

# Prebiotics and probiotics in infant nutrition

## Promotoren

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Wageningen

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Hoogleraar in Voeding tijdens Groei en Ontwikkeling, Wageningen  
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Rijksuniversiteit Groningen

Prof. Dr. Ir. M.W.A. Verstegen  
Animal Nutrition Group, Wageningen Universiteit, Wageningen

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# Prebiotics and probiotics in infant nutrition

Astrid Maria Bakker-Zierikzee

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Astrid Maria Bakker-Zierikzee

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# Abstract

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## Introduction

In general breast-fed infants suffer less from infection, which could be partly explained by the specific composition and metabolic activity of their intestinal microflora. During the last two decades, many attempts have been made to mimic the intestinal flora of breast fed infants in formula fed infants. Both prebiotics and probiotics based concepts have been developed to beneficially change the intestinal microflora and thus induce positive health effects. We conducted two infant nutrition studies with the objective to compare the effects of infant formulas containing either prebiotics or probiotics in infants on the composition (% bifidobacteria and lactobacilli) and metabolic activity (short chain fatty acid profile, lactate concentration and pH) of the intestinal microflora and on indicators of development of the secretory immune response (faecal SIgA concentration).

## Study design

In both studies, infants were enrolled within 3 days after delivery and followed during the first 32 weeks of life. Except for the intervention, the design of both studies was identical. Infants of whom the mothers decided not to breast-feed, were at random and double blindly allocated to one of the formula groups. A group of breast-fed infants was included as a reference. The first study, included 63 infants that were breast fed, 19 fed a regular, non-supplemented infant formula, 19 received infant formula containing a mixture of 0.6 g/100ml GOS (90%) and FOS (10%), 19 received standard formula containing  $6.0 \times 10^9$  /100ml viable *Bifidobacterium animalis* strain Bb-12. The second study included 38 infants on breast milk, 17 on standard, unsupplemented formula and 17 on formula containing 0.6 g/100ml GOS. During intervention, parents were asked to take faeces samples from the diaper of their infants on postnatal day 5, 10, 28 and once every 4 weeks thereafter.

## Results

The GOS/FOS-, GOS-, Bb-12 formula all induced an intestinal microflora dominated by bifidobacteria ( $59.2 \pm 7.7\%$ ,  $76.5 \pm 2.6\%$  and  $69.7 \pm 2.7\%$  mean  $\pm$  SEM resp. at 16w) and no significant differences were found compared to the standard formula group ( $56 \pm 6.4\%$ ). In contrast, we did show a significant effect of GOS/FOS on the percentage of lactobacilli ( $6 \pm 2.6\%$  at 12w,  $p=0.007$ ) compared to the standard formula group ( $1 \pm 0.4\%$ ), whereas no significant effect was found for the GOS- and Bb-12 formula ( $1 \pm 0.4\%$  and  $2.4 \pm 1.7\%$  resp. at 12w).

Infants fed on GOS/FOS formula showed a metabolic activity of the flora comparable to that of breast fed infants. GOS/FOS formula induced a faecal SCFA profile (acetate/propionate/ butyrate/others) comparable to that found in breast fed infants ( $82/14/2/2\%$  vs.  $90/6/2/2\%$  at 16w), while that of GOS- and Bb-12 fed infants is more like that in standard formula fed infants ( $78/16/3/2$  and  $70/22/6/3$  vs.  $73/20/5/3$  at age 16w). We also demonstrated that the faecal lactate concentration of the GOS/FOS group was comparable to breast fed infants ( $40.9 \pm 10.7$  vs.  $45.2 \pm 9.0$  mmol lactate/kg faeces), whereas that of GOS- and Bb-12 fed infants was more like standard formula fed infants ( $12.2 \pm 5.1$  and  $6.1 \pm 4.2$  vs.  $0.8 \pm 0.7$ ). Also the faecal pH of the GOS/FOS group was highly comparable to that in breast-fed infants ( $5.6 \pm 0.2$  vs.  $5.7 \pm 0.3$ ), whereas that of GOS- and Bb-12 fed infants was more comparable to that of standard formula fed infants (pH  $6.5 \pm 0.3$  and  $6.6 \pm 0.2$  vs.  $7.1 \pm 0.2$ ).

