In Vitro Fermentation of caprine milk oligosaccharides by bifidobacteria isolated from breast-fed infants

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Keywords: Bifidobacteria, Bifidobacterial exo-α-sialidase, Caprine milk oligosaccharides, Infant formula, Prebiotic, Sialyl-oligosaccharide

Abbreviations: ARDRA, Amplified rDNA restriction analysis; CMO, Caprine milk oligosaccharides; CMOF, Caprine milk oligosaccharide enriched fraction; FOS, Fructo-oligosaccharides; GIT, Gastrointestinal tract; GOS, Galacto-oligosaccharides; HMO, Human milk oligosaccharides; RAPD, Random amplified polymorphic DNA

This study was conducted to investigate the catabolism and fermentation of caprine milk oligosaccharides (CMO) by selected bifidobacterial isolates from 4 breast-fed infants. Seventeen bifidobacterial isolates consisting of 3 different species (Bifidobacterium breve, Bifidobacterium longum subsp. longum and Bifidobacterium bifidum) were investigated. A CMO-enriched fraction (CMOF) (50% oligosaccharides, 10% galacto-oligosaccharides (GOS), 20% lactose, 10% glucose and 10% galactose) from caprine cheese whey was added to a growth medium as a sole source of fermentable carbohydrate. The inclusion of the CMOF was associated with increased bifidobacterial growth for all strains compared to glucose, lactose, GOS, inulin, oligofructose, 3-sialyl-lactose and 6-sialyl-lactose. Only one B. bifidum strain (AGR2166) was able to utilize the sialyl-CMO, 3-sialyl-lactose and 6-sialyl-lactose, as carbohydrate sources. The inclusion of CMOF increased the production of acetic and lactic acid (P < 0.001) after 36 h of anaerobic fermentation at 37°C, when compared to other fermentable substrates. Two B. bifidum strains (AGR2166 and AGR2168) utilised CMO, contained in the CMOF, to a greater extent than B. breve or B. longum subsp longum isolates, and this increased CMO utilization was associated with enhanced sialidase activity. CMOF stimulated bifidobacterial growth when compared to other tested fermentable carbohydrates and also increased the consumption of mono- and disaccharides, such as galactose and lactose present in the CMOF. These findings indicate that the dietary consumption of CMO may stimulate the growth and metabolism of intestinal Bifidobacteria spp. including B. bifidum typically found in the large intestine of breast-fed infants.

Introduction

Evidence suggests that the microbial community of the human gastrointestinal tract (GIT) has a core function in maintaining host health by preventing the colonisation of pathogens,1 degrading dietary compounds, producing metabolites able to be utilised by the host (e.g. short chain fatty acids, SCFA)2 and maintaining mucosal immunity.3 Particularly important in early life, the composition of the GIT microbiota influences the development and maturation of the foetal/ neonatal GIT and consequently the overall health of the infant. During the perinatal period, the infant GIT is colonised by a relatively simple microbial community, initially derived from vaginal microbiota and maternal faeces.4 The type of feeding (breast versus formula feeding), local environment, and antibiotic treatment may also play an important role in determining and maintaining the microbiota composition of the infant GIT.5,6 The GIT microbiota of breast-fed infants has long been thought to be dominated by bifidobacteria, compared to that of adults and formula-fed infants.7 However, there are conflicting reports regarding differences in the relative abundance of these bacteria between breast- and formula-fed infants.8-10 Moreover, although some studies have demonstrated a specific diversity of bifidobacterial species present in formula-fed infants is more like
the adult bifidobacterial diversity,\(^{11,12}\) culture or culture-independent methods, able to provide information on the identity and relative abundance of bifidobacteria to the species level, have not been used to compare the faecal microbiota of breast-fed vs. formula-fed infants. While the mechanisms for these differences in microbial colonisation and establishment are not fully understood, it is possible that the numbers of bifidobacteria species in breast-fed infants may be enhanced by the oligosaccharides in human milk (which collectively form the third largest solid component of milk (5 to 23 g l\(^{-1}\))\(^{13}\) after fat and protein).

