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Infant formula with galacto-oligosaccharides (OM55N) stimulates the growth of indigenous bifidobacteria in healthy term infants

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RESEARCH ARTICLE

Abstract

The objective of the study was to investigate whether an infant formula supplemented with galacto-oligosaccharides (GOS; OM55N) was able to stimulate the growth of indigenous bifidobacteria and to establish microbiota similar to that of breastfed infants. A randomised, double-blind, placebo-controlled trial was performed using 35 healthy term infants (31-54 days of age; 42±6 days) to determine whether infant formula with 0.3 g/dl GOS (OM55N) stimulated the growth of bifidobacteria in the infants' guts. At the trial onset and 2 weeks after, the infants' faecal samples were examined for microbiota composition (bacterial abundance and α-diversity) and faecal characteristics. Among the 35 infants, 5 were withdrawn and 8 were excluded from the final evaluation before breaking the blinding since the indigenous bifidobacteria were not detected at the trial onset. After 2 weeks, the abundance of *Bifidobacteriaceae* was significantly increased in the GOS feeding group compared to the control (+11.6±24.1% vs -3.9±13.0%; P=0.043). The Shannon index, which accounts for both abundance and evenness of the present species, was significantly decreased with GOS supplementation (-0.1±0.4 vs +0.4±0.4; P=0.014). Faecal characteristics such as pH and organic acids were similar in both groups, with no statistical differences. No adverse side effects related to the formula consumption were reported. Although the concentration of GOS was relatively low, the infant formula with GOS increased the abundance of bifidobacteria and resulted in a reduced α-diversity of the microbiota.

Keywords: galacto-oligosaccharides, Oligomate® 55N (OM55N), infant formula, *Bifidobacteriaceae*, 16S rRNA gene amplicon

1. Introduction

Human beings are sterile at birth; however, bacterial colonisation starts immediately, and highly dense microbial ecosystems (microbiota) are established in the gut within a few days. The composition of an infant's microbiota has been intensely studied using cultivation, and more recently, culture-independent 16S rRNA sequence-based methods. Studies have reported that the development of neonate gut microbiota showed large intra-individual differences, but bifidobacteria are the most common and predominant bacteria in infants of several weeks of age (Benno *et al.*, 1984; Harmsen *et al.*, 2000; Palmer *et al.*, 2007; Penders *et al.*, 2006; Stark and Lee, 1982). It is generally accepted that the microbiota of breastfed infants are dominated by bifidobacteria; alternatively, the microbiota of formula-fed infants is more diverse with bifidobacteria often being the predominant species but in lower numbers than in breastfed infants, and it contains substantial quantities of *Enterobacteriaceae*, *Bacteroides*, and *Clostridium* species (Scholtens *et al.*, 2006). An important determinant in the development of a bifidogenic microbiota are human milk oligosaccharides, which escape digestion and absorption from the mammalian host and are fermented by bifidobacteria in the gastrointestinal tract (Sela and Mills, 2010).

Breast-feeding has been the primary choice for nursing new-borns, although supplementation or substitution with infant formula has been introduced for decades. Hence, improvements in infant formulas have attempted to emulate the beneficial effects of breast milk by supplementing prebiotics that selectively stimulate the growth of the indigenous beneficial bacteria.

A number of clinical studies have suggested that some prebiotics have an effect on enhancing the growth of bifidobacteria. Among the trials, galacto-oligosaccharides (GOS) have emerged as the major prebiotic oligosaccharides for application in infant formulas. GOS have been used as food ingredients for at least 30 years, and their application has expanded rapidly. GOS contain a mixture of short-chain oligosaccharide (2-6 DP, >95%) and are produced from lactose and β-galactosidase. Different methods have been utilised in the production of GOS; the consequence of these process changes is a difference in the final product with respect to the profile of the oligosaccharide polymers and the position of the chemical bonds between the saccharide units. Oligomate® 55N (OM55N) is one of the most popular GOS for use in infant formula. Studies have shown that OM55N is safe (Kaneko *et al.*, 2014; Kobayash *et al.*, 2013; Kobayashi *et al.*, 2009, 2013), selectively consumed by bifidobacteria and lactobacilli (Matsumoto *et al.*, 2004), and increases the population of bifidobacteria in adult gut microbiota (Matsumoto *et al.*, 2004).