Finally we showed that the GOS/FOS formula group showed a marked trend towards higher faecal SigA levels compared to the standard formula group ( $0.84$  ( $0.6-1.8$ ) vs.  $0.39$  ( $0.1-0.9$ ), median ( $P_{25}-P_{75}$ ),  $p=0.015$  at age 16w), which could not be demonstrated in the GOS and Bb-12 infant formula groups.

## Conclusion

Although, more research is needed to elucidate the effects of GOS/FOS formula on hard clinical endpoints, based on our findings it can be reasonably assumed that infants fed on GOS/FOS will have a health benefit compared to infants fed on standard infant formula.

Voor Jeroen en Niels

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## List of abbreviations

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Cfu	Colony forming units
CS	Caecarean Section
DP	Degree of polymerization
FOS	Fructo-oligosaccharides
GALT	Gut associated lymphoid tissue
GOS	Galacto-oligosaccharides
SCFA	Short chain fatty acids
SIgA	Secretory immunoglobulin A.

1

Introduction

Breast feeding is the best nutrition for infants. Human milk contains all necessary ingredients to optimally support the infants' growth and health. Complex oligosaccharides are one of the most abundant solutes in human milk, accounting for 5-8 g per litre (1). Non-digestible oligosaccharides in human milk may protect the infant by acting as a competitive receptor for potential pathogens and by stimulating the growth of bifidobacteria in the intestinal microflora (2)(3). In breast fed infants, the intestinal microflora usually consist of 80 to 90% bifidobacteria (4-6). It is generally accepted that an intestinal microflora dominated by bifidobacteria is beneficial for health and might partly explain why breast fed infants suffer less often from gastrointestinal illnesses compared to formula fed infants (7,8).

During the last two decades many attempts have been made to mimic the intestinal flora of breast fed infants in formula fed infants. Prebiotics and probiotics are novel concepts in the nutritional area that are used to selectively change the intestinal microflora and thus induce positive health effects.

In this thesis I describe two intervention studies on the effects of infant formula containing prebiotics and probiotics, on the composition and metabolic activity of the intestinal microflora, and the development of the intestinal secretory immune response in infants during the first months of life.

## Health effects of an intestinal microflora dominated by bifidobacteria

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Bifidobacteria are gram-positive, saccharolytic bacteria that were first isolated by Tissier, who named them *Bacillus bifidus* (11). Bifidobacteria dominate the large intestine of breast fed infants where they ferment undigested carbohydrates.

A high percentage of bifidobacteria in the intestinal microflora is associated with a lower percentage of potential pathogens. Bifidobacteria can either indirectly or directly inhibit the growth of pathogens and can therefore have an important protective effect on the infant.

Bifidobacteria can indirectly suppress pathogenic growth by the production of short chain fatty acids (SCFA). SCFA are metabolic end products of carbohydrate fermentation by intestinal bacteria and include acetate, propionate and butyrate. Protein fermentation leads to the production of branched-chain fatty acids such as iso-butyrate and iso-valerate, and ammonia, amines and phenols. Each bacterial species has its own characteristic SCFA profile (relative amounts of acetate, propionate, butyrate and others). Bifidobacteria mainly produce acetic and lactic acid (12). Thus, in breast fed infants, where the intestinal microflora is dominated by bifidobacteria, large amounts of acetate and lactate are produced in the intestine and consequently recovered in the faeces (13). Acetate and lactate in combination with the low faecal pH create conditions that are unfavourable for many potential pathogens and putrefactive bacteria (14,15). Bifidobacteria can also act directly on the growth of potential pathogens like *Salmonella*, *Listeria*, *Campylobacter* and *Shigella*, by excreting antimicrobial substances (16).

Because of the beneficial properties of bifidobacteria, there have been attempts to increase their relative proportion in the intestinal microflora.

In recent years, the concepts pre- and probiotics have been developed and tested to accomplish this bifidobacteria dominated flora.

