as determined by high-performance anion-exchange chromatography with pulsed amperometric detection, was evident for both lactobacilli and bifidobacteria.

Significance and Impact of the Study: These results may allow for the rational prediction of lactobacilli and/or bifidobacteria to be used in conjunction with prebiotics, such as GOS, as synbiotics.

Introduction

Since the pioneering work of Élie Metchnikoff advocated the consumption of fermented milk to eradicate putrefactive intestinal bacteria (Metchnikoff 1908), there has been an increased interest in the potential health benefits of probiotics. Currently, the most popular food-based strategy to (transiently) modulate the composition and/or metabolic activity of the intestinal microbiota, including bifidobacteria and lactobacilli, for the purpose of imparting beneficial effects, is *via* the dietary intake of 'functional foods' containing probiotics, prebiotics or their combination, synbiotics (Hoppu *et al.* 2001; Stanton *et al.* 2005; Macfarlane *et al.* 2008; Bosscher *et al.* 2009).

Prebiotic, a term first coined in 1995 by Gibson and Roberfroid, has been (re)defined to describe nondigestible dietary components that undergo selective colonic fermentation, 'thus causing significant changes in the composition of the gut microflora with increased and reduced numbers of potentially health-promoting bacteria and potentially harmful species, respectively' (Roberfroid 2007). Breast-fed infants harbour a colonic microbiota

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that is rich in bifidobacteria, a predominance, which is believed to be due to their ability to metabolize human milk oligosaccharides (HMOs) (Sela *et al.* 2008; Roger *et al.* 2010). This is believed to have beneficial effects on host health through the enhancement of defence mechanisms (i.e. antipathogenic properties) and the education and modulation of the immune system (Bode 2009; Bosscher *et al.* 2009; Fanaro *et al.* 2009; Fukuda *et al.* 2011; Kau *et al.* 2011; Fanning *et al.* 2012). In fact, a recently defined minimal human gut metagenome describes the bacterial functions involved in gut homoeostasis to include activities responsible for complex polysaccharide degradation, as well as the synthesis of short-chain fatty acids (SCFAs), vital amino acids and vitamins (Qin *et al.* 2010). Consequently, significant research efforts are currently focusing on the identification of oligosaccharides that can stimulate growth and/or metabolic activity of beneficial bacteria. Several prebiotic substrates, in particular fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin and lactulose, have already obtained scientific credibility due to their inclusion in human trials where they have demonstrated prebiotic effects (Menne *et al.* 2000; Kolida *et al.* 2002; Bosscher *et al.* 2006; Davis *et al.* 2010; Veereman-Wauters *et al.* 2011). For example, Fanaro *et al.* (2009) demonstrated that the supplementation of infant formula with 5 g l^{-1} GOS resulted in an increase in bifidobacterial numbers isolated per gram of stool. Ben *et al.* (2008) also demonstrated that the addition of low levels of GOS (0·24 g 100 ml^{-1}) to the formula of infants significantly increased the numbers of both intestinal bifidobacteria and lactobacilli relative to infant formula without GOS, following 3 months of feeding, while no significant differences were observed between the GOS formula- and human milk-fed groups. A recent study by Maathuis *et al.* (2012) demonstrated the prebiotic attributes of a purified Vivinal GOS formulation in an *in vitro* colon model. The authors observed an increase in numbers of lactobacilli and bifidobacteria as well other beneficial bacteria, with a concomitant decrease in numbers of members of the genera *Bacteroides*, *Prevotella* and *Lactococcus*.

In the present study, we evaluated the ability of specific carbohydrates, including commercially available products, to support the growth of representatives of two well-known groups of gut commensals, namely lactobacilli and bifidobacteria, and analysed strain diversity in this regard. The data gathered in this study provide a significant amount of complementary and comparative information on the growth-promoting properties of a range of carbohydrates, while also investigating interspecies differences between lactobacilli and/or bifidobacteria with regard to their carbohydrate utilization abilities.

Materials and methods

Bacterial strains, media and culture conditions

Sixty-eight strains representing a variety of mostly human-derived lactobacilli and bifidobacteria (representing 21 and 12 different species, respectively) were included in this study (Table 1). For inoculum preparation and storage purposes, the *Lactobacillus* strains were cultivated anaerobically in Lactobacilli de Man-Rogosa and Sharpe (LMRS; Difco™, France) broth at their optimal growth temperature (30 or 37°C; specified in Table 1), from stock cultures. Cells from an overnight LMRS culture were then subcultured (1%, v/v) into fresh LMRS and incubated anaerobically for a further 16 h, after which they were collected by centrifugation at 5000 ***g*** for 5 min, washed twice with phosphate-buffered saline (PBS), before a growth experiment was undertaken (see below). Bifidobacterial strains were grown under anaerobic conditions in Reinforced Clostridium Medium (RCM) (Oxoid, Hampshire, England) at 37°C, from stock cultures. Cells from an overnight RCM culture were then subcultured (1%, v/v) into fresh RCM and incubated anaerobically for an additional 16 h before use in a growth experiment (see also below). The overnight cultures were inoculated into modified MRS (mMRS) medium made from first principles (de-Mann *et al.* 1960): trypticase peptone: 10 g l^{-1}; granulated yeast extract: 2·5 g l^{-1}; tryptose: 3 g l^{-1}; K_2HPO_4: 3 g l^{-1}; KH_2PO_4: 3 g l^{-1}; triammonium citrate: 2 g l^{-1}; pyruvic acid: 0·2 g l^{-1}; cysteine HCl: 0·3 g l^{-1}; Tween-80: 1 ml; $MgSO_4.7H_2O$: 0·575 g l^{-1}; $MnSO_4.4H_2O$: 0·12 g l^{-1}; $FeSO_4.7H_2O$: 0·034 g l^{-1}. The pH of the medium was adjusted to 6·8 before autoclaving (121°C for 15 min). This mMRS medium is a complex growth medium, which does not support the growth of any of the strains in the absence of a supplemented carbohydrate. Solid LMRS medium was prepared by the addition of 1·5% (w/v) agar (Oxoid, Basingstoke, UK) to the LMRS broth. The purity of the lactobacilli and bifidobacterial cultures was routinely monitored by plating on LMRS agar or Reinforced Clostridium agar (RCA) (Oxoid), respectively. All strains were maintained at −80°C in the appropriate medium supplemented with 20% (v/v) glycerol as a cryoprotectant.

