Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants

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Background Oligosaccharides in human milk may protect infants by improving the intestinal micro-flora and fermentation. This study was to investigate effects of infant formula milk consisting of galacto-oligosaccharide (GOS) on intestinal microbial populations and the fermentation characteristics in term infants in comparison with that of human milk.

Methods The test formula (Frisolac H, Friesland, Netherland) was supplemented with GOS at a concentration of 0.24 g/dl. Human milk and another formula without oligosaccharides (Frisolac H, Friesland, Netherland) were used as positive and negative control respectively. Growth, stool characteristics, and side effects of the recruited infants were recorded after 3 and 6 months' follow-up, and the fecal species were collected for the analysis of intestinal micro-flora, short chain fatty acid (SCFA) and pH.

Results At the end of 3- and 6-month feeding period, intestinal *Bifidobacteria* and *Lactobacilli* were significantly increased in infants fed with GOS supplemented formula and human milk when compared with infants fed with negative control formula; however, there was no statistically significant difference between GOS supplemented formula and human milk groups. Stool characteristics were influenced by the supplement and main fecal SCFA (acetic), and stool frequency were significantly increased in infants fed with GOS supplemented formula and human milk, while the fecal pH was significantly decreased as compared with that of negative control ($P<0.05$). Supplementation had no influence on incidence of side effects (including crying, regurgitation and vomiting).

Conclusions Supplementing infant formula with GOS at a concentration of 0.24 g/dl stimulates the growth of *Bifidobacteria* and *Lactobacilli* in the intestine and stool characteristics are similar to in term infants fed with human milk.

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Breast-feeding is a golden standard for infant feeding. However, many infants are being fed in another way, cow's milk-based formula. Amongst multiple differences between human and cow's milk regarding the development of the intestinal flora, the flora of breast-fed infant is richer in *Bifidobacteria* and *Lactobacilli*.[1,2] both of which are known to be potentially beneficial for health of hosts.[3,4] In cow's milk, the absence of oligosaccharides, the third most abundant solid constituent of human milk, is likely to account for differences in intestinal flora.[5,6]

Human milk contains many kinds of oligosaccharide with different molecules.[5,6] Some are linear, others are branched; some are acidic, and others are neutral. Oligosaccharide in human milk may protect infant by acting as receptor homologues, inhibiting binding of

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enteropathogens to host receptors.[7] Neutral oligosaccharide causes inhibition of bacterial adhesion.[8,9] Ongoing research is linking specific carbohydrate structures with protection against specific pathogens. Recently, human milk oligosaccharide has been shown to be resistant to enzymatic digestion in the upper gastrointestinal tract.[10] Nondigestibility and selective fermentation by potentially beneficial bacteria in the colon are prerequisites for oligosaccharide to act as a prebiotic in its modulation of intestinal micro-flora.

Oligosaccharide concentration and composition in breast milk is a dynamic process.[11] The highest amount of oligosaccharide, 2 g/dl milk, should be reached on the fourth day of life. On days 30 and 120 of lactation, there is a decrease of 20% and 40%, respectively, in comparison to day 4. Amount of oligosaccharide in human milk differs at different stage of lactation. Its composition varies among different samples, and many factors combine for the final composition.[12] However, most of the previous studies reported the oligosaccharide in human milk consisting of approximately 60% – 90% galacto-oligosaccharide (GOS) and 10% – 40% fructo-oligosaccharides (FOS) in the first few months of lactation.

The dynamic process of oligosaccharide concentration and composition in breast milk makes it impossible for industry to mimic nature. Even though the composition cannot be mimicked, its effect and function can be imitated. Previous research showed that minimum addition of oligosaccharide mixture 0.4 g/dl to infant formula was effective in improving intestinal micro-flosa,[13-15] and human milk consisting of approximately 60% – 90% GOS[11,12] meant the minimum GOS supplementation 0.24 g/dl be physiological. Therefore, the current study was designed to investigate whether the supplementation of GOS (0.24 g/dl) in infant formula imitates effects and functions of oligosaccharide in human milk regarding intestinal microflora colonization and fermentation in infants.