We conducted a randomised, double-blind, placebo-controlled trial using 35 healthy term infants to assess whether infant formula with GOS (OM55N) stimulates the growth of indigenous bifidobacteria in the infant gut. A 16S metagenome method was used to evaluate the change in the gut microbiota. In addition, changes of faecal characteristics, such as pH, organic acids, and frequency of defection were assessed.

2. Materials and methods

Participants

We enrolled 35 healthy term infants aged 42±6 days old (range, 31-54 days) who were nursed with infant formula. The supplementation of breast milk of up to 20% was permitted. This study was approved by the ethical committee of the Teikyo University, and written informed consent was obtained from the infants' parents before enrolment. Parents were instructed to record their infants' faecal frequency, changes in diet and infants' health condition. Infants who had any antibiotic or probiotic treatment before and during the study period were excluded. Infants were randomly assigned to the control or GOS feeding group, and their baseline characteristics are shown in Supplementary Tables S1 and S2. Infants in both groups received a test formula for 2 weeks. Faecal samples were collected by the parents at the trial onset and at 2 weeks after the intervention. The samples were immediately transported to the laboratory and were stored at -80 °C until evaluation. A separate randomisation schedule was prepared by an external independent agent. The investigators and volunteers were blinded to the group allocation, and the blinding was broken after all the analyses were completed.

Composition of the galacto-oligosaccharides and test formula

Oligomate® 55N (OM55N; Yakult Pharmaceutical Industry, Tokyo, Japan) was used as the test GOS. As shown in Supplementary Table S3, the major saccharide was a 4' galactosyl lactose (4'-GL, Gal β1-4 Gal β1-4 Glc). The compositions of the GOS and control formulas are summarised in Table 1. The GOS formula was supplemented with 0.3 g/dl of GOS while the control formula was supplemented with dextrin. The test infant formulas were provided in powder form, and they had identical sensorial characteristics and the same label. The formulas were produced and codified by Wakodo Co., Ltd. (Tokyo, Japan).

DNA extraction

DNA was extracted according to the protocol described by Matsuki *et al.* (2004). Briefly, 20 mg of the faecal samples were suspended in a 500 µl extraction buffer (100 mM Tris-HCl [pH 9.0], 40 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulphate in the final concentration) in a 2 ml screw-cap tube. Then 300 mg of 0.1 mm diameter glass beads and 500 µl of a buffer-saturated phenol were added to the suspension. The microbial cells were lysed by mechanical disruption using a FastPrep FP 120 (BIO 101; Vista, CA, USA) at a power level of 5.0 for 30 s. The mixtures were centrifuged at 4,500×*g* for 5 min, and the upper layers were subjected to phenol/chloroform/isoamyl alcohol extraction followed by isopropanol and ethanol precipitation. Finally, the dried DNA samples were suspended in 1 ml Tris-EDTA buffer and stored at -20 °C until they were subjected for pyrosequence and quantitative PCR analysis.

Table 1. Composition of the formulas used in the study.

	Control formula (per dl)	GOS formula (per dl)
Energy (kcal)	67.7	67.3
Protein (g)	1.5	1.5
Carbohydrate (g)	7.2	7.3
Lactose (g)	6.5	6.5
Galacto-oligosaccharides (g)	0.0	0.3
Maltodextrines (g)	0.8	0.2
Fat (g)	3.7	3.6
Inositol (mg)	5.9	5.9
Linoleic acid (mg)	429	429
a-linolenic acid (mg)	52.0	52.0
Docosahexaenoic acid (mg)	10.4	10.4
Nucleotides (mg)	1.0	1.0
Lactoferrin (mg)	13.0	13.0