## Prebiotics

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Prebiotics are defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health' (17). In order to be classified as a prebiotic, a food ingredient should; (1) not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract (including the small intestine), (2) be a selective substrate for one or a limited number of potentially beneficial bacteria that are commensally to the colon and are stimulated to grow and/or be metabolically active, (3) be able to alter the colonic flora in favour of a potentially more healthy composition and (4) induce luminal or systemic effects that are beneficial to the host health.

Several food ingredients like non-digestible carbohydrates, some peptides, proteins and lipids, have been proposed as prebiotics. Although all non-digestible carbohydrates are fermented by intestinal microflora, not all of them can be classified as prebiotics because they have been found to also stimulate the growth and/or activity of potentially harmful bacteria. Fructo-oligosaccharides and galacto-oligosaccharides are two non-digestible oligosaccharides that can fulfil all criteria for prebiotics and are currently used in different products including infant formula.

### Fructo-oligosaccharides

Fructo oligosaccharides (FOS) and inulin are prebiotics naturally present in plants such as onion, chicory and asparagus (18). Inulin and FOS are mostly linear polymers of fructose with glucose as the terminal sugar. FOS have a degree of polymerization (DP; number of monosaccharide units) between 2 and 7 whereas inulin has a DP of 2 to 60.

In humans, FOS and inulin are not hydrolyzed by endogenous digestive enzymes but are rapidly fermented by bacteria in the large intestine (18-20). *In vitro* studies, showed that FOS are fermented by several strains of lactobacilli and bifidobacteria (19,21,22), but not by potential pathogens like *Clostridium perfringens* and *Escherichia coli* (15,23).

Several studies show that ingestion of a daily dose FOS can significantly stimulate the growth of bifidobacteria in the intestinal microflora and lower faecal pH (19,24-26). Mitsuoka *et al.*, demonstrated that a daily intake of 8 g FOS for two weeks, significantly increased the number of faecal bifidobacteria from 8.8 to 9.7log<sub>10</sub> and decreased mean faecal pH from 6.9 to 6.0 (19). In a study by Gibson *et al.*, it was found that 15 g FOS or inulin per day, significantly increased bifidobacteria from 8.8 to 9.5 log<sub>10</sub> and from 9.2 to 10.1 log<sub>10</sub> per g faeces respectively (26). Bouhnik *et al.* concluded that 10g per day is the most optimal and well-tolerated dose of short chain FOS that can significantly increase faecal bifidobacteria in healthy adults consuming their usual diet (24). Although most intervention studies focussed on the effects of FOS on the composition of the intestinal microflora, only few studied the potential health effects of FOS, like stimulation of mineral absorption and lowering of serum cholesterol levels (27-29).

## Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides that, in some forms, are present in human milk, but are mainly industrially produced from lactose (30). The degree of polymerisation of GOS is usually 2-7. GOS, like FOS, survive passage through the gastrointestinal tract and are completely fermented in the large intestine (31,32) by several intestinal bacteria like bifidobacteria, *Bacteroides*, enterobacteria and lactobacilli (30).

Although not consistent in all studies (32,33), it is demonstrated by several researchers that GOS have a dose dependent stimulating effect on the number of faecal bifidobacteria and lactobacilli in adults (30,31,34).

When the initial number of indigenous bifidobacteria of the subject is low, a daily intake of 2.5 g GOS is enough to lead to an increase in faecal bifidobacteria (35). Next to stimulating the growth and activity of healthy bacteria, ingestion of GOS may have several other positive health effects including improvement of defecation (36), elimination of ammonia (37), stimulation of mineral absorption (38) and effects on cholesterol and lipid metabolism (39).

