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Colon Microflora in Infants Fed Formula with Galacto- and Fructo-Oligosaccharides: More Like Breast-Fed Infants

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ABSTRACT

Objectives: The intestinal flora of breast-fed infants is generally dominated by Bifidobacteria. We aimed to investigate whether an infant formula supplemented with galacto-oligosaccharides and fructo-oligosaccharides (GOS/FOS) is able to establish a bifido-dominant microflora, not only in numbers but also with respect to the metabolic activity in the colon.

Methods: Two groups of infants fed infant formula with 0.8 g/100 ml GOS/FOS in a ratio of 9:1 (OSF group), or control formula (SF group) were evaluated in a randomised, double blind, placebo controlled intervention study. A breast-fed group was studied in parallel. At study onset and after 4 and 6 weeks, faecal samples were examined for the number of bifidobacteria, pH, short chain fatty acids and lactate.

Results: After 6 weeks, the mean proportion of bifidobacteria was significantly higher in the OSF group (59.6% versus 49.5% in the SF group; $P < 0.05$). Compared with controls, infants in the OSF group had a lower stool mean pH and an increased proportion of acetate and a decreased proportion of propionate. The mean pH in the OSF and SF groups were 5.7 and 6.3, respectively ($P < 0.001$).

Conclusions: The addition of the prebiotic GOS/FOS mixture to an infant formula has a stimulating effect on the growth of bifidobacteria and on the metabolic activity of the total intestinal flora. The changes in short chain fatty acids, lactate and pH in the prebiotic group represent a fermentation profile that is closer to that observed in breast-fed infants compared to infants fed control formula. ***JPGN 40:36–42, 2005.*** **Key Words:** Infant-formula—Prebiotics—Intestinal flora—Short chain fatty acids—Bifidobacteria. © 2005 Lippincott Williams & Wilkins

INTRODUCTION

At birth the gut is sterile. Microbial colonization starts as soon as the infant is exposed to the environment. During the first months of life diet has a significant influence on the development of the intestinal flora (1,2). The developing indigenous microflora serves as an important defense mechanism that protects the infant against colonization by invading pathogenic microorganisms, also called colonization resistance (3,4).

Several studies have examined the bacterial flora of breast-fed and bottle-fed infants using conventional plating techniques and molecular techniques. These studies have shown that the large bowel flora of breast-fed infants is generally dominated by bifidobacteria and lactic acid bacteria (5–7). The flora of formula-fed infants is more diverse, less stable and often contains more *Bacteroides*, *Clostridium* and *Enterobacteriaceae* (5–9). In formula fed infants, bifidobacteria have also been demonstrated to be a numerically important species, but they generally occur in lower numbers than in breast-fed infants of the same age (10).

Breast milk (11), contains a high level of complex oligosaccharides (10–12 g/l) which function as natural prebiotics and promote the growth of bifidobacteria (12). Despite great efforts, it has not been possible to modify the flora of formula-fed infants to be like that of breast-fed infants. Recently, however, the bifidogenic effect of a prebiotic mixture of 90% galacto-oligosaccharides (GOS) and 10% fructo-oligosaccharides (FOS), chosen to mimic the molecular size distribution of human milk oligosaccharides has been demonstrated (8,9,13–15). Although structurally not identical with human milk oligosaccharides, the GOS/FOS mixture also appears to have a bifidogenic effect.

An important activity of the bacterial microflora is the fermentation of non-digestible carbohydrates in

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the colon. Fermentation results in the production of short chain fatty acids (SCFA), lactate, and the gases hydrogen, carbon dioxide and methane. SCFA's are rapidly absorbed by the colon, salvaging energy which would otherwise be lost through excretion in faeces (10,16,17). SCFA's also play a significant role in stimulating water and sodium absorption. Other functional properties claimed for SCFA's in humans include the prevention of diarrhoea, lowering colonic pH, prevention of overgrowth by potentially pathogenic microorganisms and protection against colonic carcinogenesis by reducing the bioavailability of toxic amines (16,18,19). Although the production of specific SCFA's is not limited to one bacterial species, and different species produce similar SCFA's, the SCFA pattern in faeces reflects the metabolic activity in the colon. Changes in the SCFA pattern often reflect changes in the bacterial composition of the colon. Several studies have shown major differences in the profile of SCFA in faeces of breast-fed infants compared to formula-fed infants. Faeces of breast-fed infants contain mainly acetic and lactic acid and little or no propionic acid or butyric acid, whereas faeces of formula-fed infants mainly contain acetic- and propionic acids with small amounts of butyric acid (10,20–25).