Sources, reported purity and preparation of carbohydrate solutions

The commercial sources of the carbohydrates tested were as follows: glucose (served as a positive control as it supports good growth of all strains used in this study), lactose and lactulose were obtained from Sigma-Aldrich

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Table 1 Carbohydrate utilization by strains of *Lactobacillus* and *Bifidobacterium*

Strain	Growth Temp (°C)	Glucose	Lactose	Lactulose	Malt-odextrin	Polyde-xtrose	Fructo-oligo sa-ccharides	Fructo-oligo sa-ccharides /inulin	Inulin	Galacto-oligo sac-charides /Inulin	Galacto-oligo sac-charides	Lactobacilli de Man-Rogosa and Sharpe / Reinforced Clostridium Medium	mMRS
Lactobacillus acidophilus ATCC4356	37	+++	+++	+	+	+	+	+	–	+	+	+++	–
Lact. acidophilus LA5®*	37	+++	+++	–	–	+	++	++	–	+++	+++	+++	–
Lact. acidophilus NCFM*	37	+++	+++	++	–	+	++	–	–	+++	+++	+++	–
Lactobacillus amylovorus DSM20552	37	+++	+++	+	++	+	+	+	–	+++	+++	+++	–
Lactobacillus antri DSM16041	37	+++	+++	–	+	+	+	+	–	+++	+++	+++	–
Lactobacillus brevis DSM20054	30	+++	–	–	–	–	+	+	–	–	–	+++	–
Lactobacillus bulgaricus	37	+++	++	+	–	–	–	–	–	–	–	+++	–
Lactobacillus casei DN-144-001*	37	+++	+++	+++	+	+	+	+	–	+++	+++	+++	–
Lact. casei Shirota*	37	+++	+++	+++	+	++	+	++	+	+++	+++	+++	–
Lactobacillus curvatus NCDO2739	30	+++	–	–	–	–	–	–	–	–	–	+++	–
Lactobacillus delbrueckii subsp. *lactis* DSM20073	37	+++	+++	–	–	+	+	+	–	+++	+++	+++	–
Lactobacillus fermentum CECT5716*	30	+++	+++	–	–	+	+	+	–	+++	+++	+++	–
Lact. fermentum DSM20055	30	+++	–	–	–	–	–	+	–	–	–	+++	–
Lactobacillus gasseri CR159	37	+++	+++	–	+	+	+	+	–	+++	+++	+++	–
Lact. gasseri DW001	37	+++	+++	+++	–	+	+	++	–	+++	+++	+++	–
Lactobacillus gastricus DSM16045	37	+++	+++	+++	+	+	+	+	–	+++	+++	+++	–
Lactobacillus johnsonii LA-1*	37	+++	+++	+++	–	+	–	–	–	++	++	+++	–
Lactobacillus kalixensis DSM16043	37	+++	+++	+++	+	+	+	+	–	+++	+++	+++	–
Lactobacillus oris DSM4864	37	+++	+++	+++	–	+	+	+	–	+++	+++	+++	–
Lactobacillus parabuchneri DSM5707	30	+++	+++	–	–	+	+	+	–	+++	+++	+++	–
Lactobacillus paracasei CRL431	37	+++	+++	+++	–	+	+++	+++	–	++	+++	+++	–

(Continued)

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Table 1 *(Continued)*

Strain	Growth Temp (°C)	Glucose	Lactose	Lactulose	Malt-odextrin	Polyde-xtrose	Fructo-oligo sa-ccharides	Fructo-oligo sa-ccharides /inulin	Inulin	Galacto-oligo sac-charides /Inulin	Galacto-oligo sac-charides	Lactobacilli de Man-Rogosa and Sharpe / Reinforced Clostridium Medium	mMRS
*Lactobacillus plantarum*NCDO326	30	+++	–	–	–	–	–	+	–	–	–	+++	–
Lactobacillus reuteri ATCC 55730	37	++	+++	+++	–	+	+	++	–	+++	+++	+++	–
Lact. reuteri DSM 17938	37	+++	+++	+++	–	+	+	+	–	+++	+++	+++	–
Lact. reuteri DSM20016	37	+++	+++	+++	+	+	+	++	–	+++	+++	+++	–
Lact. reuteri DSM20053	37	+++	+++	+++	–	+	–	+	–	+++	+++	+++	–
Lactobacillus rhamnosus GG®*	37	+++	++	++	+	++	++	++	+	++	+++	+++	–
Lactobacillus sakei DSM20100	30	+++	++	++	++	++	–	++	–	–	–	+++	–
Lactobacillus ultunensis DSM16047	37	+++	+++	–	–	+	+	+	–	+++	+++	+++	–
Bifidobacterium adolescentis CIP64·61	37	+++	+++	+++	+++	+	+++	+++	+	+++	+++	+++	–
Bif. adolescentis DSM20083	37	+++	+++	++	–	+	++	+	++	+++	+++	+++	–
Bif. adolescentis LMG10502	37	+++	+++	+++	++	++	+++	+++	++	+++	+++	+++	–
Bif. adolescentis NCFB2204	37	+++	+++	+++	+++	+	++	++	+	++	++	+++	–
Bif. adolescentis NCFB2229	37	+++	+++	+++	+++	+	+++	++	+	+++	++	+++	–
Bifidobacterium animalis DSM20105	37	+++	+++	+++	+++	–	++	++	–	+	+++	+++	–
Bif. animalis JCM20097	37	+++	+++	+++	+++	–	+++	++	–	++	++	+++	–
Bif. animalis subsp. *lactis* Bb12®*	37	+++	+++	+++	+++	++	+++	+++	++	++	+++	+++	–
Bif. animalis subsp. *lactis* Bl818*	37	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++	–
Bifidobacterium bifidum LMG11041	37	+++	+++	+++	+++	++	+++	+	–	+++	+++	+++	–
Bif. bifidum NCIMB8810	37	+++	+++	+++	++	++	+++	+++	–	+++	+++	+++	–
Bifidobacterium breve CCUG43878	37	+++	+++	+++	++	–	+	+	–	–	+	+++	–
Bif. breve JCM7017	37	+++	+++	+++	–	–	++	–	+	+++	+++	+++	–

(Continued)