### Prebiotics in infant nutrition

During the last decade, the interest in the effects of prebiotic infant formulas on faecal flora of infants increased. Addition of several non-digestible components to infant formula was found to stimulate bifidobacteria in the intestinal flora. Nagendra *et al.*, found that infant formula containing either 0.5% or 1.0% lactulose significantly increased the number of bifidobacteria in the faeces of 6 infants of 2 to 10 weeks of age. Lactulose is often prescribed as a laxative to infants suffering from constipation. Surprisingly, no information was given on the occurrence of any possible laxative effects of the lactulose containing formulas. These laxative effects may shorten transit time and therefore might influence bacterial counts per gram of wet faeces and faecal pH (40). Rueda *et al.*, studied the influence of supplementing an adapted milk formula with gangliosides and found that this milk significantly modified faecal flora of preterm infants. However, changes in flora composition only consisted of lower numbers of *E coli* on day 3 and 7 and higher numbers of bifidobacteria on day 30 (41). Guesry *et al.* found that an infant nutrition containing 200, 400 or 600 mg FOS did not have a distinct effect on the number of bifidobacteria. Unfortunately, the participating infants of this study were already between 7 and 20 days old at start of the study and no information on previous nutrition was given. Therefore, possible confounding effects of age or previous breast- or formula feeding on composition of the flora cannot be completely ruled out (42).

In the last decade, several double blind intervention studies have been conducted to investigate the effect of infant formula containing a mixture

90% GOS and 10% high molecular mass (DP>10) FOS on the composition of the intestinal microflora. This GOS/FOS mixture was combined to mimic the molecular size distribution of human milk oligosaccharides and to benefit from a possible synergistic effect of both compounds to stimulate the growth of bifidobacteria (43). In a study by Boehm *et al.*, it was demonstrated that infant formula containing 1.0g/100ml GOS/FOS, significantly increased the numbers of bifidobacteria (44). In a study by Moro *et al.*, it was found that feeding infants different doses of this GOS/FOS mixture (0.4 or 0.8 g/100ml) resulted in a dose dependent, significantly higher amount of bifidobacteria compared to infants fed a standard formula (45). In contrast to Boehm *et al.*, and Moro *et al.*, who used traditional plating methods to determine intestinal flora, Knol *et al.*, studied the effect of GOS/FOS on the composition of the flora by using fluorescent in situ hybridization (FISH). With FISH, 16S rRNA targeted oligonucleotide probes are used to determine the relative numbers of specific bacteria in the faecal microflora. It was confirmed that after 6 weeks, infants fed the GOS/FOS formula had a significantly higher percentage of bifidobacteria in the intestinal flora compared to standard formula fed infants. Additional Polymerase Chain Reaction (PCR) in which target DNA is multiplied *in vitro*, separated by gel-electrophoresis and determined by hybridisation, showed that the pattern of *Bifidobacterium* species in the prebiotic group was highly comparable to that found in breast fed infants. In another study using FISH, it was demonstrated that in 0 to 2-week-old infants, GOS/FOS also alters the microflora by reducing the levels of certain groups of potential pathogenic micro-organisms (*Clostridium*, *E. coli* and *Eubacterium*) (46). In a recent, study by Schmelzle *et al.*, healthy infants of 2 weeks or younger, were fed a formula containing partially hydrolysed whey protein, modified vegetable oil with high  $\beta$ -palmitic acid content and the GOS/FOS mixture for 12 weeks. After 6 weeks, it was found that the intervention group had significantly higher bifidobacterial counts compared to a placebo group. However, because of the many differences in composition between the intervention formula and standard formula, specific effects of the GOS/FOS mixture cannot be clearly identified (47).

Most of the studies on GOS/FOS formula included groups of infants that were already several weeks old or showed a large variation in age at the start of the intervention. Additionally, part of the participating infants was breast fed whereas the others were formula fed before the start of the intervention. It is possible that full or partial breast feeding for days or weeks can have long term effects on the development of the intestinal micro-flora and therefore might have influenced the study results. Additionally, because the infants showed a wide variation in age at the beginning of the study, any age effects cannot be completely ruled out and might have interfered with the outcome variables.