In this study we investigated whether an infant formula supplemented with GOS/FOS was associated with a bifido-dominant flora, not only in bacterial numbers but also with respect to the metabolic activity of the colonic microbiota, as measured by faecal pH, short chain fatty acid profile and and lactate.

MATERIALS AND METHODS

Study Design

The study was a randomised, double blind, placebo controlled intervention study with two intervention groups. Fully formula-fed infants, aged 7 to 8 weeks, were recruited by four paediatric practitioners in Lubbecke, Detmold, Hamburg and Espelkamp (Germany). Infants were included in the study if they had a birth weight between 2600 and 4500 g, and were fully formula-fed for at least 4 weeks before the start of the intervention. Infants with congenital abnormalities, or with proven or suspected cow's milk allergy, infants of multiple gestations, infants who had received antibiotics less than 2 weeks before the start of the study and infants who were fed any infant formula containing pro- or prebiotics less than a month before the start of the study were excluded. Eligible infants were randomly allocated to one of two infant formula groups: a group receiving a formula (Aptamil, Milupa Germany) supplemented with 0.8 g/100 ml galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) in a ratio of 9:1 (oligosaccharide formula, OSF-group) and a standard formula group receiving the same infant formula without prebiotics (standard formula, SF-group). The macronutrient composition of the formulas is shown in Table 1. A group of breast-fed infants of the same age was included as a reference group (BF-group). Faecal samples were collected as freshly as possible within 3 days of the start of the intervention, at 4 weeks and at the end of the study period (6 wks). The samples were immediately frozen at −20°C until evaluation. During the study, information on the number of stools, stool-characteristics, and general well being was recorded on days 0, 28 and 42. Stool consistency was recorded on a five-point scale (1 = watery, 2 = soft pudding-like, 3 = soft formed, 4 = dry formed and 5 = dry hard pellets). Other stool characteristics and general well being (cramps, nausea, flatulence) were recorded on four-point scales (1 = none, 2 = mild, 3 = moderate, 4 = severe). The study was approved by the local medical ethical committee and written informed consent was obtained from the parents before the start of the study.

TABLE 1. *Macronutrient composition of the study formulas (per 100 ml ready to use formula)*

	Oligosaccharide-supplemented formula	Standard formula
Energy (kcal)	72	72
Protein (g)	1.5	1.5
Carbohydrate (g)	8.5	8.5
Lactose (g)	7.5	7.5
Starch (g)	1	1
Non-digestible oligosaccharides (g)	0.8	0
Galacto-oligosaccharides (g)	0.72	0
Fructo-oligosaccharides (g)	0.08	0
Fat (g)	3.6	3.6

Preparation of faecal samples

For determination of SCFA, samples were thawed in ice water, diluted 10x (w/v) in MilliQ and homogenised for 10 minutes using a stomacher (IUL Instruments, Barcelona, Spain). Three-hundred fifty μl homogenised faeces were mixed with 200 μl 5% (v/v) formic acid, 100 μl 1.25 g/l 2-ethylbutyric acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 350 μl MilliQ. Samples were centrifuged for 5 minutes at 14,000 rpm to remove large particles and the supernatant was stored at −20°C.

For FISH analysis, DNA isolation and lactic acid measurements, samples were thawed in ice water, diluted 10x (w/v) in phosphate buffered saline, pH 7.4 (PBS) and homogenised for 10 minutes using a stomacher. The homogenised faeces were stored at –20°C. For FISH analysis 1 ml of homogenised faecal suspension was fixed in 3 ml freshly prepared 4% (w/v) paraformaldehyde in PBS and incubated overnight at 4°C.