Milk oligosaccharides (sugar polymers, typically 3 to 10 units) have been studied extensively because of their marked influence on the GIT microbiota (i.e. prebiotic activity). Human milk contains a variety of oligosaccharides able to stimulate the growth of specific commensal GIT microbiota,\(^{14}\) as well as stimulate the development of the immune system\(^{15}\) and prevent adhesion of pathogens to epithelial tissues.\(^{16}\) Infant-type bifidobacterial species such as \(B.\ longum\) subsp. \(longum\), \(B.\ longum\) subsp. \(infantis\), \(B.\ bifidum\) and \(B.\ breve\), for example, contain enzymes specifically involved in the metabolism of human milk oligosaccharides (HMO).\(^{17}\)

Modern infant formulas are increasingly supplemented with a mixture of plant derived oligosaccharides, such as fructo-oligosaccharides (FOS) and inulin (DP = 10 to 60), and lactose derived oligosaccharides, such as galacto-oligosaccharides (GOS).\(^{18}\) These substrates elicit non-specific bifidobacterial growth (the "bifidogenic effect"),\(^{19}\) and lack the complexity and diversity of HMO, so are unlikely to successfully mimic the structure specific effects of HMO. Fucosylated and sialylated HMO, for example, are complex oligosaccharides known to act as bacterial adhesin analogs and/or to mimic the receptors used by enteric pathogens to adhere to the surface of host epithelial cells.\(^{16}\)

Sialyloligosaccharides support the growth of breast-fed, neonate specific commensal bacteria, such as \(Bifidobacterium\ longum\) subsp. \(infantis\) ATCC15697.\(^{21}\) It has been suggested that these acidic oligosaccharides, in addition to eliciting a more specific bifidogenic effect, may combat influenza infections\(^{22}\) and ulcers caused by \(Helicobacter pylori\).\(^{23}\) Other activities reported include regulation of inflammation by reducing adhesion of human leukocytes on activated endothelial cells, and promotion of commensal enteric bacterial proliferation.\(^{24,25}\) The predominant forms of sialyloligosaccharides found in human milk, 3'-, and 6'-galactosyl-lactose are also the most prevalent oligosaccharides in caprine colostrum, milk and whey. Therefore, the supplementation of infant formula with caprine milk oligosaccharides (CMO) is likely to stimulate the growth and metabolism of bifidobacterial strains typically found in breast-fed infants, and also to mimic the structure specific effects of HMO with associated infant health benefits. Understanding whether bifidobacterial strains from breast-fed infants are able to utilize CMO as a fermentable substrate is an important factor in the potential use of CMO as an infant formula supplement. To explore the effects of CMO on the growth and metabolism of specific postnatal bifidobacteria, this study aimed to investigate the ability of bifidobacterial strains, isolated from exclusively breast-fed neonates, to ferment a caprine milk oligosaccharide enriched fraction (CMOF) (especially sia
yloligosaccharides), prepared from caprine cheese whey, using a recently published method,\(^{26}\) and to produce SCFA (compared with other prebiotics or milk sugars).

### Results

**Genetic characterization of bifidobacterial strains**

A total of 17 bifidobacterial strains were isolated from faecal samples of 4 exclusively breast-fed infants. The strains were positively identified by amplification and sequencing of a 498 bp region of the 16S rRNA gene corresponding to the V2 to V3 variable regions as \(B.\ bifidum\) (n = 4), \(B.\ longum\) (n = 6) and \(B.\ breve\) (n = 7) (Fig. 1). For the 6 \(B.\ longum\) strains, digestion of a partial 16S rRNA gene amplicon with Sau3AI was consistent with \(B.\ longum\) subsp. \(longum\).

RAPD sub-typing of the 17 bifidobacterial strains was undertaken to provide an indicative level of genetic heterogeneity that could be explored with further phenotypic assays. The dendrogram separated the 17 strains into 4 main clusters, broadly corresponding to the 3 different \(Bifidobacteria\) species (Fig. 1). A clear distinction between \(B.\ longum\) subsp. \(longum\) strains isolated from infant 2 (AGR2170 to AGR2174) and infant 3 (AGR2176) was discernible by RAPD analysis.

**Fermentation profile, SCFA and lactate analysis**

All 17 strains were assessed for growth in media supplemented with glucose, CMOF (50% oligosaccharides, 10% GOS, 20% lactose, 10% glucose and 10% galactose),\(^{26}\) combo, oligofructose, lactose, GOS, 3'-sialyl-lactose, 6'-sialyl-lactose, or no carbohydrate (Fig. 2). No significant growth (OD\(_{600} < 0.5\)) was observed from any bifidobacterial strains in the medium supplemented with inulin or with no added carbohydrate (data not shown).