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Table 1 *(Continued)*

Strain	Growth Temp (°C)	Glucose	Lactose	Lactulose	Malt-odextrin	Polyde-xtrose	Fructo-oligo sa-ccharides	Fructo-oligo sa-ccharides /inulin	Inulin	Galacto-oligo sac-charides /Inulin	Galacto-oligo sac-charides	Lactobacilli de Man-Rogosa and Sharpe / Reinforced Clostridium Medium	mMRS
Bif. breve JCM7019	37	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	–
Bif. breve NCFB11815	37	+++	+++	+++	+++	+	++	+	–	++	++	+++	–
Bif. breve NCFB2257	37	+++	+++	+++	+++	++	+++	+++	+	+++	+++	+++	–
Bif. breve NCFB2258	37	+++	+++	+++	+++	+	+++	++	–	+++	+++	+++	–
Bif. breve NCIMB8815	37	+++	+++	+++	+++	+	++	++	+	+++	+++	+++	–
Bif. breve UCC2003	37	+++	+++	+++	++	++	++	+	++	+++	+++	+++	–
Bif. breve Yakult*	37	+++	+++	+++	++	++	+	++	+	+++	+++	+++	–
Bifidobacterium dentium NCFB2843	37	+++	+++	+++	+++	+	+++	+++	+	+++	+++	+++	–
Bifidobacterium globosum JCM5820	37	+++	+++	++	+	+	+	++	+	+	++	+++	–
Bif. globosum JCM7092	37	+++	+++	+++	+++	+	+	–	+	+	+	+++	–
Bifidobacterium infantis NCDO2205	37	+++	+++	+++	+++	–	+++	+++	+	+++	+++	+++	–
Bifidobacterium longum CCUG15137	37	+++	+++	+++	++	++	+++	+	++	+++	+++	+++	–
Bif. longum CCUG30698	37	+++	+++	+++	+	–	+++	++	+	+++	+++	+++	–
Bif. longum CIP64·63	37	+++	+++	+++	+++	+	+++	++	+++	+++	+++	+++	–
Bif. longum JCM7050	37	+++	+++	+++	+	+	++	+++	+	+++	+++	+++	–
Bif. longum JCM7052	37	+++	+++	+++	+++	+++	+	++	+	+++	+++	+++	–
Bif. longum JCM7053	37	+++	++	+++	–	–	+++	++	–	+	++	+++	–
Bif. longum JCM7056	37	+++	+++	+++	+	–	+++	++	–	+	+++	+++	–
Bif. longum NCIMB8809	37	+++	+++	+++	+++	–	+++	++	–	++	++	+++	–
Bif. longum Onken*	37	+++	+++	+++	+++	+	+++	++	+	+++	+++	+++	–
Bif. longum/infantis CCUG18157	37	+++	+++	+++	+++	–	+++	+++	+	+++	+++	+++	–
Bifidobacterium pseudocatenulatum LMG10505	37	+++	++	+++	+++	+	++	++	–	+++	+++	+++	–
Bif. pseudocatenulatum NCIMB 8811	37	+++	+++	+++	+++	+	+++	+++	–	+++	+++	+++	–

(Continued)

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Table 1 *(Continued)*

Strain	Growth Temp (°C)	Glucose	Lactose	Lactulose	Malt-odextrin	Polyde-xtrose	Fructo-oligo sa-ccharides	Fructo-oligo sa-ccharides /inulin	Inulin	Galacto-oligo sac-charides /Inulin	Galacto-oligo sac-charides	Lactobacilli de Man-Rogosa and Sharpe / Reinforced Clostridium Medium	mMRS
Bifidobacterium pseudolongum DSM20095	37	+++	+++	+++	+++	+	+++	++	+	++	++	+++	–
Bif. pseudolongum NCIMB2244	37	+++	+++	++	+++	–	+++	++	–	+	+	+++	–
Bifidobacterium thermophilum JCM7027	37	+++	+++	+++	+++	+	+++	+++	++	++	++	+++	–

ATCC, The American Type Culture Collection; CCUG, Culture Collection of the University of Goteborg; CIP, Collection de l'Institut Pasteur; CRL, CERELA culture collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan collection of micro-organisms; LMG, Belgian co-coordinated collection of micro-organisms; NCDO, National collection of dairy micro-organisms; NCFB, National collection of food bacteria; NCIMB, National collection of industrial and marine bacteria; NCTC, National collection of type cultures; UCC, University College Cork, Collection.

*Strains isolated from commercial products. *Lactobacillus acidophilus* LA-5 and *Bif. animalis* subsp. *lactis* Bb-12 were obtained from a commercial product containing both strains. Bb-12 and LA-5 are registered trademarks of Chr. Hansen A/S. A minus sign (–) indicates that final OD_{600} <0·3; + indicates final OD_{600} = 0·3–0·5; ++ indicates final OD_{600} >0·5–0·8; and +++ final OD_{600} >0·8.

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(Steinheim, Germany); maltodextrin (potato-derived) from Cargill-Cerestar; polydextrose (Litesse®; 90% pure, 10% glucose and sorbitol) obtained from Danisco, Wellingborough, UK; FOS (Raftilose P95®; 5% glucose, fructose and sucrose), inulin (Raftiline HP, 100% pure) and FOS/inulin mixture (Synergy 1; ratio 50 : 50 FOS : inulin; contains 8% glucose, fructose and sucrose) sourced from Orafti, Tienen, Belgium; GOS (which represents purified Vivinal® GOS; contains 97% GOS, 0·7% galactose, 1·6% glucose and 0·7% lactose) and GOS/inulin [ratio 9 : 1, GOS : inulin (Raftiline HP)] supplied by FrieslandCampina Domo, Amersfoort, the Netherlands. For the preparation of GOS, Vivinal® GOS (typical composition: 59% GOS, 21% lactose, 19% glucose, 1% galactose) was enzymatically treated with a lactase to hydrolyse the lactose into glucose and galactose, after which the monosaccharides were removed by nanofiltration.

A 5% stock solution of each of the carbohydrates to be tested was prepared by dissolving the particular sugar in distilled water. The obtained stock solution was then sterilized by membrane filtration, using ministart filters (pore size, 0·45 μm; Sartorius AG, Göttingen, Germany), and stored at 4°C. Inulin proved difficult to dissolve and required heating to 50°C prior to filter sterilization.

Evaluation of bacterial growth on oligosaccharides

Carbohydrate-dependent growth by a given culture was evaluated in 96-well microtitre plates. Overnight cultures (10 ml) of *Lactobacillus* strains were prepared as described above. An aliquot (representing 1×10^7 CFU ml^{-1}) was inoculated into 5 ml of mMRS medium supplemented with 0·5% final concentration (v/v) of an individual carbohydrate solution, representing the sole carbon source in the medium. The mMRS without any supplemented carbohydrate source and commercially obtained LMRS (Difco) were used as negative and positive controls, respectively. A similar procedure was employed to test growth potential and characteristics of the bifidobacterial strains, albeit that they were not subjected to a wash step. In this case, the freshly prepared mMRS medium was supplemented with 0·5% final concentration (v/v) of an individual carbohydrate solution plus 0·05% (v/v) L-cysteine HCl (a redox potential-lowering compound). For the bifidobacteria, mMRS without any added carbohydrate and commercially obtained RCM (Reinforced Clostridial Medium; Oxoid) were used as negative and positive controls, respectively.

All cultures were incubated under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy Ltd, Dublin, Ireland), at the desired temperature (30 or 37°C; as indicated in Table 1) for 24 h. The optical density at 600 nm (final OD_{600}) was monitored using a Powerwave™ Microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) in conjunction with Gen5™ Microplate software for data collection and analysis. The plate reader was run in discontinuous mode, with absorbance readings captured at 30-min intervals, and each read was preceded by 10-s shaking step at medium speed to mix the culture. Cultures were grown in biologically independent duplicates, and the resulting growth data were expressed as the mean of these replicates.