Fluorescent *in situ* hybridisation (FISH)

Fixed samples were thawed and applied to gelatine-coated glass slides (PTFE coated 8-wells {1 cm^2/well} object slides, CBN labsuppliers, Drachten, the Netherlands) and air-dried. Fluorescent enumeration was performed essentially as described previously (5) and samples were hybridised with 10 ng/μl *Bifidobacterium* specific probe Bif164 (26). For staining all bacteria the samples were incubated with 0.25 ng/μl 4',6-diamidino-2-phenylindole (DAPI). After staining, slides were washed, dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA) and covered with a coverslip. The

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slides were automatically analysed using an Olympus AX70 epifluorescence microscope with image analysis software (Analysis 3.2, Soft Imaging Systems GmbH, Münster, Germany). The percentage of bifidobacteria per sample was determined by analysing 25 randomly chosen microscopic positions. At each position the percentage of bifidobacteria was determined by counting all cells with a DAPI filter set (SP100, Chroma Technology Corp., Brattleboro, VT) and counting all bifidobacteria using a Cy3 filter set (41007, Chroma Technology Corp., Brattleboro, VT).

Short chain fatty acids

Acetic, propionic, *n*-butyric, iso-butyric and *n*-valeric acids were quantitatively determined by a Varian 3800 gas chromatograph (GC) (Varian, Inc., Walnut Creek, CA) equipped with a flame ionization detector. 0.5 μl of the sample was injected at 80°C in the column (Stabilwax, 15 m x 0.53 mm, film thickness 1.00 μm, Restek Co.) using helium as carrier gas (3.0 psi). New columns were conditioned overnight at 200°C. After injection of the sample, the oven was heated to 160°C at a speed of 16°C/min, followed by heating to 220°C at a speed of 20°C/min and finally maintained at a temperature of 220°C for 1.5 minutes. The temperature of the injector and the detector was 200°C. After every ten samples the column was cleared by injection of 0.5 μl 1% (v/v) formic acid to avoid memory effects of the column, followed by injection of 0.5 μl standard SCFA mix (1.77 mM acetic acid, 1.15 mM propionic acid, 0.72 mM *n*-butyric acid, 0.72 mM iso-butyric acid, 0.62 mM *n*-valeric acid obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands) to monitor the occurrence of memory effects. The levels of SCFA were determined using 2-ethylbutyric acid as an internal standard.

Lactic Acid

Homogenised faeces were thawed on ice and centrifuged for 5 minutes at 14,000 rpm. One-hundred μl supernatant was heated for 10 minutes at 100°C to inactivate all enzymes. Concentrations of L-lactic acid and D-lactic acid were determined enzymatically using a L-lactic acid detection kit and D-lactate-dehydrogenase (Boehringer Mannheim, Mannheim, Germany).

pH Measurements

After storage at −20°C, faecal samples were thawed and the pH was measured directly at room temperature using a Handylab pH meter (Schott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler-Toledo, Columbo).

Statistical Analysis

The sample size was based on the described concentration of bifidobacteria in faecal samples (5). With a sample size of at least 13 infants per group, it is possible to detect a mean difference of 30% in bifidobacteria, with a probability of 80% and a significance level of 0.05. Normal distribution was determined by visual inspection of normal probability plots and tested with the Shapiro-Wilk test of normality. Logarithmic transformations were used for variables that did not show a normal distribution. Because of the absence of true baseline values and a large variation around the pre-specified sample time points between the subjects, differences between the two treatment groups were calculated with a general linear model, assuming a linear relation between the subjects and the sample moments. In the model, the variation in sample moments was entered as a covariate. Also, the effects of the type of delivery (caesarean or vaginal) and the use of breast milk in the first few months of life (yes or no) were entered as covariables in the model. Estimated marginal means were used to calculate the estimated values on day 42 for all variables. The results of the percentage of bifidobacteria, faecal pH, short-chain fatty acids and lactate were expressed as estimated mean values after 6 weeks of intervention (day 42), calculated from the general linear model. Differences between the groups were calculated with pairwise comparisons from the model. The breast-fed group has not been included in the significance tests, since it is impossible to randomly assign infants to this feeding group and a selection bias may occur due to social and educational differences between breast- and formula-fed infants. Geometric estimated means are given for variables that have been transformed in the model because of a non-normal distribution. Data on stool characteristics are presented as medians. Statistical analyses were performed with the statistical package SPSS version 11.0.1 (SPSS Inc., Chicago, IL) (27).