16S rRNA gene amplification and pyrosequencing

The 16S rRNA genes of each sample were amplified using a 66F-TAG-linker A forward primer and a 338Rm-linker B reverse primer. The sequence of the 66F-TAG-linker A primers was 5′-*CCATCTCATCCCTGCGTGTCTCCGAC*-TCAG-NNNNNNNNNN-GCYTAAYACATGCAAGTMGA-3′. The italicised sequence represents the 454 Life Sciences linker A; TCAG was inserted as a key sequence as recommended by the manufacturer; NNNN... represents the unique 10-base barcode for tagging each PCR product; and the underlined sequence is the broadly conserved bacterial primer 66F. The 66F forward primer was designed based on the 63F primers (Marchesi *et al.*, 1998) to detect common intestinal bacteria in infants. The sequence of the 338Rm-linker B primers was 5′-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*-GCTGCCWCCCGTAGGWGT-3′. The italicised sequence represents the 454 Life Sciences linker B, and the underlined sequence is the broadly conserved bacterial primer 338R (Lane, 1991) with a modification to detect *Akkermansia*, which has a two-base mismatch in this target region.

The PCR mixture (50 µl) contained 1×SYBR Premix Ex Taq (Takara Bio, Osaka, Japan), 100 nM primers, and 1 µl of DNA. Thermal cycling consisted of initial denaturing at 95 °C for 5 min followed by 25-33 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Amplification was performed by using an ABI PRISM7500 (Applied Biosystems, Framingham, MA, USA), and thermal cycling was terminated before the amplification curve reached the plateau to avoid the bias of the composition and the generation of chimera. Amplicons were purified with Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) and then quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Leek, the Netherlands). After quantification, the amplicons were pooled in equal amounts and sequenced using a Roche 454 GS Junior pyrosequencer with a GS FLX Titanium emPCR Kit (Lib-L) according to the manufacturer's protocols (Roche Applied Science, Bavaria, Germany).

16S rRNA gene sequence-based microbiota analysis

The sequences generated from the 454 GS Junior platform were analysed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). The extracted high-quality 16S rRNA gene sequences were assigned to operational taxonomic units (OTUs) using Usearch (Edgar, 2010) under the threshold of 97% identity. Coincidentally, UCHME (Edgar *et al.*, 2011) was used to remove the potential chimeric sequences in the representative set of OTUs with the reference mode. The taxonomy of each OTU representative sequence was assigned using the Ribosomal Database Project classifier with a minimum bootstrap threshold of 50% (Wang *et al.*, 2007). A single representative from each OTU was aligned using MUSCLE (Edgar, 2004), and a phylogenic tree was constructed using FastTree (Price *et al.*, 2009).

Enumeration of predominant bacteria by real-time PCR

The population of predominant bacteria were enumerated by real-time PCR as described previously (Matsuki *et al.*, 2004). The real-time PCR analyses were performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Each reaction mixture (10 µl) was composed of 1×SYBR Premix Ex Taq, 0.25 µM of the specific primers (Supplementary Table S4) (Matsuda *et al.*, 2007; Matsuki, 2007; Matsuki *et al.*, 2004; Rinttila *et al.*, 2004), and 1 µl of 1×-, 10×-, and 100×-diluted template DNA. The amplification program consisted of one cycle of 94 °C for 5 min and then 40 cycles of 94 °C for 20 s, 55 or 50 °C for 20 s, and 72 °C for 50 s and finally one cycle of 94 °C for 15 s. A melting curve analysis was performed after amplification to distinguish the targeted PCR product from the non-targeted PCR product.

