

Faecal SIgA secretion in infants fed on pre- or probiotic infant formula

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Secretory immunoglobulin A (SIgA) plays an important role in the defence of the gastrointestinal tract. The level of faecal SIgA antibody is associated with increased neutralization and clearance of viruses. Formula-fed infants who lack the transfer of protective maternal SIgA from breast milk may benefit from strategies to support maturation of humoral immunity and endogenous production of SIgA. We aimed at studying the effects of standard, prebiotic and probiotic infant formulas on the faecal SIgA levels. At birth, infants of whom the mother had decided not to breastfeed were allocated to one of three formula groups in a randomized, double-blind fashion. Nineteen infants received standard infant formula; 19 received prebiotic formula containing a specific mixture of 0.6 g galacto-oligosaccharides (GOS)/fructo-oligosaccharides (FOS)/100 ml formula and 19 received probiotic formula containing 6.0×10^9 cfu *Bifidobacterium animalis*/100 ml formula. Faecal samples were taken on postnatal day 5, day 10, wk 4 and every 4 wk thereafter until wk 32. SIgA in faeces was determined by an enzyme-linked immunosorbent assay. During the intervention, infants fed on prebiotic formula showed a trend towards higher faecal SIgA levels compared with the standard formula-fed infants reaching statistical significance at the age of 16 wk. In contrast, infants fed on the probiotic formula showed a highly variable faecal SIgA concentration with no statistically significant differences compared with the standard formula group. Formula-fed infants may benefit from infant formulas containing a prebiotic mixture of GOS and FOS because of the observed clear tendency to increase faecal SIgA secretion. Adding viable *B. animalis* strain Bb-12 to infant formula did not reveal any sign for such a trend.

Key words: prebiotics; probiotics; infant; formula; SIgA

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Secretory immunoglobulin A (SIgA) is one of the most abundant immunoglobulin in the human body and is the predominant immunoglobulin in mucosal surfaces. SIgA plays a key role in the gastrointestinal defence mechanism against dietary and microbial antigens. It inhibits adherence and invasion of potentially harmful antigens into mucosal tissues and neutralizing toxins and virulence factors from microbial pathogens (1). It is well established that the level of faecal SIgA antibody correlates with higher virus-neutralizing capacity and increased viral clearance (2). IgA deficiency in humans is one of the most common

immunodeficiencies and is associated with frequent gastrointestinal infections (3). There is accumulating evidence that the intestinal SIgA production is highly influenced by the intestinal microflora. Indeed, the development of the IgA-producing plasmablasts (intermediate stage of the development of a B-lymphocyte into IgA-producing plasma cell) in the intestinal mucosa seems to be affected by components of the intestinal microflora (4).

During the first few weeks after birth, the mucosal humoral immunity is not in a mature state. Passive immunity in this phase is provided

by breast milk, which contains high levels of SIgA and antimicrobial peptides. SIgA in breast milk are mainly directed against the mother's previous and current gut microflora (5). Breast milk SIgA protects the maternal mammary gland against mastitis, protects the neonatal mucosa against early exposure to microbes and limits bacterial translocation. In breast milk, SIgA levels are highest during the first days after birth (human colostrum contains 2–5 mg SIgA/ml) and then gradually decrease to 0.5–1 mg/ml (6).

Although many factors may influence SIgA survival in the large intestine, measuring SIgA levels in faeces gives a good representation of the amount available in the colon (7). In the first 2–4 wk of life, the concentration of IgA in faeces of breast-fed infants is substantially higher compared to that found in formula-fed infants in whom SIgA is basically undetectable (8). Between 4 wk and 6 months of life, faecal IgA concentrations in both breast-fed and formula-fed infants converge towards similar levels. At age 1–2 yr, when weaning is completed, the production of SIgA reaches adult levels (9).

It is generally recognized that intestinal microflora may play an active role in the ontogeny of the newborn's immune response. Studies performed in germ-free animals showed that colonization leads to the development of the gut-associated lymphoid tissue, including SIgA secretion in the intestine (10, 11). Moreau and Baforieu-Routhiau have shown that in particular bifidobacteria in the infant's intestine are important for the synthesis of IgA against viral enteropathogens. Therefore, they suggested that foods promoting bifidobacteria in the intestine could be instrumental in stimulating endogenous SIgA production and hence promote resistance in infants (12). Although the mechanism of immune stimulation by bifidobacteria is largely unknown, it is thought that the cell walls of Gram-positive bacteria, which are rich in peptidoglycans, may play a role.