RESULTS

Study Population

Sixty-eight infants were entered in the study. 60 infants completed the study and 8 dropped out. After excluding subjects who did not meet inclusion criteria, 53 infants were included in the final analyses. A flowchart of the study population is given in Figure 1. Mean age of all infants at includion was 7.7 weeks and the mean period of formula-feeding before the start of the intervention was 5.5 weeks (SD = 2.4 wks). The baseline characteristics of all infants are shown in Table 2.

Bifidobacteria

Figure 2 shows the proportion of faecal bifidobacteria, expressed as a percentage of the total number of bacteria in faeces of the two study groups 6 weeks after the start of the intervention. Baseline values for the percentage bifidobacteria in the OSF group and the SF group were 45.2% and 47.3%, respectively (without correction for the model). After 6 weeks, the percentage of bifidobacteria was significantly higher ($P = 0.046$) in the OSF group compared to the SF group (59.6% and 49.5%, respectively, with a SE of the difference of 4.1%). The difference in absolute numbers of bifidobacteria did not reach statistical significance (geometric means of 5.3×10^9 and 4.0×10^9 cells per gram wet weight faeces for the OSF group and the SF group, respectively; $P = 0.44$).

pH

Faecal pH of infants in the OSF group was significantly lower than that of the SF group (5.7 and 6.3, respectively, with a SE of the difference of 0.1,

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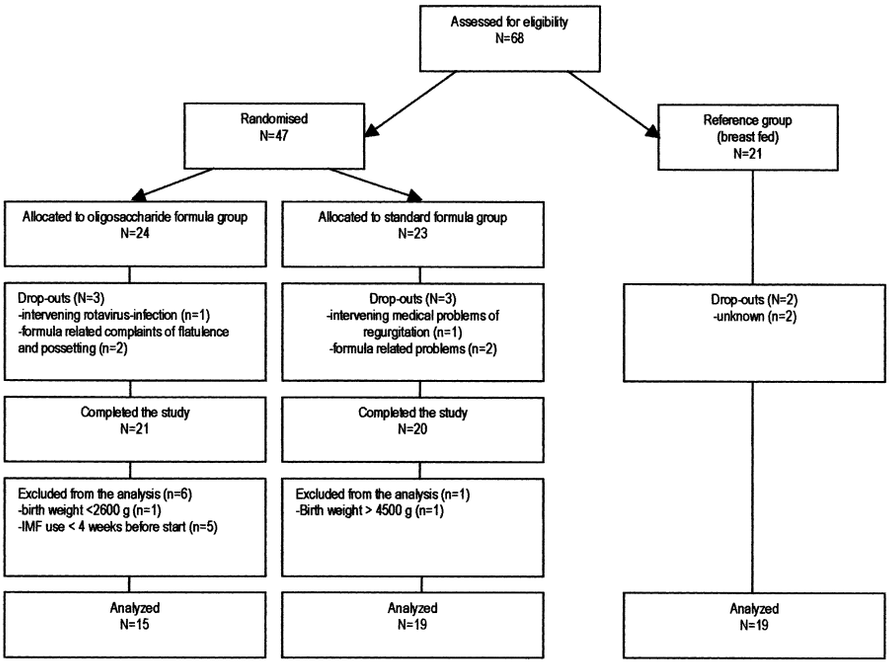

FIG. 1. Randomisation and eligibility chart.