Total bacterial counts

100 µl of 10-fold diluted (v/w) faecal homogenate was added to three volumes of 4% paraformaldehyde in phosphate buffered saline, which were incubated at 4 °C for 16 h. The paraformaldehyde-treated samples were smeared over a MAS-coated slide glass (Matsunami, Osaka, Japan) and stained with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The fluorescent cells in the samples were automatically counted using a Leica MM AF microscope (Leica, Wetzlar, Germany) and Image-Pro Plus image analysis software (version 5.1; Media-Cybernetics, Silver Spring, MD, USA). The bacterial count was expressed as the mean value of 10 fields for each smear sample.

pH and organic acids measurements

The pH of the faecal samples was measured directly using a handheld pH meter (model IQ150) equipped with a PH17SS electrode (IQ Scientific Instruments, San Diego, CA, USA). To determine the concentrations of short-chain fatty acids (SCFA), a portion of the samples was diluted 5× in phosphate buffered saline and was homogenised. An amount of 180 µl of homogenised faeces was mixed with 20 µl of 10% $HClO_4$ and incubated at 4 °C overnight. The samples were centrifuged for 5 min at 14,000×*g* and filtrated with Centricut W-MO MF (0.45 µm; Kurabo, Tokyo, Japan), according to the manufacturer's protocol. The concentration of SCFA was determined by the high performance liquid chromatography system equipped with 432 electro-conductivity detectors (Waters, Milford, MA, USA) and a

Rspak KC-811 column (Showa Denko KK, Tokyo, Japan), as previously described (Matsumoto *et al.*, 2006).

Statistical analysis

Statistical analyses of the data were performed using the open source package R, version 3.1.0 (R Core Team, 2014). A Wilcoxon rank sum test was used to detect differences in the abundance of bacterial taxon, pH, concentration of SCFA, and faecal frequency. Differences in the number of categorical data between the groups were evaluated by Chi-square test. The number of volunteers was enumerated to be >25 by the R package (power.anova.test command) with a power of 0.8 and a significance level of 0.05.

3. Results

Subjects

We randomised and enrolled 35 infants into the study. Eighteen were allocated to the control group and 17 to the GOS group (Figure 1). Five infants withdrew because of the introduction of probiotics, change in feeding, or a parental decision. Thus, 14 infants in the control and 16 in the GOS group successfully completed the study. At enrolment, there were no significant differences between the two groups in terms of birth weight, gestational age, sex, and age at onset (Supplementary Table S1). However, there were more infants delivered via Caesarean section in the GOS group than in the control (P=0.014). Adverse side effects related to the consumption of the test formula, such as regurgitations, gassiness, or rejections, were not reported.

16S metagenome analysis overview

To characterise the microbiota of the 60 faecal samples from 30 infants, we performed multiplex pyrosequencing of the V1 and V2 variable regions of the 16S rRNA gene with a 454 GS Junior system (16S metagenome). We generated a dataset consisting of 139,393 filtered, high-quality 16S rRNA gene sequences with a mean average (± standard deviation) of 2,323±777 sequences per sample (Supplementary Table S6). On average, 92±16% of the sequences belonged to the 7 most populated bacterial families, namely *Bifidobacteriaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Streptococcaceae*, *Veillonellaceae*, *Bacteroidaceae*, and *Staphylococcaceae*. *Bifidobacteriaceae* was the predominant bacterial lineage in many infants (found in 22 out of 30 [73%]; abundance, 60±41% on average). However, the lineage was not found in some infants (8 out of 30 [27%]). Results of the 16S metagenome analysis at onset in both feeding groups are shown in Supplementary Tables S5 and S6. Before the intervention, there were significantly more infants with *Bifidobacteriaceae* in their gut in the control group than in the GOS group (93 vs 56%, P=0.039).

Effects of the test formula with galacto-oligosaccharides

The main objective of this study was to evaluate the effects of GOS on the growth of indigenous bifidobacteria. There were no bifidobacteria at onset, GOS may have no effect on the growth of the bacterial lineage. Therefore, we decided to eliminate 8 infants whose bifidobacteria were below the detection limit at onset from the final evaluation before breaking the blinding (Figure 1, Table 2).