Both prebiotics and probiotics change the intestinal microflora by increasing the numbers of bifidobacteria and lactobacilli. Bifidobacteria and lactobacilli have various beneficial effects in health and disease, including maintenance of intestinal barrier integrity and mucoprotection, stimulation of protective immune responses and protection against harmful pathogens.

During the last decade, interest on the immune effects of probiotics has increased markedly. Experimental studies showed that probiotics have strain-specific effects on immunity, for instance in the prevention or treatment of allergic disease. The reported effects of probiotics include

enhancement of gut barrier function and induction of regulatory and pro-inflammatory immune responses (13). Additionally, several studies reported that supplementation of food with prebiotics or probiotics can increase SIgA response to viruses and bacteria. However, most of these studies were performed in animals or *in vitro* and the mechanisms for this immune stimulation are largely unknown (14–18).

As infants not receiving breast milk have lower SIgA levels during the first months of life, they would potentially benefit from strategies to support maturation and production of mucosal SIgA. Therefore, we studied the effects of infant formula with added probiotics or prebiotics on the faecal SIgA levels in infants. We hypothesized that infants on probiotics or prebiotics will have higher levels of total faecal SIgA compared with infants fed on a standard, unsupplemented infant formula.

Materials and methods

Subjects

Sixty-three pregnant women who had decided to breastfeed and 57, who chose not to, were recruited during their last trimester of pregnancy. Infants with normal birth weight, no congenital abnormality, congenital disease or gastrointestinal disease were enrolled within 3 days after delivery. The study was approved by the Medical Ethics Committee of the University Medical Centre St Radboud (Nijmegen). Written informed consent was obtained from the parents before enrolment in the study.

Feeding groups

Infants of mothers who decided not to breastfeed were allocated to one of three formula groups (GOS/FOS, Bb-12 or standard) in a randomized, double-blind fashion. Randomization included a block size of 3 and was carried out by a logistics manager who was not involved in the study in any other way. The formula tins containing the different products were coded by the logistics manager, using the number the infants received at inclusion. The study formulas were fed *ad libitum* during the study period and were supplied on request.

The standard formula group ($n = 19$) received a regular, non-supplemented infant formula (Nutrilon I; Nutricia, Zoetermeer, the Netherlands). The composition of the standard formula at a standard dilution of three scoops per 100 ml formula is given in Table 1. The prebiotic formula

Table 1. Composition of the standard formula per 100 ml

	Standard formula
Energy (kcal)	67
Protein (g)	1.4
Casein/whey ratio	40/60
Fat (g)	3.5
Carbohydrates total (g)	7.5
Lactose	7.5
Glucose	–
Minerals (mg)	
Calcium	54
Phosphorus	27
Magnesium	5
Sodium	19
Potassium	64
Chloride	43
Iron	0.5
Zinc	0.5

group (GOS/FOS; $n = 19$) received the same standard infant formula supplemented with a mixture of 0.6 g/100 ml GOS (Vivinal GOS; Borculo Domo Ingredients, Zwolle, the Netherlands) and FOS (Raftiline HP; Orafit active food ingredients, Tienen, Belgium). The mixture comprised 90% GOS and 10% FOS in order to closely resemble the spectrum of molecular masses of the neutral oligosaccharide fraction in human breast milk (19). The probiotic formula group (Bb-12; $n = 19$) received the standard infant formula supplemented with 6.0×10^9 cfu *Bifidobacterium animalis* per 100 ml formula (Bb-12; Christian Hansen Ltd, Hørsholm, Denmark). The shelf-life of the probiotic formula was tested during storage. After 12 months of storage $1.0 \times 10^9 \pm 0.5 \times 10^9$ cfu of vital *B. animalis* could still be recovered.

Mothers were instructed to heat the water to a maximum temperature of 45°C before adding the milk powder to avoid hot spots in the liquid milk during micro waving, which could kill the probiotic bacteria. Mothers who decided to breastfeed were stimulated to continue breastfeeding during the course of the study and were supported by a lactation consultant when needed.

Questionnaires

Demographic, clinical and anthropometrical data of the mother were collected prior to delivery. Information on delivery was obtained from the mother at day 5 after delivery. Information on the infants' food intake, formula tolerance, stool characteristics, health and anthropometrics was obtained from questionnaires

at postnatal days 5, 10, 28, and once every 4 wk thereafter.