Maximum growth rate determinations and pH determinations

Growth rates (μ) were calculated for strain–carbohydrate combinations that demonstrated good growth (final OD_{600} >0·8) according to a previously published formula (Rada *et al.* 2008): $\mu = (\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x_2 and x_1 were absorbance values measured within the exponential phase of growth at times t_2 and t_1, respectively. The pH measurements were performed on the culture medium following 24 h of growth using a standard pH meter.

Analysis of GOS or FOS utilization by HPAEC-PAD

In order to demonstrate possible selective metabolic utilization of particular GOS or FOS components by lactobacilli and bifidobacteria, the carbohydrate composition profile of postfermentation cell-free supernatants was determined using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), employing a Dionex ICS-3000 system (Dionex, Sunnyvale, CA, USA). Oligosaccharide fractions (25 μl aliquots) were separated on a CarboPac PA1 (Dionex) analytical-exchange column with dimensions 250 mm × 4 mm with a CarboPac PA1 guard column (Dionex) with dimensions 50 mm × 4 mm and a Dionex ED40 detector in the pulsed amperometric detection (PAD) mode. Elution was performed at a constant flow rate of 1·0 ml min^{-1} at 30°C using the following eluents for the analysis: (A) 200 mmol l^{-1} NaOH, (B) 100 mmol l^{-1} NaOH, 550 mmol l^{-1} sodium acetate and (C) Milli-Q water. The following linear gradient of sodium acetate was used: 100 mmol l^{-1} NaOH: 0–50 min, 0 mmol l^{-1}; 50–51 min, 16 mmol l^{-1}; 51–56 min, 100 mmol l^{-1}; 56·1–61 min, 0 mmol l^{-1}. The obtained chromatographic profile of nonfermented GOS or FOS was used for comparison to evaluate GOS or FOS utilization, respectively, from samples, and the CHROMELEON software Ver. 6·70 (Dionex Corp.) enabled the integration and evaluation of the chromatograms.

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Bioinformatics used for heat maps and clustering of data

Clustering of the data relating to the final OD_{600} of strains grown on various carbon sources was performed in order to search for potential correlations between strains or species and the acquired information on growth/metabolic characteristics. Hierarchical clustering was performed using the complete linkage clustering method implemented in GENESIS software and employing default settings (Sturn *et al.* 2002).

Results

Comparative growth on different carbon sources

All carbohydrates included in this study (except glucose and lactose, which were included as positive controls) were selected for this *in vitro* study as they are deemed to possess prebiotic properties and are thus expected to support growth/metabolism of beneficial gut commensals.

Growth results obtained for strains of *Lactobacillus*

All 29 tested *Lactobacillus* strains demonstrated appreciable growth (i.e. reached a final OD_{600} >0·7) on mMRS supplemented with glucose, as compared to 24 strains that were capable of equivalent growth on lactose (Tables 1 and S1). Distinctive growth profiles were produced for the lactobacilli when the remaining eight carbohydrates were used as the sole carbon/energy source in mMRS. To exemplify the strain dependency of the obtained profiles, the four tested *Lactobacillus acidophilus* species (*Lactobacillus johnsonii* LA-1; *Lact. acidophilus* ATCC4356; *Lact. acidophilus* LA5; and *Lact. acidophilus* NCFM) all grew well in the presence of glucose and lactose. They are unable to efficiently utilize maltodextrin, polydextrose or inulin, but do possess variable utilization capabilities for lactulose, FOS, FOS/inulin, GOS/inulin (9 : 1) and GOS. Growth of *Lactobacillus casei* Shirota was supported (final OD_{600} >0·3) by all the tested carbohydrates (as did the other commercial probiotic lactobacilli strains that are used as active ingredients in various functional foods, e.g. *Lact. casei* DN-144-001 and *Lactobacillus rhamnosus* GG), and for this reason was chosen as a representative strain to highlight the observed growth characteristics across the 10 carbohydrates (Fig. 1a). Lactulose, a semisynthetic disaccharide produced by the isomerization of lactose (Panesar and Kumari 2011), supported intermediate to good growth for 15 of the lactobacilli strains tested. From the *in vitro* growth data obtained, it is evident that maltodextrin, polydextrose, FOS, FOS/inulin and inulin are rather poor *in vitro* growth substrates for the majority of the tested lactobacilli. Just four (*Lact. acidophilus* LA5; *Lact. acidophilus* NCFM; *Lact. casei* DN-144-001; and *Lactobacillus paracasei* CRL431) of the 29 lactobacilli tested were capable of growth (final OD_{600} >0·5) in the presence of FOS, while *Lact. paracasei* CRL431 was the only tested *Lactobacillus* strain capable of reaching a higher final OD_{600} on FOS compared to that obtained for GOS (1·380 ± 0·093 *vs* 0·865 ± 0·078, respectively). Another interesting observation is that FOS/inulin performs marginally better as a growth substrate as compared to its individual components, FOS and inulin (e.g. *Lactobacillus gasseri* DW001; Fig. 1b). *Lactobacillus casei* Shirota reached a final OD_{600} on GOS of 1·454 ± 0·499, GOS/inulin (9 : 1) of 1·094 ± 0·117 and inulin of 0·397 ± 0·023. These final OD_{600} values suggest that this strain is utilizing the GOS component of GOS/inulin as it displays poor growth on inulin alone. *Lactobacillus casei* Shirota was shown to reach a final OD_{600} of 0·474 ± 0·033 when grown on FOS, and together, these findings indicate that GOS imparts a superior growth-promoting ability to 23 of the tested lactobacilli strains as compared to FOS or inulin (additional examples of this growth are observed for *Lact. gasseri* DW001, *Lactobacillus bulgaricus* and *Lactobacillus antri* DSM16041, and displayed in Fig. 1b–d).

Growth results obtained for strains of *Bifidobacterium*

One major difference between lactobacilli and bifidobacteria is that the majority of the bifidobacterial strains were capable of reasonable to good growth (final OD_{600} ≥0·5) on eight of the 10 tested substrates, representing a higher number as compared to the lactobacilli, where the latter were able to support reasonable to good growth (final OD_{600} ≥0·5) on just four carbohydrates (Table 1, S1 and S2). Lactulose, maltodextrin, FOS, GOS and the GOS/inulin (9 : 1) mixture supported the most prolific growth abilities, while inulin and polydextrose appeared to be rather poor substrates for bifidobacterial growth (Table 1). All bifidobacterial strains tested were capable of growth reaching a final OD_{600} ≥0·7 in mMRS supplemented with glucose, lactose or lactulose. This is exemplified by *Bifidobacterium longum* JCM7050 that reaches a final OD_{600} of 1·320 ± 0·108 on glucose, 1·414 ± 0·132 on lactose and 1·203 ± 0·001 on lactulose (Fig 2a and Table S2). Merely 11 of the bifidobacterial strains tested demonstrated appreciable levels of growth (final OD_{600} >0·5) on polydextrose, while inulin appears to be a rather poor growth substrate for most bifidobacterial strains tested, supporting the growth of just nine strains to a final OD_{600} >0·5. Just two strains, *Bifidobacterium breve* JCM7019 and *Bif. longum* CIP64·63, exhibited good growth (final OD_{600} values of 1·009 ± 0·031 and