METHODS

Two hundred and seventy-one term infants were recruited for this study. They were admitted to Nanjing Children's Hospital of Nanjing University (n = 144), Nanjing Maternity Hospital of Nanjing Medical College (n = 62), and Affiliated Hospital of South East University (n = 65). The ethics committees of the three hospitals approved the study, and all written informed consents were available.

Enteral nutrition was started with breast-feeding for all infants. When mothers were not able to or decided not to breast-feed, infants were randomly assigned to one of two formula groups within the first week after birth. The test formula (Frisolac H, Friesland, Netherland) was supplemented with GOS at a concentration of 0.24 g/dl. Another formula without oligosaccharide (Frisolac Advanced, Friesland, Netherland) was used as a negative control. Apart from the supplemented oligosaccharide, composition of the two formulas was identical. Among 271 term infants recruited, 69 fed fully with test formula, 52 fed fully with negative control formula and 26 fed fully with breast-milk. One hundred and twenty-four infants received mixed feed (test formula and breast milk). Growth, stool characteristics, and side effects were recorded at 3 and 6 months' follow-up, and the faecal species were collected for analysis of intestinal micro-flora, short chain fatty acid (SCFA) and pH. All recruited infants were excluded from the study while receiving antibiotic treatment.

Microbiologic analysis was as follows.[13,14] For the incubation of *Bifidobacteria* and *Lactobacilli*, 1 g fresh faecal sample was homogenized and diluted in 10 ml prereduced brain heart infusion broth in an anaerobic glove box within 1 hour of collection, portions (10 μl) of each dilution were spread onto the surfaces of the plates that contained Rogosa SL agar (Difco) and were incubated anaerobically at 37℃ (2 days for *Lactobacilli* and 4 days for *Bifidobacteria*, after *Lactobacillus* colonies were marked at days 2). For the incubation of *Escherichia coli*, 1 g fresh faecal sample was homogenized and diluted in 10 ml prereduced brain heart infusion broth in clean air cupboard, portions (10 μl) of each dilution were spread onto the surfaces of the plates that contained MacConkey agar (Difco) and were incubated aerobically at 37℃ (1 day for *Escherichia coli*). Colonies were counted to determine colony-forming units per gram of wet feces (CFU/g). A colony-forming unit was defined as a distinct colony measuring at least 1 mm in diameter.

Concentrations of SCFA were determined by using gas chromatography.[15] Briefly, weighed faecal samples were diluted (1∶10) in 0.1 mol/L sodium phosphate broth (pH 6.5), and suspensions were used to determine the concentrations of acetate, propionate and butyrate using a Hewlett-Packard 5890A Series Ⅱ gas chromatograph (Agilent, Wilmington, DE) and a glass column (180 cm ×4 mm) packed with 10% SP-1200 or 1% H_3PO_4 on 80/100 mesh Chromosorb WAW (Supelco, Bellefonte, PA). Nitrogen was the carrier gas and a flow rate of 75 ml/min was used. Oven, detector and injector temperatures were 125℃, 175℃ and 180℃, respectively. Blank tube SCFA concentrations were used

to correct for nonsubstrate SCFA production.

The pH value was measured in fresh stool samples using a multicolor indicator paper (accuracy ± 0.2, Spezialindikatorpapier Merck Eurolab GmbH, Darmstadt, Germany). Stool characteristics were recorded with respect to consistency (score 1 – 4: 1 = watery; 2 = loose/mushy; 3 = soft; and 4 = hard) and frequency were recorded on the basis of mothers' interview. Incidence of crying (score 1 – 3: 1 = no cry; 2 = cry when fed; and 3 = cry independent on meals), regurgitation (score 1 – 3: 1 = no regurgitation; 2 = one or two regurgitations; and 3 = more than two regurgitations per day), and vomiting (score 1 – 3: 1 = no vomiting; 2 = one episode of vomiting; and 3 = more than one episodes of vomiting per day) were recorded based on mothers' interview.