Faecal samples

Parents were asked to take faecal samples from their infants, at postnatal days 5, 10, 28, and once every 4 wk thereafter. The samples were taken from the diaper, as soon as possible after defecation, collected in faeces containers (Greiner Labortechnik, Alphen aan den Rijn, the Netherlands) and stored immediately at -20°C by the parents. During the study period, the investigators visited the participants regularly to collect faecal samples and questionnaires. Faecal samples were transported in a portable freezer (minimal temperature -15°C , MRFD-015; Veba Meditemp, Uden, the Netherlands) to the laboratory.

Faecal homogenates

For the determination of the SIgA concentration by an enzyme-linked immunosorbent assay (ELISA), 10% (w/v) faecal homogenates were prepared according to standard procedures. Briefly, the frozen (-20°C) faecal samples were defrosted on ice. Suspensions were made by adding 1 g faeces to 9 ml of phosphate-buffered saline (PBS) and homogenizing for 10 min using a stomacher. The homogenates were stored at -80°C until further processing.

Enzyme-linked immunosorbent assay

ELISA plates (NUNC 384 well Immuno-Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with Mouse α Human Secretory Component (clone GA-1; Sigma, Zwijndrecht, the Netherlands), 1:50,000 in PBS. After thoroughly washing the plates five times with buffer (0.005% Tween-20 in PBS), the plates were incubated for 1 h at room temperature with PBS containing 1% (w/v) of bovine serum albumin (BSA) (Sigma) to block non-specific protein binding sites. After blocking, the plates were again washed thoroughly. The supernatants of the faecal homogenates (defrosted on ice, vortexed and spun down at 1500 g for 5 min at 4°C) were serially diluted 10 times in PBS/BSA. Purified human IgA isolated from colostrum (I-1010; Sigma) was used as a positive standard. The samples and standards were incubated for 2 h at room temperature. Plates were then washed five times and biotin-conjugated mouse anti-human IgA_{1/2} monoclonal antibody (clone G20-359; Pharmingen, Alphen aan den Rijn, the Netherlands) was added to the

plates at a concentration of 0.2 µg/ml PBS/BSA. After 1 h of incubation, the plates were washed five times and incubated with streptavidin-conjugated horseradish peroxidase (CLB, M2051) 1:50,000 diluted in PBS/BSA for 30 min at room temperature. The wells were then washed and incubated for 5–10 min with 50 µl of a tetramethylbenzidine (TMB) substrate solution. Enzymatic colour development was stopped by adding 1.8 M H₂SO₄. The absorbance was measured at 450 nm by using a plate reader. Concentrations of SIgA were calculated from the standard curve.

Data analysis

Because the concentration of SIgA was not normally distributed, results are expressed as median and the range between the 10th (P₁₀) and 90th (P₉₀) percentiles. SIgA concentrations of the Bb-12 and GOS/FOS groups at all ages were separately compared with the standard formula group using the Mann–Whitney *U*-test (two-tailed). Because it is not possible to randomize and double blindly assign breastfeeding, statistical analysis was performed only on the formula-fed groups.

Results

In total, 120 infants were included in the study between January 2000 and May 2003. The characteristics of the study subjects are shown in Table 2. Results on the composition and metabolic activity of the intestinal microflora are published elsewhere (20). Of the 63 infants fed on breast milk directly after birth, 40 switched to complete or partial formula feeding, 12 were completely breastfed and 11 dropped out during the intervention period. Fifty-seven infants started on formula feeding directly after

birth and were equally divided among the formula groups. In the formula groups, 16 infants dropped out of the study: five in the standard group, five in the GOS/FOS group and six in the Bb-12 group. Reasons for drop-out included colics, suspicion of cow's milk allergy, constipation and practical problems.

The median and the range between P₁₀ and P₉₀ of the faecal SIgA concentration found in the infants between birth and 32 wk of age are shown in Table 3. As expected, the SIgA concentrations of breast-fed infants were higher than in standard formula-fed infants during the complete study period. From birth until the age of 8–12 wk, faecal SIgA levels show a decrease towards levels that are slightly higher compared with those in formula-fed infants. In the GOS/FOS formula group, when integrated over the complete intervention period, faecal SIgA concentrations tended to be higher compared with infants fed standard formula. In the Bb-12 formula group, there was no marked trend towards different SIgA secretion compared with the other formula groups.