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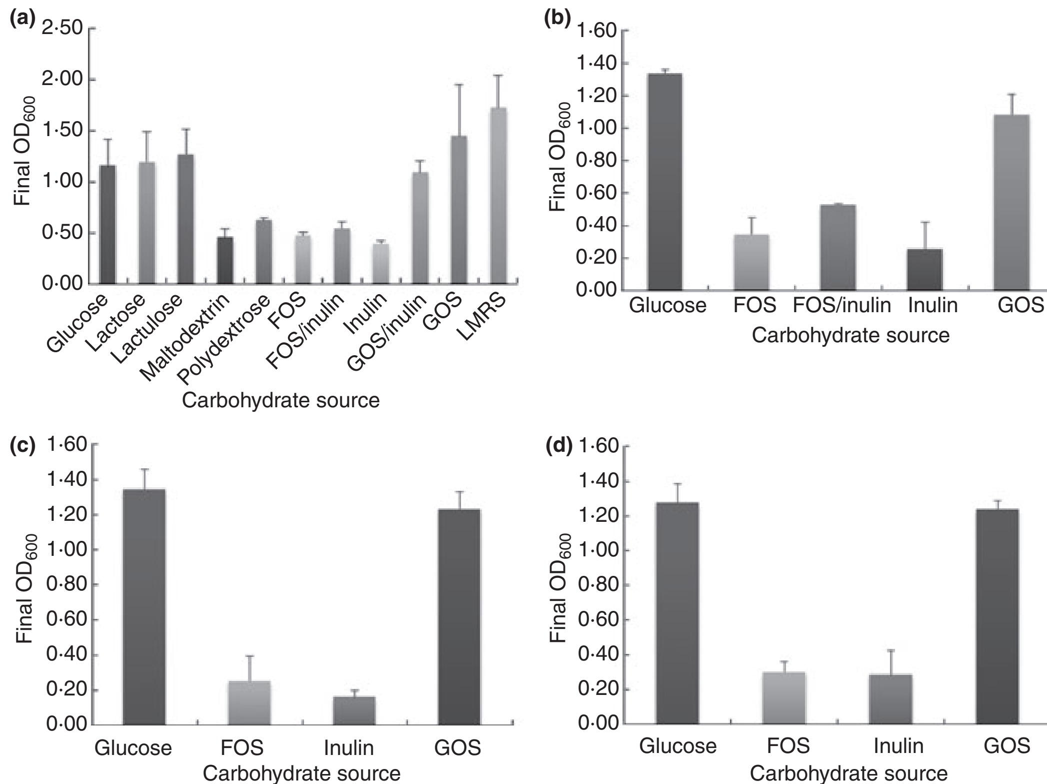

Figure 1 Final OD_{600} reached by *Lacobacillus casei* Shirota (a) grown on modified MRS supplemented with 0·5% of the relevant carbohydrates as the sole carbon source. *Lacobacillus gasseri* DW001 (b) reaches a higher final optical density on 0·5% fructo-oligosaccharides (FOS)/inulin (50 : 50) compared to 0·5% FOS and 0·5% inulin when used as separate supplements. Carbohydrate utilization fingerprint of *Lacobacillus bulgaricus* (c) and *Lacobacillus antri* DSM16041 (d), grown separately on mMRS supplemented with either 0·5% of glucose, FOS, inulin or galacto-oligosaccharides (GOS) as the sole carbon source, highlighting excellent growth-promoting properties of GOS as compared to FOS or inulin.

0·840 ± 0·159, respectively) on inulin. Growth of the bifidobacterial strains on maltodextrin, FOS, FOS/inulin, GOS/inulin (9 : 1) and GOS all produced easily interpretable and differentiating growth profiles. These profiles were, again, as expected and rather strain dependent as illustrated by the nine *Bif. breve* strains included in this study. All strains demonstrated variable growth on these carbohydrates ranging from no/very poor growth (-) to good growth (+++) depending on the substrate and strain involved (Table 1). Thirty-two strains exhibited reasonable to good growth (final OD_{600} >0·5) in maltodextrin (an oligosaccharide consisting of D-glucose linked in chains of varying lengths), while 34 of the bifidobacterial strains showed good growth in the presence of FOS. This is mirrored in the growth-sustaining properties observed for the FOS : inulin mixture (Synergy 1), where 30 of the bifidobacterial strains exhibited similar growth abilities. Finally, 36 strains grew to high final optical densities in GOS, and 31 strains in GOS/inulin (9 : 1). As an example, using the same representative strain as above, *Bif. longum* JCM7050 reached a final OD_{600} on GOS of 1·000 ± 0·136, GOS/inulin (9 : 1) of 0·965 ± 0·175 and inulin of 0·407 ± 0·069 (Fig. 2a). These final OD_{600} values again highlight this strain's preference for the GOS components of GOS/inulin as it does not grow on inulin alone, but does reach a similar final OD_{600} when grown in the presence of the GOS or GOS/inulin (9 : 1). Another strain that illustrates that GOS is on par with FOS, with regard to its growth-sustaining abilities, is *Bif. longum* CCUG18157, which reached a final optical density of 0·950 ± 0·052 on FOS and an equally high final OD_{600} of 0·916 ± 0·201 on GOS (Fig. 2b).

Final pH values and growth rates

The pH value of the growth medium following 24 h of growth was determined, which demonstrated the expected inverse correlation between the final OD_{600} value reached for a particular strain and the final pH of the corresponding medium in which the strain was grown: the higher the final OD_{600} value obtained, the lower the final pH of the medium (Tables S3 and S4). *Lactobacillus johnsonii* LA-1 exemplifies the fact that in the presence of maltodextrin this strain reaches a final

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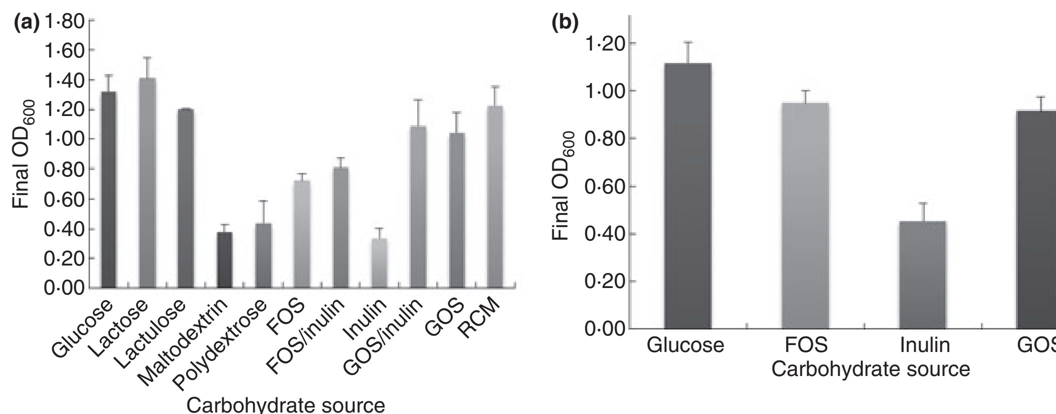