In general, all strains reached higher optical densities at 16 h with CMOF as the sole carbohydrate source, compared to all the other carbohydrate sources (\(P < 0.001\)). The oligosaccharides 3'-sialyl-lactose and 6'-sialyl-lactose were fermented only by \(B.\ bifidum\) strain AGR2166 (Fig. 2a). \(B.\ bifidum\) strain AGR2166 fermented all tested carbohydrate substrates except oligofructose. The remaining \(B.\ bifidum\) strains (AGR2165, AGR2167 and AGR2168), were only able to ferment CMOF as a carbohydrate source (Fig. 2b). The \(B.\ longum\) subsp. \(longum\) AGR2170, AGR2171, AGR2172, AGR2173, AGR2174 (Fig. 2c), AGR2176 (Fig. 2d) and \(B.\ breve\) strain AGR2169, AGR2177, AGR2178, AGR2179, AGR2181, AGR2183 (Fig. 2e) AGR2175 (Fig. 2f) strains reached an intermediate optical density (OD\(_{600} = 1.5\)) with oligofructose, and increased optical densities (OD\(_{600} \geq 2\)) with GOS, lactose, combo and glucose as the sole carbohydrate source, but with a different growth profile during the 48 h of fermentation.

A single strain representative of each fermentation profile was selected for quantifying SCFA production (Fig. 3, \(B.\ bifidum\) AGR2166 (a), AGR2168 (b); \(B.\ longum\) AGR2173 (c),...
AGR2176 (d); B. breve AGR2177 (e), AGR2175 (f)). Only acetic and lactic acid were produced after 16 h and 36 h incubation for all the strains tested (Fig. 3). Acetate was shown to be present at time 0, due to the presence of sodium acetate (0.5%) in the basal media. The same media batch was used to investigate the growth of the different bacterial strains/carbohydrates for comparison purposes. All strains (except AGR2168), produced higher concentrations ($P < 0.001$) of acetic and lactic acid in medium supplemented with CMOF at 36 h post-inoculation compared to the medium supplemented with the combo preparation (Fig. 3). AGR2168 only grew in the medium supplemented with CMOF, thus comparisons with the combo preparation were not possible. Formate was measured over the 36 h fermentation period but was produced at concentrations that were too low to quantify using our HP-LC methods.

Production of acetic and lactic acid by all the strains tested was associated with a decrease in overall pH of the culture over 36 h with the CMOF (Fig. 4). The drop in pH from 6.5 to 4.2 - 4.9 was correlated with the final OD of the culture ($r = -0.95; P = 0.001$), and the concentration of acetic ($r = -0.85; P=0.02$) and lactic acid ($r = -0.83; P = 0.03$) produced.

Bifidobacterial catabolism of oligosaccharides from CMOF

The initial relative abundance of several oligosaccharides associated with the CMOF, measured by LC-MS, was approximately 13% 3'- and/or 6'-galactosyl-lactose, 27% 3'- and/or 6'-sialyl-lactose, 32% 6'-Glycolyl-neuraminyl-lactose, 9% lacto-N-hexaoe and less than 11% disialyl-N-lactose and 8% 6'-N-acetyt-glucosaminyl-lactose. All strains preferentially catabolised the 3'- and/or 6'-galactosyl-lactose and 3'- and/or 6'-sialyl-lactose (Fig. 5). B. bifidum AGR2166 had the highest levels of depletion (35 to 55%) of 3'- and/or 6'-galactosyl-lactose, 3'- and/or 6'-sialyl-lactose and 6'-glycolyl-neuraminyl-lactose. Disialyl-N-lactose and 6'-N-acetyt-glucosaminyl-lactose had the lowest depletion levels ($<10\%$, similar among all strains). The depletion rate of all oligosaccharides, however, was different between strains from the same species (Fig. 5). B. bifidum AGR2166, for example, had the higher catabolism level among all strains, utilizing 33% of the total oligosaccharides identified in the CMOF, more pronounced then the reduction by B. bifidum AGR2168 (13%). A similar strain dependant variation of oligosaccharide catabolism was noted for B. longum subsp. longum AGR2173 (7%), AGR2176 (14%) and B. breve AGR2175 (5%) and AGR2177 (16%) strains. No correlation between the catabolism of CMO and bacterial growth were found after 36 h of inoculation. This may have been due to the fermentation of other carbohydrates (e.g., mono- and di-saccharides) present in the CMOF that may have stimulated the bifidobacterial growth independent of CMO.