Discussion

In this study, we found indications that adding non-digestible oligosaccharides to infant formula results in a trend towards higher levels of faecal SIgA when compared with unsupplemented infant formula.

Although our results are very promising, variations of the SIgA concentrations of the groups were large and statistical significance was reached only once on comparing the GOS/FOS and the standard formula group. We believe that the high variation that was observed might have masked a possible effect of GOS/FOS on faecal SIgA secretion. Nevertheless, we feel this to be an important observation as this additional effect of prebiotics on endogenous SIgA production occurs in a critical period of maturation (9). Several reasons for the high variation of the faecal SIgA concentrations between samples can be suggested. First, disease frequency and vaccination of the infant can have an effect on the levels of faecal SIgA (21, 22). The health questionnaire, primarily designed to report formula tolerance, however did not indicate major differences between the groups as regards frequency of vomiting (2–13% of all questionnaires reported vomiting), fever (4–8%) and diarrhoea (3–8%) during the course of the study. Moreover, omitting infants suffering from any illness did not affect our results. In the Netherlands, infants are vaccinated in accordance with the guidelines of

Table 2. Characteristics of study subjects

	Standard formula (n = 19)	GOS/FOS formula (n = 19)	Bb-12 formula (n = 19)	Breast milk (n = 63)
Sex				
Male	5	12	10	33
Female	14	7	9	30
Place of birth				
At home	7	8	10	40
Hospital	12	11	9	23
Mode of delivery				
Vaginal	14	16	18	59
Caesarean section	5	3	1	4
Birth weight (g)	3601 ± 501	3318 ± 602	3481 ± 524	3651 ± 601

GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides.

Table 3. Median faecal SIgA concentrations (mg/g wet weight faeces) in infants from birth until the age of 32 wk, fed on standard, GOS/FOS or Bb-12 formula or breast milk

Age	Standard formula			GOS/FOS formula			Bb-12 formula			Breast milk		
	n†	Median	P ₁₀ –P ₉₀	n†	Median	P ₁₀ –P ₉₀	n†	Median	P ₁₀ –P ₉₀	n†	Median	P ₁₀ –P ₉₀
Day 5	13	0.000	0.000–0.000	15	0.000	0.000–0.000	19	0.000	0.000–0.000	43	10.918	1.604–33.013
Day 10	19	0.000	0.000–0.000	18	0.000	0.000–0.000	17	0.000	0.000–0.000	44	7.722	2.365–21.230
Week 4	16	0.230	0.048–1.414	17	0.737	0.111–2.380	17	0.346	0.044–4.203	36	4.243	1.229–12.434
Week 8	15	0.704	0.048–3.949	18	0.785	0.315–3.712	17	0.616	0.103–2.086	30	3.175	1.154–6.807
Week 12	15	0.754	0.056–3.484	13	1.141	0.116–5.878	16	0.519	0.059–2.858	24	1.576	0.375–5.561
Week 16	14	0.292	0.028–3.712	14	0.841*	0.433–4.445	15	0.358	0.044–2.702	22	1.103	0.141–5.992
Week 20	14	0.385	0.011–2.410	14	0.524	0.164–2.291	12	0.439	0.032–8.451	18	0.937	0.100–3.906
Week 24	13	0.449	0.042–0.882	13	0.654	0.085–2.510	14	0.573	0.023–4.693	12	0.767	0.027–7.948
Week 28	13	0.157	0.036–1.384	12	0.328	0.067–3.164	12	0.168	0.018–0.362	15	0.288	0.042–4.965
Week 32	12	0.165	0.024–1.426	12	0.140	0.036–1.269	13	0.255	0.045–3.799	12	0.265	0.087–7.098

GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides.

*p < 0.05 compared with the standard formula group.