Table 3 summarises the change in the gut microbiota of the target infants (13 in the control and 9 in the GOS group; total 22) at the trial onset and 2 weeks after. The GOS group showed an increase in *Bifidobacteriaceae* abundance (+11.6±24.1 in the GOS group vs -3.9±13.0 in the control; P=0.043) (Supplementary Figure S1). *Streptococcaceae* tended to decrease in the GOS group compared to the control (-0.8±1.9 vs +1.8±5.3; P=0.071). The Shannon index, which accounts for both abundance and evenness of the species present, was significantly lower in the GOS group than in the control (-0.1±0.4 vs +0.4±0.4; P=0.014). The Chao 1 index represents a species' richness, whereas the phylogenetic diversity (PD) index represents the phylogenic diversity of the microbiota, and these tended to be lower in the GOS group than in the control group. The effects of GOS were also evaluated on a species level, but no significant change was observed in bifidobacteria (Supplementary Table S7 and Figure S2). Results of the real-time PCR confirmed the increase of *Bifidobacterium* in the GOS feeding group (log 0.4±0.9 vs -0.1±0.3; P=0.022), whereas there were no significant differences observed in the other genera (Table 4).

The effects of the GOS on the faecal SCFA, pH, and frequency of stool were also assessed, and the results are summarised in Supplementary Figure S3 and Table S9. No significant change was observed in the control or GOS feeding groups.

4. Discussion

The current study demonstrated that infant milk formula with 0.3 g/dl GOS (OM55N) stimulates the growth of indigenous *Bifidobacteriaceae* and results in reduced α-diversity (Shannon index) of the infants' gut microbiota.

To date, the effects of GOS on infant gut microbiota have been investigated using conventional cultivation techniques (Ben *et al.*, 2008; Moro *et al.*, 2002). Fluorescence in situ hybridisation or quantitative PCR with targeted 16S rRNA gene-specific primers/probes have also been employed in some studies (Knol *et al.*, 2005; Sierra *et al.*, 2015). However, these techniques allow the identification of a limited number of bacterial lineages. In the present study, the effects of GOS supplement were evaluated using an integrated molecular approach based on a high throughput