For all infants, growth parameters were measured. For example, body weight was measured using a scale with an accuracy of ± 5 g. The crown-heel length and head circumference were recorded using a special board for newborn infants, which had an accuracy of ± 1 mm.

StatView 5.0 (SAS Institute Inc., USA) was used for data analyses. All data were expressed as mean ± standard deviation (SD). An overall group effect on a measured variable was evaluated by one way analysis of variance (ANOVA). If significant, it was followed by q test for single factor group comparisons. A P value less than 0.05 was considered statistically significant.

RESULTS

At the end of 3 and 6 months' feeding period, intestinal *Bifidobacteria* and *Lactobacilli* were significantly increased in infants fed with GOS supplemented formulas and human breast milk in comparison with infants fed with negative control formula, but no significant difference between the first two groups was found. Intestinal *Escherichia coli* did not show any significant difference among those three groups (Tables 1 and 2).

Table 1. Intestinal micro-flora at the end of 3 months' feeding

Groups	Intestinal micro-flora (Log_{10} CFU/g)		
	Bifidobacteria	*Lactobacilli*	*Escherichia coli*
Test formula (n = 69)	9.0 ± 1.8	6.1 ± 0.8	6.8 ± 0.8
Mixed formula (n = 124)	8.9 ± 1.0	5.9 ± 0.6	6.5 ± 1.1
Human milk (n = 26)	9.2 ± 0.4	5.5 ± 0.3	6.4 ± 0.8
Formula without supplement (n = 52)	7.2 ± 1.2 *	3.9 ± 1.6 *	5.7 ± 0.6
F value	3.15	6.10	1.44
P value	< 0.05	< 0.01	> 0.05

* $P < 0.05$, compared with other three groups by q test.

Table 2. Intestinal micro-flora at the end of 6 months' feeding

Groups	Intestinal micro-flora (Log_{10} CFU/g)		
	Bifidobacteria	*Lactobacilli*	*Escherichia coli*
Test formula (n = 69)	7.9 ± 1.3	6.3 ± 1.0	6.1 ± 1.3
Mixed formula (n = 124)	7.1 ± 1.0	5.3 ± 0.6	6.5 ± 1.1
Human milk (n = 26)	7.5 ± 1.4	6.3 ± 0.8	6.2 ± 1.4
Formula without supplement (n = 52)	6.0 ± 0.9 *	4.3 ± 1.2 *	7.0 ± 0.9
F value	4.04	6.92	1.60
P value	< 0.05	< 0.01	> 0.05

* $P < 0.05$, compared with other three groups by q test.

Fecal SCFA (acetic) and stool frequency were also significantly increased in infants fed with GOS supplemented formulas and human breast milk, while fecal pH was significantly decreased when compared with that of negative control (Table 3). Supplementation had no influence on the incidence of side effects (including crying, regurgitation, and vomiting). Weight gain and length increments were similar among the three groups.

Table 3. Fecal concentration of SCFA and pH value at the end of 3 and 6 months' feeding

Groups	SCFA ($\mu mol \cdot L^{-1} \cdot g^{-1}$)		pH value	
	3-month	6-month	3-month	6-month
Test formula (n = 69)	26.3 ± 7.9	22.2 ± 4.7	5.3 ± 0.3	5.2 ± 0.3
Mixed formula (n = 124)	25.3 ± 5.3	21.9 ± 8.9	5.3 ± 0.3	5.3 ± 0.3
Human milk (n = 26)	27.1 ± 5.3	19.7 ± 5.6	5.3 ± 0.3	5.4 ± 0.3
Formula without supplement (n = 52)	11.9 ± 5.8 *	12.3 ± 4.6 *	5.8 ± 0.5 *	5.8 ± 0.5 *
F value	13.56	7.67	10.06	12.8
P value	< 0.01	< 0.01	< 0.01	< 0.01

SCFA: short chain fatty acid; * $P < 0.05$, compared with other three groups by q test.