The concentrations of galactose, glucose, lactose, and GOS in a media supplemented with 1% CMOF were determined after 36 h of bifidobacterial incubation and compared to the concentrations immediately after inoculation (Fig. 6). These data indicate that lactose was depleted by all bifidobacterial...
strains tested. The degradation of lactose, GOS, and CMOF were likely to have increased the overall concentrations of glucose and galactose in the media, and were not fully utilised by the end of the 36 h incubation. *B. bifidum* AGR2166 and AGR2168 had lower levels of glucose and higher levels of galactose in their media compared to pre-incubated media and to the other strains after CMOF fermentation. AGR2166 also had higher levels of lactose, compared to other strains, which may have been associated with oligosaccharide catabolism by this strain. The GOS concentration in the *B. longum* AGR2173 and *B. breve* AGR2175 media after incubation did not differ from uninoculated media; however, 50% of the GOS present in the pre-incubated media was fermented by the other strains.

Figure 2. Growth profile of (A) *B. bifidum* AGR2166; (B) *B. bifidum* AGR2168 (similar to AGR2165 and AGR2167); (C) *B. longum* AGR2173 (similar to AGR2170, AGR2171, AGR2172 and AGR2174); (D) *B. longum* AGR2176; (E) *B. breve* AGR2177 (similar to AGR2169, AGR2178, AGR2179, AGR2181, AGR2183); (F) *B. breve* AGR2175. Each time point is an average of 3 replicates and the errors bars indicate standard deviation.

Identification of genes encoding for exo-α-sialidase and associated sialidase activity

Sialidase activity of the bifidobacterial isolates on CMOF was assessed using molecular methods to demonstrate the presence of sialidase-encoding genes and by measuring sialidase activity using a fluorogenic substrate. The presence of 3 reported sialidase genes (BBPR_1793 and BBPR_1794 from *B. bifidum* PRL2010 and HMPREF92 28_0182 from *B. breve* ACS-071-V-Sch8b), were confirmed by PCR amplification and DNA sequencing in the *B. bifidum* and *B. breve* strains from this study (Fig. 1). Sialidase enzyme activity was also analyzed in 6 selected isolates (highlighted in Fig. 1); 2 *B. breve*, 2 *B. bifidum* and 2 *B. longum*. Only the *B. bifidum* isolates (AGR2166 and AGR2168) had cellular sialidase activity when grown in the presence of CMOF.
Fig. 7, although \textit{B. bifidum} AGR2168 had only limited ability to ferment the CMOF sialyloligosaccharides, 3'- and 6'-sialyl-lactose as a sole source of carbon (Fig. 2). Sialidase activity from \textit{B. bifidum} was mainly cell-associated, although approximately 12% residual activity was also detected in the culture supernatant of these 2 strains (Fig. 7A). \textit{B. bifidum} cellular sialidase activity in AGR2166 and AGR2168 was induced by all 4 substrates examined, and was significantly enhanced ($P \leq 0.01$) in bacterial cell preparations taken from cultures grown in the presence of 3'- and 6'-sialyl-lactose when compared to CMOF and combo. Despite the presence of 3' and 6'-sialyl-lactose in CMOF, there was no increase in sialidase activity of AGR2166 and AGR2168 grown in the presence of CMOF compared to the combo (Fig. 7B). Similar sialidase activity was observed from the culture supernatant taken from AGR2166 grown in the presence of 6'-sialyl-lactose and CMOF, and this sialidase activity was higher than combo and 3'-sialyl-lactose ($P < 0.001$). Despite the presence of a sialidase-encoding gene in the \textit{B. breve} isolates examined, no sialidase activity was observed. However, in contrast to the sialidase proteins from \textit{B. bifidum} that have signal peptide cleavage sites and transmembrane helices, no such structural characteristics were associated with the sialidase from \textit{B. breve}, suggesting a potential intracellular localization. Intracellular sialidase activity was not determined.

**Discussion**

This study investigated the \textit{in vitro} effects of a CMOF on the growth of selected bifidobacteria isolated from 4 exclusively breast-fed infants. The CMOF (containing high concentrations (46%) of sialyloligosaccharides), supported enhanced growth of...
selected bifidobacteria strains isolated from breast-fed infants, and stimulated the in vitro production of lactate and SCFA, such as acetate. These results confirm the hypothesis, bifidobacteria isolated from the faeces of breast-fed infants are able to ferment CMOF, increasing bifidobacterial growth and metabolism.