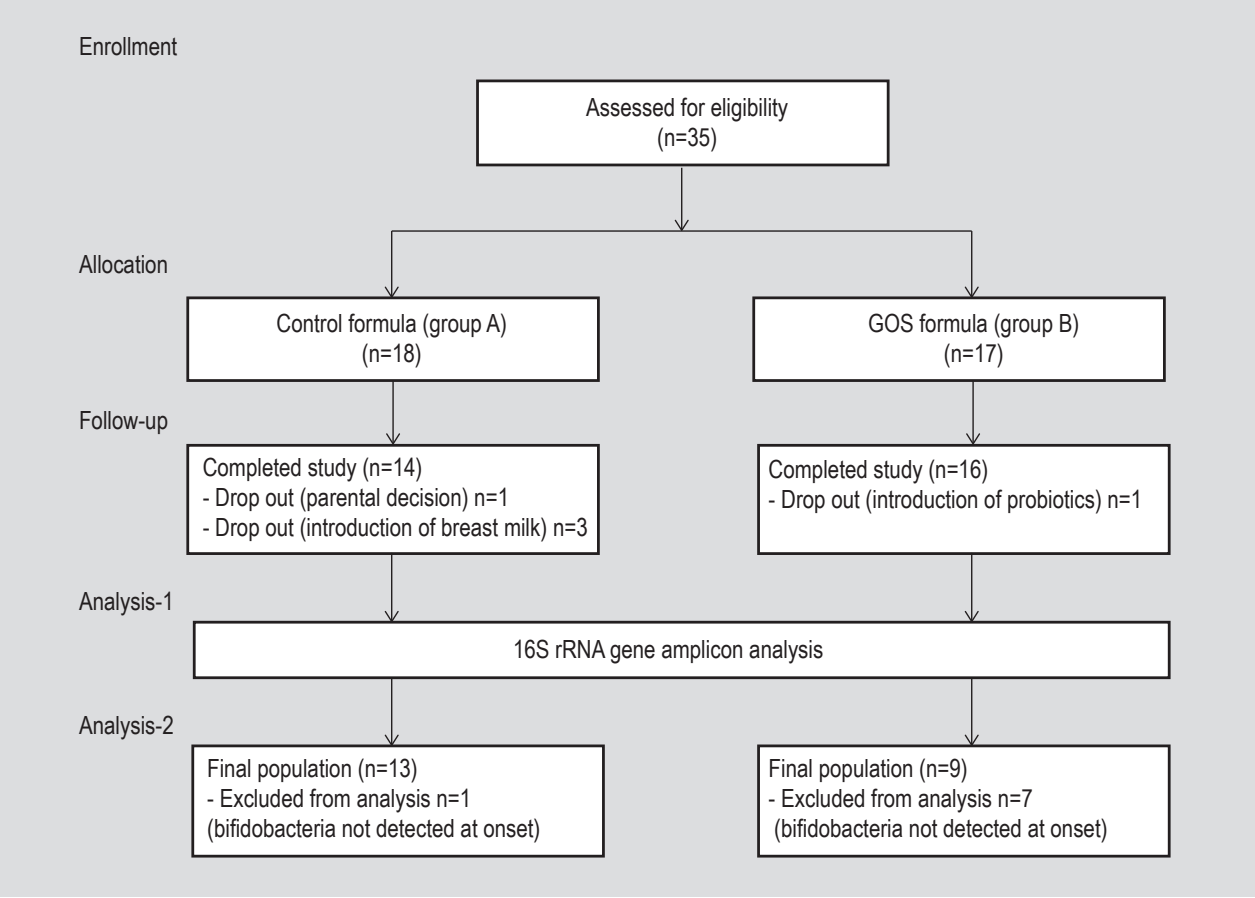

Figure 1. Flow chart of infants enrolled in the study and the disposition of subjects throughout the study.

Table 2. Summary of the baseline characteristics of the infants in terms of evaluating the effects of the galacto-oligosaccharides (GOS).

	Control group (n=13)	OM55N group (n=9)	*P*-value[1]
Sex (male/female)	8/5	6/3	1.000
Delivery (vaginal/Caesarean)	11/2	4/5	0.074
Sibling (+/-)	11/2	5/4	0.178
Birth weight (g)	3,108±380	3,036±461	0.696
Gestation (weeks)	39±1	39±2	0.530
Feeding started (day)	42±6	43±7	0.693

[1] Differences between the control and GOS groups at trial onset were determined using the Wilcoxon rank sum or Pearson chi-square tests.

of 16S rRNA-based techniques (16S metagenome analysis). The new DNA sequencing technology, which was firstly used to assess the effects of prebiotics in infant formula, allowed the evaluation of the abundance of change in all bacteria present in the microbiota at different taxonomic levels. In addition, the technique allows one to assess the change in diversity of the microbial community. Currently, α-diversity indexes, such as the Chao 1, Shannon index, the observation of species/OTUs, and PD have been widely used. In this study, we found that the Shannon index was significantly decreased with GOS supplementation. It has been reported that gut microbiota of breastfed infants showed a lower diversity than that of formula-fed infants (Scholtens *et al.*, 2006). Therefore, the supplementation of GOS (OM55N) was able to induce microbiota more similar to that of breast-fed infant. It is necessary to investigate its physiological significance further.

In general, the gut microbiota of infants mainly contains bifidobacteria at the age of 1 month; however, some studies

Table 3. Change in the infant gut microbiota using the test formula (16S metagenome analysis).