$P < 0.001$). The pH of faeces of the breast-fed reference group was 5.8. Baseline pH values were 6.1 and 6.3 for the OSF group and the SF group, respectively (without correction for the model). The results are shown in Figure 3.

Short Chain Fatty Acids

The calculated mean percentages of acetate, propionate and butyrate in the stools of the two study groups and the breast-fed group are shown in Figure 4. The percentages are the percent of total SCFA accounted for by acetate, propionate, butyrate, isovalerate and isobutyrate. The main SCFA found in the stools of all infants was acetate, and this fatty acid was present in a significantly higher proportion in the OSF group (85.2%) than in the SF group (77.2%) ($P < 0.001$, SE of the difference = 1.7%). In the breast-fed group, the level of acetate was 89.9%. The proportion of propionate was significantly lower in the stools of the OSF group, with levels of 12.0% and 17.8% in the OSF and SF groups, respectively ($P < 0.001$, SE of the difference = 1.3). The proportion of butyrate was relatively low and the difference between the groups was not statistically significant. The branched chain short-chain fatty acids isovalerate and isobutyrate were also not significantly different between the groups and were present in small amounts (<1%).

D- and L-Lactate

Lactate was produced in large amounts by all infants and the highest levels occurred in the OSF group (with a geometric mean of 22.2 mmol/kg wet weight faeces in the OSF group, and 5.2 mmol/kg wet weight faeces in the SF group) after 6 weeks intervention. This difference was statistically significant ($P < 0.001$). In the breast-fed group the geometric mean lactate after 6 weeks was 14.2 mmol/kg wet weight faeces. The proportion of L-lactate, expressed as a percentage of the total lactate was significantly higher in the OSF group compared to the SF group (geometric means of 59.8% and 34.0%, respectively, $P = 0.001$). The geometric mean proportion L-lactate in the BF group was 48.2%.

Stool Characteristics

The median stool frequency in the OSF group increased slightly with time (with 1, 1 and 2 stools on

TABLE 2. *Baseline characteristics*

	OSF group (n = 15)	SF group (n = 19)	BF group (n = 19)
Gender (n male/n female)	8/7	10/9	12/7
Birth weight (g ± sd)	3482 ± 407	3561 ± 430	3617 ± 417
Age at enrollment (d ± sd)	57 ± 21	54 ± 22	52 ± 21
Use of breast milk before the study (y/n)	9/6	11/8	19/0
Type of delivery (n caesarian/n vaginal)	4/11	4/15	2/16

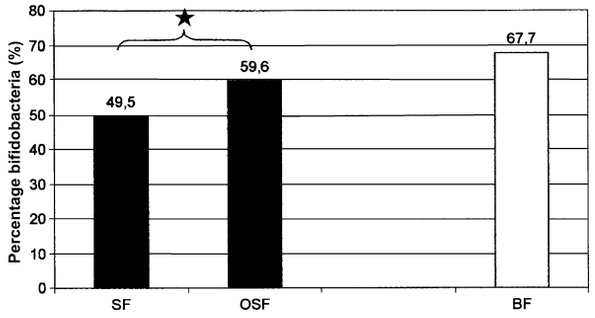

FIG. 2. Percentage of faecal bifidobacteria after six weeks intervention. The percentage bifidobacteria represents the absolute number of bifidobacteria per g wet weight faeces to the total number of bacteria per g wet weight faeces. Baseline percentages were 45.2% and 47.3% for the OSF group and the SF group respectively without correction for the model. The difference between the OSF group and the SF group is statistically significant ($P = 0.046$), with a SE of the difference of 4.1%.

days 0, 28 and 42, respectively). In the SF group, there was a slight decrease with time, with a median frequency on days 0, 28 and 42 of 2, 1 and 1, respectively. The median stool consistency for the SF and OSF groups was mushy/soft and this remained constant in both study groups throughout the study. Symptoms of flatulence and possetting were reported mild in all study groups during the study.