In a recent study, CMO was shown to increase the growth of human faecal Bifidobacterium spp in anaerobic batch culture, although the specificity of bifidobacterial CMO consumption was not investigated. The B. breve, B. longum and B. bifidum strains isolated in this work, together with B. longum subsp infantis and B. adolescentis, are among the most prevalent species found in infants independent of their feeding regime.

Although only a small number of bifidobacterial strains were selected from each infant, previous work suggests that the infant microbiota in the GIT is heterogeneous but is dominated by 3-5 different bifidobacterial species. The RAPD analysis undertaken in this study broadly agrees with the well-recognized level of clonal heterogeneity demonstrated among the B. breve, B. longum and B. bifidum strains as determined by genetic fingerprinting methods such as ribosomal intergenic spacer analysis (RISA) and RAPD.

Among the bifidobacterial species tested, B. bifidum (AGR2166) was shown to utilize both 3'- and 6'-sialyl-lactose as a sole carbon source to support growth (Fig. 5) with associated depletion of these same oligosaccharide isomers present in CMOF (Fig. 5). Enhanced depletion of 3'- and 6'-sialyl-lactose from CMOF by B. bifidum (AGR2166) was likely through cell-associated sialidase expression after induction with the same oligosaccharides (Fig. 7). B. bifidum (AGR2168), in contrast, displayed intermediate growth on 3'- sialyl-lactose (Fig. 2b) with partial (20%) utilization of 3'- and 6'-sialyl-lactose from CMOF (Fig. 5) with cell-associated sialidase expression (Fig. 7). These data largely agree with previous work that suggests a surface or intracellular location for the sialidase enzyme on B. bifidum. The residual activity of sialidase found in the B. bifidum (AGR2166 and AGR2168) culture supernatant therefore may be associated with cell wall debris and/or released enzyme present within the culture supernatant.

B. longum and B. breve strains were unable to utilize 3'- and 6'-sialyl-lactose as a growth substrate when included as the only carbon source (Fig. 2c to 2f) and no expression of cell-associated sialidase was observed (Fig. 7). Limited depletion (5-15%) of these oligosaccharides by B. longum and B. breve strains was detected (Fig. 5) however, when grown in a CMOF enriched media. This partial depletion may have occurred through incomplete catalysis of 3’- and 6’-sialyl-lactose without fermentation of any resulting breakdown products or that these sugars were selectively adsorbed to the bacterial cells that were present in the media, which were then removed prior to analysis.

Contrasting oligosaccharide depletion observed between strains of the same species was in accordance with inter-strain heterogeneity shown in the RAPD analyses. B. breve AGR2175 and AGR2177, for example, both isolated from the same infant (3) and with similar growth profiles (Fig. 2), showed different oligosaccharides catabolism profiles (Fig. 5) which might indicate differential regulation or expression of enzymes involved in carbohydrate metabolism.

Augmented microbial biomass associated with enhanced growth and fermentation of CMOF increased microbial fermentation end products such as acetate and lactate. These data agree with a previous study, where CMO was shown to increase the production of acetate, lactate and propionate in anaerobic batch culture inoculated with human faeces. Formate may also be produced as an end-product of bifidobacterial fermentation with the inclusion of fructose or OF as the main carbohydrate source but was produced at levels that were too low to detect using HP- LC.