	Control group (n=13)		OM55N group (n=9)		*P*-value[1]	
	Onset week	2 weeks after	Onset week	2 weeks after	Control vs GOS at onset	Change from onset to 2 weeks after
Abundance (%)						
Bifidobacteriaceae	83.1±12.7	79.2±16.2	78.8±30.0	90.4±6.9	0.695	0.043
Enterobacteriaceae	4.5±5.2	6.1±7.3	8.9±19.5	2.9±3.3	0.709	0.186
Enterococcaceae	2.5±4.9	4.3±6.4	2.3±2.9	2.4±3.3	0.844	0.216
Streptococcaceae	2.1±3.1	3.9±5.9	2.3±2.6	1.5±1.5	0.744	0.071
Veillonellaceae	2.1±5.0	0.7±1.1	2.6±4.4	0.9±1.1	0.215	0.707
Bacteroidaceae	1.6±2.8	1.8±2.7	2.4±6.5	0.2±0.5	0.324	0.486
Staphylococcaceae	0.4±0.8	0.5±1.3	0.3±b0.5	0.2±0.3	1.000	0.555
Alpha-diversity						
Chao1 index	30.8±8.3	32.4±9.0	27.2±8.9	25.1±11.0	0.209	0.096
Phylogenetic diversity	5.2±1.4	5.4±1.6	5.5±1.6	5.0±1.3	1.000	0.082
Shannon index	2.1±0.8	2.5±0.6	1.7±0.5	1.6±0.6	0.262	0.014

[1] Differences between the control and galacto-oligosaccharides (GOS) (OM55N) group were determined using the Wilcoxon rank sum test.

Table 4. Changes in the infant gut microbiota using the test formula (real-time PCR analysis with group-specific primers).[1]

	Control group (n=13)		OM55N group (n=9)		*P*-value[2]	
	Onset week	2 weeks after	Onset week	2 weeks after	Control vs GOS at onset	Change from onset to 2 weeks after
qPCR						
Bifidobacterium	9.7±0.2	9.6±0.4	9.4±1.1	9.8±0.3	0.896	0.022
Enterobacteriaceae	8.3±1.0	8.7±0.4	8.4±1.2	8.5±0.7	0.794	0.384
Enterococcus	8.0±2.0	8.6±1.5	8.8±1.5	8.8±1.7	0.470	0.403
Streptococcus	9.3±0.7	9.5±0.5	9.2±0.6	9.2±0.7	0.324	0.242
Veillonella	7.1±1.5	6.7±1.4	7.9±0.9	7.7±0.7	0.234	0.744
Bacillus fragilis group	6.8±1.7	6.9±1.6	6.2±1.7	5.9±1.4	0.549	0.588
Staphylococcus	7.2±0.9	6.9±1.0	7.1±0.9	6.8±0.8	0.948	0.431

[1] The population of each bacterial taxon was expressed in log bacteria per g of faeces.
[2] Differences between the control and galacto-oligosaccharide (GOS) (OM55N) groups were determined using the Wilcoxon rank sum test.

did not detect bifidobacteria in limited populations (e.g. 12% in the report by Tsuji *et al.*, 2012). In this study, bifidobacteria were not detected in 8 out of 30 infants (27%). One reason for this may be that this study included more infants delivered via Caesarean section than in the previous studies. Many studies have demonstrated that Caesarean-section delivered infants showed a lower population of bifidobacteria compared with vaginally delivered infants (Penders *et al.*, 2006). The difference of microbiota between the vaginally and Caesarean-section delivered infants in this study are shown in Supplementary Table S10.

In this intervention, Caesarean-section infants tend to be assigned to the GOS-feeding group, although there was no significant difference between the evaluated groups (P=0.074, Table 2). However, the biased assignment of Caesarean-section infants was observed in original group (P=0.014, Supplementary Table S1), which might have affected the outcome of the study. However, it should be mentioned that four Caesarean-section infants who did not harbour detectable levels of *Bifidobacteriaceae* before the trial show this group of bacteria in their faeces after two weeks GOS supplementation. Even though these infants

were excluded from analysis, this strengthens our notion that the GOS stimulate and/or facilitate the colonisation of *Bifidobacteriaceae*.