DISCUSSION

The current study demonstrates that an infant milk formula with a mixture of GOS/FOS in a 9:1 ratio was able to selectively stimulate the growth of bifidobacteria with associated changes in faecal pH and SCFA content in fully formula-fed infants aged 4 to 12 weeks. Although the GOS/FOS mixture has previously been shown to stimulate the growth of bifidobacteria in term and preterm infants (13–15), this study is the first

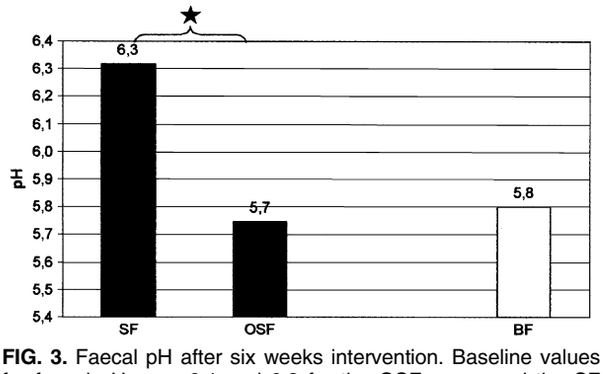

FIG. 3. Faecal pH after six weeks intervention. Baseline values for faecal pH were 6.1 and 6.3 for the OSF group and the SF group respectively. The difference between the OSF group and the SF group is statistically significant ($P < 0.001$), with a SE of the difference of 0.1.

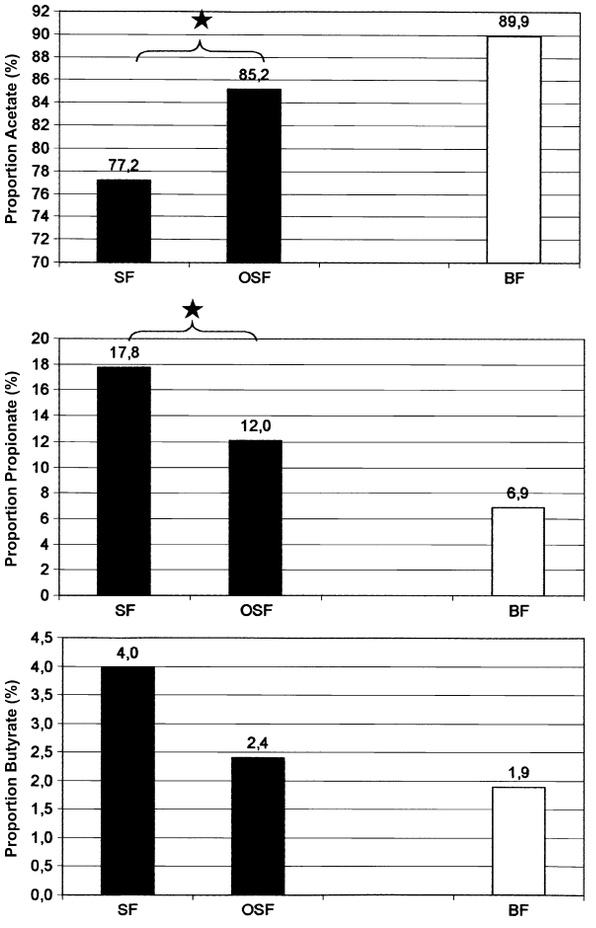

FIG. 4. Proportions of faecal short chain fatty acids after six weeks intervention. The proportions represent the absolute amount of each SCFA to the total amount of analyzed SCFA's (acetate, butyrate, propionate and, isovalerate and isobuterate). The difference between the OSF group and the SF group is significant for the ratio acetate ($P < 0.001$) and the ratio propionate ($P < 0.001$), with a standard error of the difference of 1.7% and 1.3% respectively.

to demonstrate this bifidogenic effect and an associated change metabolic activity in infants with an already established microflora associated with conventional milk formula.