An absolute measurement of CMO utilization in this study was impossible due to the high concentrations (50%) of lactose, GOS, glucose and galactose in the CMOF. However, when used as a sole carbohydrate source, lactose, GOS, glucose and galactose did not support enhanced bacterial growth when compared to the CMOF. It is likely that the CMO component of the overall CMOF, is not only a fermentable substrate, but also stimulates the utilization of other simpler carbohydrates. The GlcNAc-containing oligosaccharides (6'-N-acetyl-glucosaminyl-lactose and lacto-N-hexaose), for example, have been reported as a growth factor stimulating lactose utilization by B. bifidum. The mechanism through which these oligosaccharides are used remains to be identified, but studies on the utilization of HMO may provide some clues. Certain bifidobacterial strains such as B. bifidum NCIMB41171 have the ability to synthesize long chain carbohydrates (such as GOS) from lactose and galactose using the transglycosylactic activity of β-galactosidase. Thus, it is difficult to precisely determine how much lactose was degraded to glucose and galactose through the hydrolytic activity of β-galactosidase, and how much GOS, if any, was produced by transglycosylactic activity of β-galactosidase.
Lactose, the core of all HMO and CMO, and the main structure of galactosyl-lactose, is likely to be degraded to galactose and glucose in a catabolic reaction that requires β-galactosidase activity. *B. bifidum*, for example, contains both extracellular and intracellular β-galactosidases.43 *B. breve* and *B. longum subsp. longum*, on the other hand, have been reported to contain only intracellular β-galactosidases, and the high utilization of lactose by these strains may indicate that lactose is likely to be actively transported into the cells by a yet unidentified transporter. Although GOS is also hydrolysed to glucose and galactose by β-galactosidase, different strains have been shown to have differential consumption of selected GOS with different DPs.44 The infant isolates (*B. longum subsp. infantis* and *B. breve*) are able to more efficiently consume the GOS species with DP from 3 to 8, while *B. adolescentis* and *B. longum subsp. longum* exhibited differential consumption of selected DP.44 These contrasting HMO and CMO utilisations suggest that niche specific adaptation abilities exist among various bifidobacterial species and strains, and with other components of the microbiota of the GIT. These complex effects cannot be reproduced by simple carbohydrate structures most often used as prebiotics.

The ability of the bifidobacterial strains to utilize the different carbohydrates present in CMOF and/or stimulate the consumption of other carbohydrate sources is important to determine the effects of these milk components in the GIT microbiota. Although more than 8% of the identified genes from bifidobacterial genomes are predicted to be involved in carbohydrate metabolism, the ability to metabolise certain complex milk oligosaccharides is species and strain specific.45,46 Analysis of the genes involved in carbohydrate utilization indicate that *B. bifidum* (JCM1254 and JCM7004) contain genes that encode specialized enzymes associated with the extracellular deglycosylation of milk oligosaccharides, including extracellular α-fucosidases,47 β-galactosidases, β-N-acetylglucosaminidases48 and α-sialidases,49 which efficiently remove monosaccharides from complex milk oligosaccharides. *B. bifidum* and *B. longum* also contain a membrane enzyme, lacto-N-biosidase, responsible for the cleavage of the bifidogenic HMO lacto-n-tetraose to lacto-n-biose50 and lactose. The mono- and disaccharides released by this endoglycosidase (especially lacto-n-biose), are internalised by family 1 solute binding proteins, and metabolised. Family 1 solute binding proteins are part of a gene cluster conserved across all infant GIT-associated bifidobacteria, including *B. bifidum*, *B. infantis*, *B. longum* and *B. breve* isolates.50,51 The same enzyme degradation mechanism may be responsible for the utilization of lacto-N-hexaose present in CMO.

None of the selected bifidobacterial strains were able to utilize inulin as the sole carbohydrate source, but all except the *B. bifidum* strains were able to utilize oligofructose. The DP (oligofructose DP 2-10; HP inulin DP 11-60) is likely to influence the ability of

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**Figure 5.** Percentage of oligosaccharide depletion by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains after 36 h of growth in semi-synthetic broth supplemented with 1% of caprine milk oligosaccharide (determined from caprine milk oligosaccharide enriched fraction), when compared to the uninoculated control media. The oligosaccharides are represented by their abbreviation and their relative initial abundance is shown in brackets. 3’- and/or 6’-galactosyl-lactose (3-GL and/or 6-GL), 3’- and/or 6’-sialyl-lactose (3-SL and/or 6-SL), 6’-glycyl-neuraminyl-lactose (NGL), lacto-N-hexaose (LNH), disialyl-N-lactose (DSL), 6’-N-acetyl-glucosaminyl-lactose (NAL). Each strain was incubated in triplicate and errors bars show the standard deviation of depletion. *a, b, c* Bars with dissimilar letters differ significantly in depletion within each oligosaccharide (P < 0.001).
bifidobacterial strains to utilize FOS as the sole carbon source. However, bifidobacteria present in breast-fed infants may also be selectively stimulated by milk oligosaccharides instead of plant derived oligosaccharides. Previous studies confirmed the poor growth of *B. bifidum* strains on inulin type fructans, but strain differences in β-fructofuranosidase production levels have been reported. After weaning, with the introduction of plant derived foods, *B. bifidum* strains are likely to benefit indirectly from the fermentation of inulin type fructans by other members of the GIT microbiota through the lowering of the GIT pH, or the increased availability of monosaccharides as substrates.