Human milk oligosaccharides (HMOs) are a natural example of prebiotics. HMOs are composed of >130 structurally distinct oligosaccharides (Castanys-Munoz *et al.*, 2013; Sela and Mills, 2010; Wada *et al.*, 2008). Lacto-N-tetraose and fucosyllactose were identified as abundant HMO components, and galactosyl-lactose is also found in human breast milk (Sumiyoshi *et al.*, 2004). GOS are a mixture of oligosaccharides that are produced by the reaction of β-galactosidase in lactose. Dependent on the trans-galactosylation activity of the β-galactosidase used, the profile of the oligosaccharide polymers and the chemical bonds between the saccharide units are different among the products (Supplementary Table S3). OM55N was produced by catalysing lactose with oligosaccharide polymers, *Sporobolomyces singularis*, and *Kluyveromyces lactis*, and its main component is 4'-galactosyllactose (4'-GL) (Kaneko *et al.*, 2014). Many other GOS on the market have been produced using β-galactosidase delivered from *Bacillus circulans* with a main component of 6'-galactosyllactose (6'-GL) (Kaneko *et al.*, 2014). It has been reported that the oligosaccharides of OM55N were selectively utilised by bifidobacteria *in vitro* and in adult gut microbiota (Matsumoto *et al.*, 2004). In this study, the effects of OM55N were evaluated and confirmed in infant gut microbiota.

With regard to the safety of the GOS (OM55N), no adverse side effects were reported in this study. OM55N is used in various food products and has been approved by the Generally Regarded as Safe Notice Inventory of the United States Food and Drug Administration (GRAS Notice No. GRN 334), and there are many reports on the safety of the product (Kobayash *et al.*, 2013; Kobayashi *et al.*, 2009, 2013). A recent study also showed that OM55N includes less allergens and is regarded as a hypoallergenic and safe GOS product (Kaneko *et al.*, 2014).

Bifidogenic effects of the GOS have been evaluated in various concentrations. The effects of a mixture of GOS and fructo-oligosaccharides in a 9:1 ratio have been evaluated to date using 0.8 g/dl (Knol *et al.*, 2005), 0.4 g/dl (Moro *et al.*, 2002), and 0.23 g/dl concentrations (Moro *et al.*, 2002). Most of these studies found that the prebiotics showed an increase population and/or abundance of bifidobacteria. More recently, Sierra *et al.* (2015) reported that the bifidobacterial population increases significantly in infants following infant formula with 0.44 g/dl of GOS. In this study, we found that infant formula with only 0.3 g/dl of GOS stimulated the growth of bifidobacteria and resulted in a decreased α-diversity of infant gut microbiota. On the other hand, no significant change in intestinal acidity, SCFA concentration, and the frequency of stool were observed. This may be due to the lower concentration of GOS and the limited number of infants enrolled in the study compared to the other trials. Therefore, the effect of OM55N on the pH, faecal concentration of SCFA, and stool frequency should be further evaluated in a larger study with more appropriate design and additional supplementation of GOS.

In summary, the supplementation of a formula in term infants with GOS (OM55N) stimulated the growth of bifidobacteria and resulted in reduced α-diversity of the microbiota.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2015.0168.

Figure S1. Change in *Bifidobacteriaceae* abundance at onset and at 2 weeks after the intervention with the test formula.

Figure S2. Heat map analysis of the *Bifidobacterium* species.

Figure S3. Faecal frequency.

Table S1. Summary of the baseline characteristics of the infants who completed the study.

Table S2. Baseline characteristics of all the enrolled infants.

Table S3. The sugar composition of OM55N (Oligomate® 55N).

Table S4. The genus- and group-specific primers used in this study.

Table S5. The 16S rRNA gene amplicon analysis of the gut microbiota in all infants at the trial onset.

Table S6. The 16S rRNA gene amplicon analysis data of all samples in this study (a family level analysis).

Table S7. Summary of the 16S rRNA gene amplicon analysis data for the *Bifidobacterium* species.

Table S8. Real-time PCR data of all samples in the study.

Table S9. The faecal pH and organic acid concentrations of all samples in this study.

Table S10. Comparison of gut microbiota between infants delivered vaginally and via Caesarean section.

Conflicts of interest

This study was designed and funded by Yakult Central Institute.

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