Figure 2 (a) Final OD_{600} reached by *Bifidobacterium longum* JCM7050 grown on mMRS supplemented with 0·5% of the relevant carbohydrates as the sole carbon source. (b) Final OD_{600} reached by *Bif. longum/infantis* CCUG18157 grown on mMRS supplemented with 0·5% of glucose, fructo-oligosaccharides (FOS), inulin or galacto-oligosaccharides (GOS) as the sole carbon source, again highlighting excellent growth-promoting properties of GOS which is on par with FOS and better than inulin for the majority of strains.

OD_{600} of 0·115 ± 0·020 and the pH of the medium is 6·48; however, in the presence of a suitable growth substrate such as glucose, the strain reaches a final OD_{600} of 1·374 ± 0·030 while the pH decreases to 4·14. This was indeed found for the majority of the strains, with just a few exceptions. Such an exception is illustrated by *Lact. antri* DSM16041, which in the presence of GOS/inulin (9 : 1) and GOS reaches a final OD_{600} of 1·228 ± 0·142 and 1·240 ± 0·045 and a pH of 6·41 and 5·20, respectively.

Growth rates (μ) (Tables S5 and S6) are critical for the evaluation of suitable prebiotic carbohydrates as they establish the substrate requirements and specificities of an individual strain. These findings provide valuable insights into how a particular prebiotic may influence a microorganism's ability to compete with other bacteria in the colon. A wide variety of growth rates were observed across the different strain and carbohydrate combinations with values ranging from 0 (when no growth on a particular carbohydrate was observed) to a maximum of 0·886 h^{-1} (*Lactobacillus reuteri* ATCC55730 grown on 0·5% glucose) for the lactobacilli, and a maximum of 1·150 h^{-1} (*Bif. longum* CCUG30698 grown on 0·5% glucose) for the tested bifidobacteria. Growth rates were compared within the results of a given test strain, rather than comparatively between strains, due to the large variability in growth rates with any carbon source between strains. Notably, 14 *Lactobacillus* strains grew more rapidly on GOS (when ignoring growth on glucose and lactose) than on any other tested carbohydrate substrate. The same trend was observed for 17 bifidobacterial strains which also achieved a higher growth rate on GOS (disregarding growth rates on glucose and lactose) as compared to other sugars, while only two bifidobacterial strains exhibited faster growth on FOS when compared to other tested carbohydrates (again with the exception of glucose and lactose).

Evaluation of GOS degradation using HPAEC-PAD

Microbial consumption of GOS was evident for most lactobacilli and bifidobacteria with no apparent selectivity for the different GOS fractions as quantified by HPAEC-PAD. The HPAEC profile of the reference GOS mixture indicated a high abundance of different oligosaccharides with different retention times in the GOS mixture (Fig. 3a). The carbohydrate identity of three peaks, that is, those corresponding to glucose, lactose and galactose, was established on the basis of reference compounds – glucose and/or galactose (both of these carbohydrates have a retention time of approximately 5·4 min under the experimental conditions used, while lactose has a retention time of ~11 min) (Fig. 3 I). Microbial consumption of GOS was evident for 23 of the lactobacilli, as indicated by the obvious reduction in GOS peak size and number. Due to the nonmolar response of the HPAEC detector, no quantitative conclusions on the rate of degradation of GOS and the consumption of its constituents can be drawn from these results; however, we can demonstrate the differential utilization of GOS components in the case of three different strains (Fig. 3). A representative *Lactobacillus* strain, *Lact. casei* DN-144-001, is able to utilize individual GOS components to achieve a final OD_{600} of 1·193 ± 0·018 (Fig. 3 II). Interestingly, eight of the 29 tested lactobacilli (*Lactobacillus amylovorus* DSM20552; *Lactobacillus delbrueckii* subsp. *lactis* DSM20552; *Lactobacillus kalixensis* DSM16043; *Lactobacillus fermentum* CETC5716; *Lact. reuteri* DSM20016;

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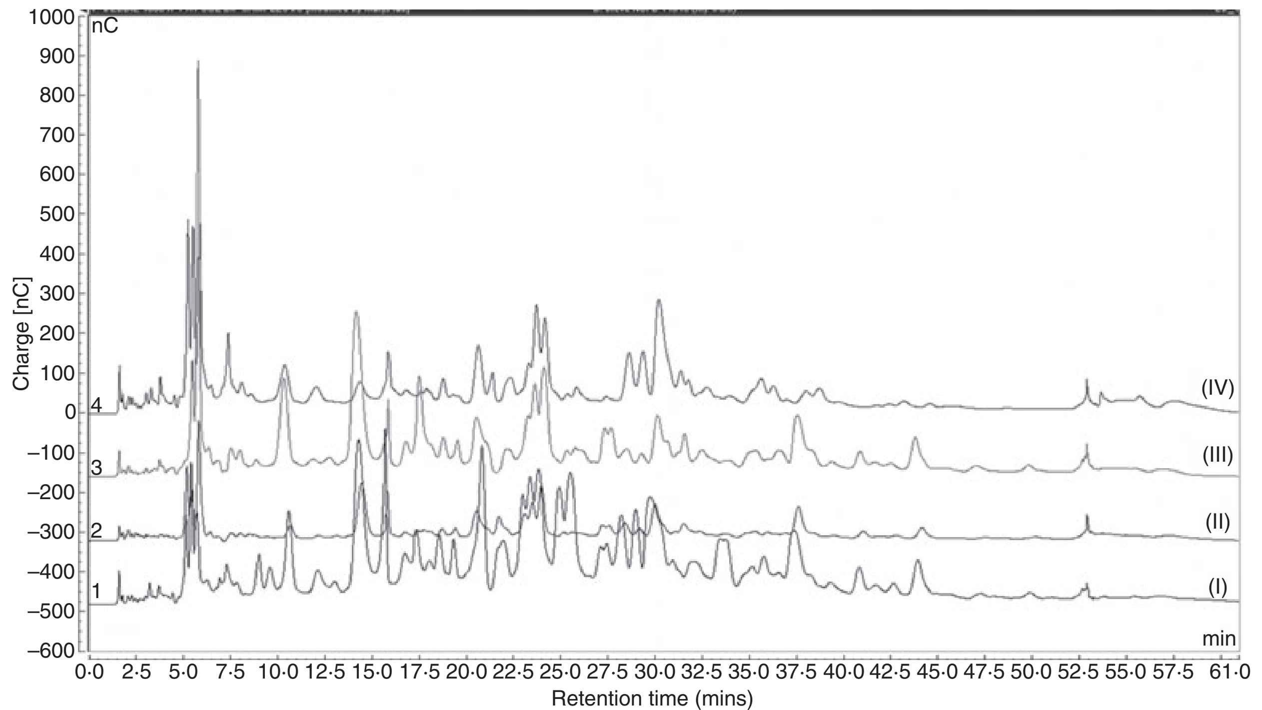