In conclusion, faecal bifidobacteria species isolated from breast-fed infants are heterogeneous at the genetic level based on RAPD profiles, but also at the level of substrate utilization through the differing depletion of certain carbohydrate components of the CMO preparation including some oligosaccharides. CMOF was able to stimulate the growth of bifidobacteria commonly found in the GIT of breast-fed infants. CMO contained in the CMOF may also have stimulated the consumption of lactose, glucose, galactose and GOS. Comparing the selected strains, *B. bifidum* were better able to ferment CMOF, especially the sialyloligosaccharides, which may indicate that in *vivo*, this strain may benefit from CMO consumption. Defining and linking the utilization of specific oligosaccharide structures, such as the sialyloligosaccharides, to cultured bacteria will provide a scientific path for targeting infant health by establishing protective microbial communities, beneficial to their hosts and potentially applicable to different stages of human life and health states.

### Material and Methods

#### Isolation of bifidobacteria

Collection of faecal samples from healthy breast-fed infants was approved by the local human ethics committee (Massey University, Palmerston North, New Zealand). Bifidobacteria were isolated from faeces obtained from freshly soiled diaper/nappy of 4 exclusively breast-fed neonates on modified TPY agar (MTPY). The plates were incubated anaerobically (93% CO₂, 7% H₂) at 37°C for 48 h. Bacterial colonies were then individually picked, subcultured on fresh MTPY agar plates to obtain single colonies and again on anaerobic De Man-Rogosa-Sharpe (MRS, Oxoid, CM0361) agar slopes (pH 6.5 – 7.0) supplemented with L-cysteine-hydrochloride (0.5 g l⁻¹), at 37°C for 48 h, for storage at −80°C until further analysis.

#### Bifidobacterial characterization

The identity of the bifidobacterial isolates was confirmed using PCR and 16S rRNA gene sequencing using bif 164 (5'-GGG TGG TAA TGC CGG ATG-3') and bif 662 (5'-CCA CCG TTA CAC CGG GAA -3') primers.57 Sequencing of the PCR product (15 ng) was performed using the bif 164 or bif 662 primers (3.2 nM) and 16S rRNA gene sequences were compared with known bacterial sequences available from GenBank database using BLAST.

#### Randomly amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) PCR was performed using 7 random decamer primers: P2 5'-GAT CGG ACG G-3', P15 5' CTG GGC ACG A 3', P16 5' TCG CCA GCC A 3', P17 5' CAG ACA AGC C 3', PER1 5'AAG AGC CCG T 3', and CC1 5'AGC AGC GTG G 3'.60 CORR1 5'-TGC TCT GCC C-3'. The amplifications were carried out using 100 ng template DNA and a dendogram was generated from the RAPD profiles generated with the 7 random primers using Bionumerics 4.0 (Applied Maths). Profiles were compared using Dice's Similarity Coefficient at a tolerance of 2%.
Amplified rDNA restriction analysis (ARDRA) of B. longum
subsp longum versus infantis

PCR amplification of a 914 bp DNA fragment and restriction endonuclease digestion with Sau3AI (New England Biolabs, R0169S) was used to differentiate B. longum subsp longum from B. longum subsp infantis as described previously.62 The B. longum subsp infantis strain JCM 10088 control DNA was kindly gifted by Professor Gerald Tannock (University of Otago, New Zealand).

Carbohydrate fermentation and measurement of SCFAs and lactate

To investigate the growth profiles of bifidobacterial strains, each strain was inoculated into MRS broth supplemented with 0.05% (w/v) L-cysteine and incubated at 37°C for 36 h until late logarithmic/early stationary phase was reached. Each strain was then subcultured (100 µl) into a defined semi-synthetic medium63 supplemented with a carbohydrate source at a final concentration of 1% (w/v). The carbohydrates added were either CMOF (5 g l⁻¹ caprine milk oligosaccharides, 1 g l⁻¹ GOS, 2 g l⁻¹ lactose, 1 g l⁻¹ glucose and 1 g l⁻¹ galactose) prepared from caprine cheese whey, and characterized as described previously26; combo, identical to CMOF but lacking CMO, (1 g l⁻¹ GOS, 2 g l⁻¹ lactose, 1 g l⁻¹ glucose and 1 g l⁻¹ galactose); 10 g l⁻¹ of glucose (BDH), lactose (BDH), oligofructose P95 (BENEO-Orafti; average degree of polymerisation [DP] of 4), inulin HP (BENEO-Orafti; average DP of 25), galacto-oligosaccharide (GOS) (TOS-100, Yakult; DP <8), 3'-sialyl-lactose (Carbosynth, OS31041) or 6'-sialyl-lactose (Carbosynth, OS04398).