†Group sizes decreased due to drop out, absence of a faecal sample and the switch from breastfeeding to formula feeding. In the latter case, samples taken after the switch were not included in the analysis.

the National Vaccination Programme (National Institute of Public Health and the Environment). In our study only one infant was not vaccinated. Therefore, we conclude that in the present study, disease and vaccination are not major confounding variables. The GOS/FOS group most likely did not show systematically higher SIgA levels due to higher disease frequency and/or vaccination rate. However, disease or vaccination results in an increased SIgA secretion that may differ between individuals and therefore may have caused the high variation of faecal SIgA concentrations within the formula groups. Another potential cause of the high variation in faecal SIgA is differences in the characteristics of the stool samples. SIgA secretion was measured in wet weight faeces and might therefore be influenced by factors like defecation rate and consistency of the stool. However, several studies showed that measuring SIgA levels in wet weight faeces gives a good representation of the amount of SIgA available in the colon (7). Based on our health questionnaires, no differences were found in defecation rate (1.8 ± 1.0 , 1.7 ± 1.0 and 1.6 ± 1.2 stools per day $\pm$ s.d. in the GOS/FOS, Bb-12 and standard formula group respectively) and reported stool consistency $\pm$ s.d. (2.6 ± 0.8 , 2.8 ± 0.9 and 2.7 ± 0.9 in the GOS/FOS, Bb-12 and standard formula group, respectively, based on a 5-point Likert scale: 1 – watery; 2 – soft pudding-like; 3 – soft formed; 4 – dry formed; 5 – dry hard pellets) between the formula groups. Although, on average, we did not find differences in stool consistency and frequency between the groups, individual differences in consistency and frequency may have an effect on faecal SIgA concentrations and there-

fore may interfere with the effect of the pre- and probiotics. A third possible cause of the high variation is that the SIgA secreted might be partly digested by certain bacteria of the intestinal microflora. Studies in dogs have shown that large differences are found between the SIgA concentrations in the ileum and the faeces (23). It was suggested that the presence of certain bacterial species with IgA-degrading capability (e.g. *Clostridium*) in the intestinal microflora may be responsible for this decrease (24). Previous findings show that in infants fed on GOS/FOS formula, the intestinal microflora contains very low percentages of *Clostridium*, *Escherichia coli* and *Eubacterium*. Theoretically, lower numbers of *Clostridium* could be associated with less degradation of SIgA in the gut, which is positive for intestinal protection. This concept will be further evaluated to determine whether particular flora components may have added to the large variation of the faecal SIgA concentrations (25). Generally, an increase in the level of SIgA is considered to be associated with a significantly faster clearance of pathogenic bacteria and viruses from the intestine. Therefore, despite the large variation of our results, which prevented us from demonstrating statistical significance, we do see a trend of higher SIgA concentrations in the GOS/FOS formula group compared with the standard formula group.

To study the effects of pre- and probiotics on faecal SIgA concentration, we statistically compared data from the GOS/FOS and Bb-12 formula groups with the standard formula group. For several reasons, we decided to exclude the breast-fed group from statistical analysis. First, in breast-fed infants it is difficult to make a

distinction between SIgA ingested with the mother's milk and SIgA produced endogenously. Second, it is not possible to randomize and double blindly assign breastfeeding. Due to differences in social and educational status between breastfeeding and formula feeding mothers, selection bias is likely to occur. By limiting statistical analysis to the formula groups, we were still able to assess the effects of the prebiotic and probiotic components on faecal SIgA concentrations.

Our results are in accordance with the current literature on the effect of pre- and probiotics on luminal SIgA production in both animals and humans with the aim of improving natural resistance (14–16, 26, 27). Roller et al. showed that a combination of prebiotics (inulin enriched with oligofructose) and probiotics (*Lactobacillus rhamnosus* GG and *B. animalis* Bb-12) fed to rats for 4 wk increased the local SIgA production in the ileum (27). Recently, it was found that feeding newborn mice a diet containing 5% FOS resulted in a twofold higher ileal IgA secretion rate and 1.5-fold polymeric immunoglobulin receptor expression compared with control mice (26).

In conclusion, the results of our study confirm the differences observed in faecal SIgA levels between breast-fed and formula-fed infants. Total faecal SIgA secretion between several infant formulas has not been compared before. Statistical analysis of the data is complicated, most likely because of the large inter-individual variations. *Post-hoc* power analysis suggests group sizes of more than 100 children per group. Alternatively, it might be more worthwhile to study the effect of pre- and probiotics on the levels of antigen or vaccine-specific SIgA, which might be less subjected to inter-individual variation.

Despite the limitations, the consistently higher faecal SIgA levels in the prebiotic group allow the conclusion that it is possible to stimulate the development of the mucosal immune response with a prebiotic mixture of 90% GOS and 10% FOS. The probiotic strain Bb-12 was found to be less effective.

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