Figure 3 (I) The high-performance anion-exchange chromatography (HPAEC) profile of the reference galacto-oligosaccharides (GOS) mixture indicated a high abundance of different oligosaccharides with different retention times (II) Qualitative GOS degradation pattern for *Lacobacillus casei* DN-144-001 which grows well on GOS. This HPAEC chromatogram highlights the ability of the strain to utilize individual GOS components to achieve a final OD_{600} of 1·193. (III) *Lactobacillus amylovorus* DSM20552 demonstrates galactose accumulation in the cultural broth. (IV) Qualitative GOS degradation pattern for *Bifidobacterium breve* NCFB11815 which also grows well on GOS. This HPAEC chromatogram highlights the ability of the strain to utilize individual GOS components to achieve a final OD_{600} of 0·752.

Lact. reuteri DSM17938; *Lact. acidophilus* NCFM; and *Lact. johnsonii* LA-1) each exhibited galactose accumulation in the culture broth when GOS was the sole carbohydrate source (as observed in the HPAEC chromatograms displayed for *Lact. amylovorus* DSM20552 in Fig. 3 III). This would suggest that the rate of galactose release in the medium was higher than the rate of galactose uptake and/or intracellular galactose metabolism, leading to a net accumulation in the medium. Three bifidobacterial strains (*Bif. breve* CCUG43878; *Bifidobacterium globosum* JCM7092; and *Bifidobacterium pseudolongum* NCIMB2244) did not exhibit good growth in the presence of GOS. However, the remaining 36 strains were all shown to grow well on GOS as the sole carbon source, as illustrated by *Bif. breve* NCFB11815 (Fig. 3 IV).

Correlations and clustering

In the case of lactobacilli, even though no absolute correlations were observed, some general tendencies could be discerned. Carbohydrate metabolism has been recognized as being encoded by hypervariable regions in lactobacilli and these areas are commonly related to niche adaptation or survival (Berger *et al.* 2007). Based on hierarchical clustering, three major divisions (Fig. 4a, clusters Lb*a*, Lb*b* and Lb*c*) can be distinguished among the *Lactobacillus* strains. The strains were assigned to clusters that occurred at the first major branching point of the dendrogram. The first of these (Fig. 4a, cluster Lb*a*) contains five of the six strains that exhibited optimal growth at 30°C; four strains (*Lactobacillus curvatus* NCDO2739; *Lact. fermentum* DSM20055; *Lactobacillus brevis* DSM20054; and *Lactobacillus plantarum* NCDO326) were clustered together, linked by their sole ability to utilize glucose. Members of clusters Lb*b* and Lb*c* are grouped based on their ability to utilize particular carbohydrates, namely lactose and GOS, and may support the notion that adaptation to growth in nutrient-rich environments is the principal driving force behind the duplication or acquisition of genes during the evolutionary process (Ochman 2005; Makarova *et al.* 2006; Makarova and Koonin 2007).

Based on hierarchical clustering, two major divisions are evident for the tested bifidobacteria (Fig. 4b, clusters Bif*a* and Bif*b*), and strains were again assigned to clusters that occurred at the first major branching point of the dendrogram. This variation suggests environmental

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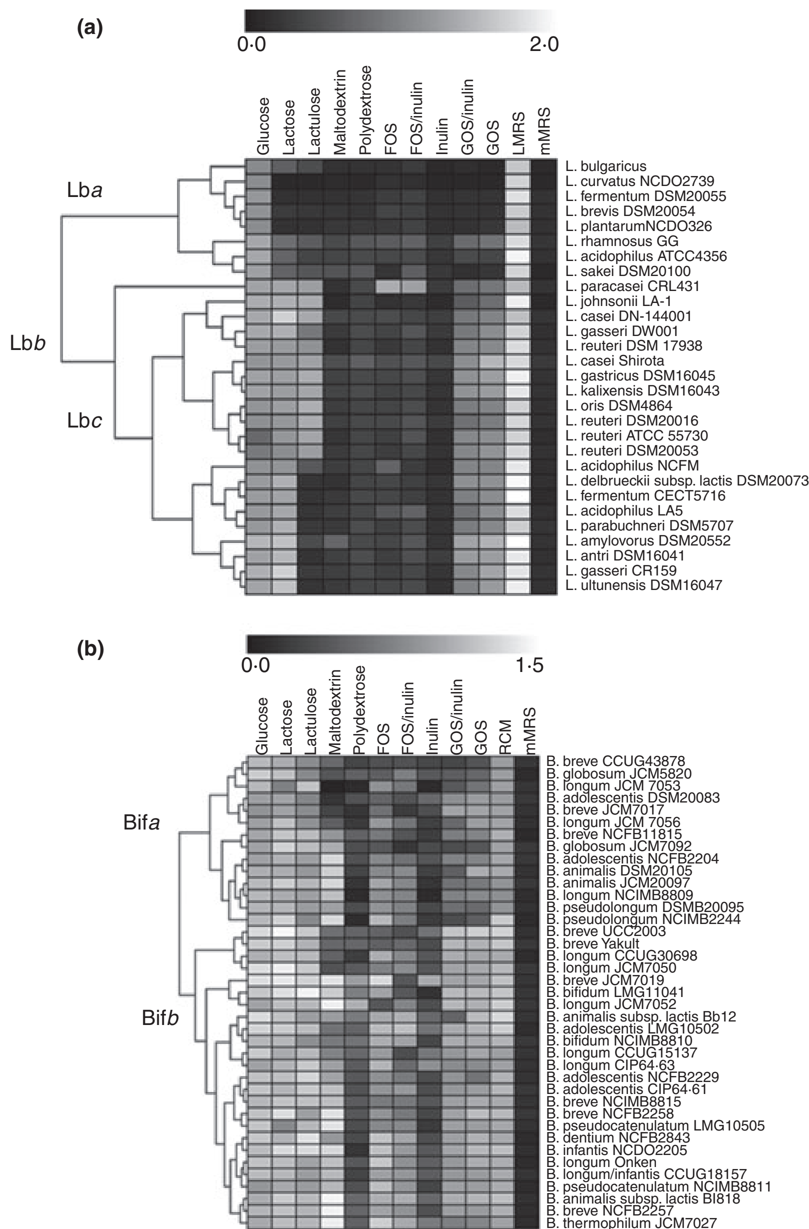