The dominant oligosaccharides present in the CMOF were 3'- and/or 6'-galactosyl-lactose (12%, DP = 3), 3'- and/or 6'-sialyl-lactose (27%, DP = 3), 6'-glycolyl-neuraminyl-lactose (32%, DP = 3), lacto-N-hexose (9%, DP = 6), disialyl-N-lactose (11%, DP = 4), 6'-N-acetyl-glucoaminyl-lactose (7%, DP = 3). Each bifidobacterial strain was grown in triplicate in 5 mL of semi-synthetic broth, and cultured anaerobically for 45 h at 37°C. Cell growth was measured 2 hourly by taking optical density measurements at a wavelength of 600 nm on a spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences).

Fermentation reactions from 6 strains (2 B. bifidum, 2 B. longum, and 2 B. breve), selected due to their optimum growth in CMO-supplemented medium, were evaluated for SCFA production. Anaerobic growth of each strain in 6 different broth cultures supplemented with CMO (10 g l⁻¹, 1% [w/v] final concentration contained in CMOF), and combo (10 g l⁻¹, 1% [w/v]), was...
B. longum subsp. infantis ATCC15697

B. breve ACS-071-V-Sch8b

B. bifidum PRL2010

B. bifidum PRL2010

Origin | Predicted localization | Primer name | Sequence | Amplicon length | Region amplified
--- | --- | --- | --- | --- | ---
B. longum subsp. infantis ATCC15697 | Intracellular | Sialil-Inf | F. 5'-TACTGTGTGCGGCAGGAAAC | 1136bp | 33-1168bp
B. breve ACS-071-V-Sch8b | Intracellular | Sialil-Br | F. 5'-GCGGGTCGCGCAGACATCTAT | 1527bp | 737-2263bp
B. bifidum PRL2010 | Extracellular | Sialil-Bf15 | F. 5'-GCCGACCCTCAGCGGACAC | 1198bp | 448-1645bp
B. bifidum PRL2010 | Extracellular | Sialil-Bf2 | F. 5'-GCTGCATCGCGTGTCGTCGTC | 1049bp | 915-1963bp

Sialidase assay

The sialidase activity of selected bifidobacteria was determined using the fluorescent substrate 4-methylumbelliferyl-α-D-N-acetyleneuraminic acid (4MU-Neu5Ac) (Sigma, M8639) as described previously. Briefly, bifidobacterial strains were grown anaerobically in 5 ml of a semi-synthetic medium supplemented with 10 g l⁻¹ CMO (contained in CMOF), combo, or of 3'-syalyl-lactose or 6'-syalyl-lactose as the sole carbohydrate source for 24 h at 37°C. Six separate cultures of each of 2 B. bifidum, 2 B. longum, and 2 B. breve strains were examined, with each biological replicate assessed in triplicate. Enzyme activities from washed bacterial cells and culture supernatant were determined by comparing absolute fluorescence units (Afu) minus the blank, as described previously.

Statistical analysis

Bacterial growth, SCFA profiles and sialidase production at 36 h of fermentation for each substrate were tested for normality and homogeneity of variances and compared by one-way analysis of variance (ANOVA) using GenStat (15th edition SPS). Differences were considered significant at $P \leq 0.05$. To identify the correlation between bacterial growth, pH and SCFA production, Pearson’s Rank correlation factors and $P$ values were calculated.

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using GenStat (15th edition SPS). Correlations were considered significant if \( P < 0.05 \).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Drs Jolon Dyer and Alison J. Hodgkinson (AgResearch) for proof-reading the manuscript. We also thank Yakult Japan for the provision of GOS (TOS-100), Invita NZ Limited for the supply of FOS and inulin, and Wayne Young for the RAPD profile analysis.

Funding
Caroline Thum acknowledges the Ministry of Business, Innovation and Employment, New Zealand (C10×0907), the Riddet Institute Centre of Research Excellence (CoRE) and AgResearch for the funding and the PhD Scholarship.

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