Bacterial fermentation of non-digestible carbohydrates and proteins in the colon results in the production of short chain fatty acids and lactate and a decrease of the luminal pH. In the current study, faecal acids were analysed and the results showed that acetic acid was the main SCFA produced accounting for about 85% (OSF) and 77% (SF) of the total SCFA's. Compared to the SF group, the corresponding percentages of propionic acid was lower in the OSF group. The SCFA pattern of infants in the OSF group, approximates the pattern of the SCFA's of infants in the BF group. The results of the infants fed a standard formula or breast milk are consistent with

findings in other studies, in which high levels of acetate, and low levels of propionate and butyrate are observed in breast-fed infants, and high levels of both acetate and propionate are found in infants fed regular infant formulas (20–25). The difference between the SCFA profile in infants fed the prebiotic mixture, and infants fed the standard formula, indicates that the composition of the microflora differs between the two groups. This is consistent with the finding of a higher percentage bifidobacteria in the OSF group compared to the SF group. The production of acetic acid and lactic acid has been attributed to bifidobacteria and lactic acid bacteria in the colon of breast-fed infants (22,24,28,29). Higher levels of propionate in faeces of infants in the SF group may indicate the presence of a more complex microbiota, since propionate (and butyrate) are, amongst others, produced by bacteria belonging to the *Bacteroides* and *Clostridium* genera, and not by Bifidobacteria (23,29–31). Acids like propionate and butyrate are commonly found in adults and the fermentation profile in the SF group indicates a more adult-like colonic microflora. Only minor amounts of the branched chain SCFA's isovalerate and isobutyrate were detected in either formula group or breast-fed group. Since these branched chain fatty acids are derived from protein fermentation, these results indicate that protein degradation in the large bowel by other proteolytic groups of bacteria did not contribute significantly to the final SCFA patterns (32).

The stools of breast-fed infants generally also have a lower pH than formula-fed infants (7,21,23,33). In a study performed by Kleessen et al., breast-fed infants at 3 months had a faecal pH of 5.8, whereas infants fed two different types of infant formula had a faecal pH of approximately 7.2 (9). After 6 weeks intervention, the OSF group had a pH of 5.7, which was similar to the pH in the BF group and significantly lower than the value of 6.3 found in the SF group, again indicating a relevant shift in the metabolic activity of the flora in the OSF group.

Compared to the SF group, the lactate content was higher in the OSF group, with a higher percentage of L-lactate compared to D-lactate. Bifidobacteria exclusively produce L-lactate, and the increase of bifidobacteria in the OSF group can explain the shift towards L-lactate in this group (32). The production of SCFA in combination with a lower pH may have an impact on the growth of other groups of bacteria, including potentially pathogenic microorganisms since these are inhibited by weak acids in combination with a low pH. In this respect L-lactate is even more effective in suppressing certain *Escherichia coli* strains than D-lactate (34–36). This shift in metabolic activity will maintain the dominance of bifidobacteria and lactobacilli, since these bacteria are relatively insensitive to these specific conditions. Additionally, it has also been reported that bifidobacteria, and other lactic acid bacteria, secrete specific compounds (e.g., bacteriocins) that directly influence the growth of other colonic bacteria, including pathogens (37). A healthy microflora is very important for the general well-being of an infant since it plays an important role in normal nutrition and metabolism. Hence, it is involved in the protection against the invasion and colonisation by pathogens through competition for adhesion sites, competition for nutrients and the production of antimicrobial products. Furthermore, the potential influence of the intestinal microflora on the development of the immune system in infants and the later appearance of allergies has been highlighted in recent scientific literature (38–41).

In conclusion, this randomised, double blind, placebo controlled intervention study has shown that feeding a formula containing prebiotic GOS/FOS oligosaccharides to formula-fed infants with an established intestinal microflora results in an increased proportion of bifidobacteria in the stools. This modification of the microflora was associated with a reduction in stool pH and a SCFA pattern containing a higher proportion of acetate and a lower proportion of propionate. The changes induced by GOS/FOS bring the intestinal flora and its metabolic activity closer to that of breast-fed infants. It is not clear that the addition of prebiotic oligosaccharides to infant formula will have beneficial effects on the frequency of gastrointestinal infections or atopic diseases.

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