Figure 4 (a) Heat map and cluster analysis of final optical density (final OD_{600}) data received following 24 h of growth of 29 different *Lactobacillus* strains (a), and 39 different bifidobacterial strains (b), grown on a variety of carbohydrate substrates as the sole carbon source. Colour range is correlated with growth rate of a given sample (ranging from OD_{600} 0 to 2·0 or 0 to 1·5 for the Lactobacilli and Bifidobacteria respectively, which correlates in colour from black to white). The result of the cluster analysis is indicated on the left of the figure as a dendrogram. Each horizontal row corresponds to one strain, and each column represents a carbohydrate. The colour gradient, which goes from black to white, depicts the level of growth achieved by the strain.

selection or adaptation in carbohydrate metabolism, even the GIT itself is a succession of varying habitats corresponding to different compartments (e.g. oral cavity, the stomach and the small/large intestine). Based on this, we observed different preferences of desirable substrates among strains isolated from the infant gut (Fig. 4b). Cluster Bif*a* contains 14 of the strains, including six strains that were originally isolated from animal faecal samples.

Discussion

Recent studies indicate that diet alterations can dramatically change the overall composition and structure of endogenous microbial communities in the gut (Muegge *et al.* 2011). The work described here represents a comparative analysis of an extensive array of *in vitro* growth and metabolic characteristics for a diverse collection of lactobacilli and bifidobacterial strains. These strains have been obtained from various culture collections and commercial sources. In terms of supporting growth of the tested bacteria, it was shown that GOS [including GOS/inulin (9 : 1)] is on par with, or better than, the prebiotic FOS, as well as other commercially available prebiotics, such as lactulose (in the case of the lactobacilli) and a mixture of FOS/inulin for both genera. This notion is re-enforced when one considers that the preparations of

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FOS and FOS/inulin still contain considerable amounts of mono- and disaccharides (glucose and fructose), which may have caused some exaggeration of the observed growth abilities. Previous *in vivo* studies have demonstrated that both lactobacilli and bifidobacteria can metabolize GOS (Ben *et al.* 2008; Konstantinov *et al.* 2008; Fanaro *et al.* 2009; Walton *et al.* 2012). These studies adopted a commercially available galacto-oligosaccharide mixture (Vivinal® GOS) containing digestible monosaccharides (i.e. galactose and glucose) and the disaccharide lactose, corresponding to 41% of dry matter content in addition to GOS. These digestible mono- and disaccharides are expected to be taken up in the human small intestine and therefore do not reach the large intestine for microbial fermentation.

From this study, microbial consumption of GOS was evident for lactobacilli and bifidobacteria with no apparent selectivity for the different GOS components. GOS is typically synthesized by reverse hydrolysis of lactose by the enzyme β-galactosidase (lactase), which is generally derived from a member of the *Bacillus* genus or from *Kluyveromyces lactis* or *Streptococcus thermophilus* (reviewed by Park and Oh 2010). A recent study demonstrated the use of recombinant β-galactosidase from *Lact. plantarum* WCFS1 to synthesize the prebiotic galacto-oligosaccharide (GOS) that is predicted to enhance the growth of lactobacilli *in vivo*. This prediction was attributed to the formation of a GOS mixture that contains an increased (relative to other analysed GOS) level of β-(1→6)-linked oligosaccharides, which supports the growth of bifidobacteria in mixed culture populations (Iqbal *et al.* 2010). The GOS used in this study is composed mainly of β-(1→4)-linked oligosaccharides (Coulier *et al.* 2009), and specific GOS mixtures may therefore possess more targeted specificities with regard to growth-promoting activity (e.g. by adjusting the amounts and ration of β-(1→4)- and β-(1→6)-linked oligosaccharides).

It is worth noting that although not all the strains have the ability to utilize (the majority of the) GOS components, this would not rule out the ability of intestinal communities to metabolize GOS, especially in the case of lactobacilli where they may cross-feed in the GIT environment. This notion is supported by the work of Xu *et al.* (2007), who found that fermentation of complex carbohydrates in the gut is due to the combined action of several bacteria. Our study shows that particular prebiotics promote the growth of certain strains/species, but not others. This is an important finding when deducing the prebiotic capabilities of various carbohydrates in order to enhance the gut microbiota diversity.

The data presented here illustrate that the success of prebiotics relies on the exploitation of differences in substrate preferences and competitive abilities among different members of the gut microbiota. Indeed, one has to keep in mind that pure culture models do not reflect the environmental experiences that confront bacteria in the mammalian host. Metabolic studies, such as the one described here, focus on the physiological implications of different oligosaccharide utilization in order to understand the bacteria. Our conclusions are at the moment based on a reasonably large number of investigated strains. Despite this, the number of strains is actually too small to decipher reliable ecological predictions and would require further, more extensive, investigations. The variations observed indicate niche-specific clustering of both bifidobacteria and lactobacilli and could have *in vivo* implications. Recently, elegant studies such as those employing *in vitro* colonic models (Maathuis *et al.* 2012) and *in vivo* clinical trials (Walton *et al.* 2012) have demonstrated that Vivinal GOS is bifidogenic while also increasing the numbers of *Lactobacillus* sp. These *in vitro* and *in vivo* studies highlight the potential for prebiotics to selectively enrich for probiotic strains and in doing so have the capacity to prevent pathogen colonization and promote gastrointestinal health. This research can pave the way towards a better understanding of the probiotic/prebiotic relationship through the facilitation of the rational design of prebiotics with a high degree of selectivity. In the current metagenomic and metatranscriptomic era, clinical trials involving prebiotic or symbiotic food formulations will undoubtedly unveil the beneficial changes in microbiota composition and activity.

Acknowledgements

This work was financially supported by FrieslandCampina. DvS and MO'CM (also a HRB postdoctoral fellow–Grant no. PDTM/20011/9) are members of the Alimentary Pharmabiotic Centre, which is a research centre funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan (grant nos. 02/CE/B124 and 07/CE/B1368).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (I) The HPAEC profile of the reference FOS mixture indicated an abundance of different oligosaccharides with different retention times. (II–V) Qualitative FOS degradation patterns for *Bif. infantis* NCDO 2205, *Bif. dentium* NCFB 2843, *Bif. pseudolongum* NCIMB 2244 and *Bif. longum* Onken.

Table S1 Final OD_{600} values reached by lactobacilli following 24 h of growth.

Table S2 Final OD_{600} values reached by bifidobacteria following 24 h of growth.

Table S3 pH values of the growth medium following 24 h of growth by lactobacilli

Table S4 pH of growth medium following 24 h of growth of various bifidobacteria.

Table S5 Growth rates for lactobacilli growing on various carbohydrate sources.

Table S6 Growth rates for bifidobacteria growing on various carbohydrate sources.

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