DISCUSSION

Breast-fed infants are known to have a gastrointestinal flora that is dominated by *Bifidobacteria* and *Lactobacilli*.[1,2] Although breast-fed and formula-fed infants have a similar gastrointestinal flora on days 3 or 4 of life, there is a significant difference in colonic flora after several weeks of life. As a consequence, some industries try to mimic gastrointestinal colonisation of breast-fed infants by adding probiotics, such as *Lacto-bacilli* to the formula, while others try to mimic mother's milk and add prebiotics, such as GOS, to the formula.

The addition of *Lactobacilli* to infant formula results in a gastrointestinal flora that is dominated by *Lactobacilli*. Not only does the flora become "breast-fed like", but some typical aspects of breast-fed infants (such as the stools) change accordingly.[16,17] These observations lead to the conclusions that "probiotic theory" is valid and

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development of gastrointestinal flora can be manipulated by adding *Lactobacilli* to infant formula. However, this concept may be regarded as unphysiological since *Lactobacilli* are of course not present in human milk. Another weakness in probiotic concept is that the bacteria need to be stored and administered in a viable way, necessitating specific guidelines of formula preparation.

According to prebiotic concept, oligosaccharide is added to infant formula. This seems to be a more physiological approach. But dynamic aspects of oligosaccharide content in mother's milk cannot be mimicked in artificial feeding. In the prebiotic concept, there are no special precautions or guidelines for preparation of the formula. It has been shown that adding oligosaccharide mixture consisting of GOS and FOS to infant formula results in a similar effect on gastrointestinal colonisation, and the absolute number of *Bifidobacteria* and the proportion of *Bifidobacteria* to total microorganisms will increase with the concentration of oligosaccharide mixture increases from 0.4 or 0.8 g/dl to 1 g/dl.[18,19]

The present study was designed to investigate the effects of 0.24 g/dl GOS supplementation to infant formula on intestinal micro-flora and fermentation in term infants. The data show that supplementation of a standard milk formula with 0.24 g/dl GOS did increase the numbers of fecal *Bifidobacteria* and *Lactobacilli*, accompanied by an increase in intestinal SCFA production and a reduction in fecal pH, which may have health promoting effects. For example, *Bifidobacteria* and Lactobacill may protect the host from colonisation of invasive microorganisms. The flora also hydrolyses proteins and therefore may play a role in decreasing allergenicity of non-digestible proteins.[19] The flora is also capable of hydrolysing or fermenting complex carbohydrates that reach the colon, and SCFA is produced as an end-product of this hydrolysis. Increased SCFA production also benefits for colonic health in that SCFA is a preferred energy source for colonic epithelial cells and has been shown to stimulate normal colonocyte proliferation. Furthermore, SCFA also has been suggested to enhance small intestinal glucose uptake.[20]

Increased frequency and softer stool consistency is the results of supplementation, which is of practical importance because it may decrease adverse effects associated with higher incidence of hard stool or constipation in infants fed with standard formula.[21] GOS of 0.24 g/dl added to the formula increases the osmolarity slightly, which cannot explain differences in stool characteristics that are probably influenced by the changes in intestinal flora.

Studies in adults indicate that dietary oligosaccharide may lead to some side effects (in particular flatulence), which mainly depends on the quantity of oligosaccharide supplementation.[22] In the present study, 0.24 g/dl GOS supplementation to the formula did not lead to flatulence, but did show an effect of intestinal micro-flora modulation and fermentation, indicating that 0.24 g/dl GOS supplementation to the formula is safe and effective.

In summary, supplementation of a term formula with 0.24 g/dl GOS stimulates *Bifidobacteria* growth and results in stool frequency and consistency similar to those found in term infants fed with human milk. Thus, prebiotic may help to improve intestinal tolerance to enteral feeding.

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