### Effects of prebiotics on faecal SIgA

Secretory immunoglobulin A (SIgA) is one of the most abundant immunoglobulins in the human body. It is the predominant immunoglobulin in mucosal surfaces and the main constituent of the humoral immune response. SIgA plays an important role in the defence of the gastrointestinal tract. It inhibits adherence and invasion of potentially harmful antigens into mucosal tissues and neutralizes toxins and virulence factors from microbial pathogens (48). It is well established that the level of faecal SIgA antibody correlates with higher virus-neutralizing capacity and increased viral clearance (49). It is thought that the intestinal SIgA response is highly influenced by the intestinal microflora. For instance, the development of the IgA producing plasmablasts (B-cell precursor) in the intestinal mucosa seems to be affected by components of the intestinal microflora (50). Studies performed in germ free animals showed that colonization leads to the development of the gut associated lymphoid tissue (GALT), including SIgA secretion in the intestine (51,52). Moreau *et al.*, indicated that especially the presence of *Bifidobacterium* in the infant's intestine is important for the synthesis of SIgA specifically directed against viral enteropathogens. They suggested that foods promoting bifidobacteria in the intestine could be instrumental in promoting a beneficial effect on health (53).

Prebiotics and probiotics are novel concepts in the nutritional area, used to selectively change the intestinal microflora towards a potentially more healthy flora mainly by increasing the number of bifidobacteria and/or lactobacilli (17,54). Several studies reported that supplementation of food with prebiotics increases SIgA response to several 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 (55-59). In a recent study 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 (pIgR) expression compared to control mice (60). Hosono *et al.* showed that FOS stimulated the growth of intestinal lactobacilli and increased IgA secretion by Peyer's patches in a dose dependent way. It is thought that FOS stimulate the mucosal immune cells by promoting an increase in bacterial components like peptidoglycan and polysaccharides, derived from gram-positive bacteria in the intestinal microflora (59). No studies on the effects of prebiotics on faecal SIgA concentrations in infants are available. Since the humoral immune system in the gut is not fully developed during their first months, infants depend on SIgA provided by breast milk. Thus, infants not receiving breast milk have lower SIgA levels during the first months of life and could potentially benefit from strategies to support maturation and production of mucosal SIgA.

## Rationale for prebiotics

Interest and knowledge on the use of prebiotics in infant nutrition is increasing. Until now, studies on prebiotic infant formula mainly focussed on the effects of a mixture of GOS and long chain FOS, which was designed to have a synergistic effect on the development of the intestinal microflora. However, we believe that a synergistic effect can only be truly determined when the effects of both components (GOS and FOS) have been carefully evaluated.

Additionally, the previous studies on GOS/FOS formula included groups of infants that showed a relatively large range in age. Additionally, of the

groups of infants that were fed the intervention formula, a part was previously fed breast milk and a part was previously fed on infant formula. Because age and nutrition are major factors in the development of the intestinal flora, the large age differences and different feeding previous to inclusion might have influenced the outcome variables. By starting the intervention directly after birth, the effect of previous feeding can completely be ruled out.

Finally, one of the requirements for prebiotics include that they should be able to stimulate growth and/or metabolic activity of bacteria commensally to the human colon. Therefore, next to studying the effect of prebiotics on the composition of the flora it might be very interesting to also evaluate their effects on the metabolic activity of the flora.

## Probiotics

Probiotics are originally defined as '*live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance*' (54). According to the 'Scientific Committee of Food', bacterial strains added to food can be considered as generally safe when they have been shown to survive passage through the gastrointestinal tract, proliferate in the gut during consumption and modify the intestinal milieu e.g. pH and SCFA) (61). Although the prerequisites for probiotics have recently been questioned, there is considerable interest in including probiotics in infant nutrition.

During the last decade, many potential probiotics have been proposed. Although it is clear that not all probiotics have the same positive health effects, many of them have an important role in normalizing the altered microflora, increasing intestinal permeability and improving the immune barrier functions (62). Among the probiotic agents, most commonly used genera are lactobacilli and bifidobacteria.

### ***Bifidobacterium animalis* (Bb-12)**

One of the most thoroughly studied probiotic *Bifidobacterium* strain currently on the market is *Bifidobacterium animalis* (strain Bb-12, sometimes referred to as *B. lactis*). Bb-12 survives passage through the gastrointestinal tract (33). Several studies showed that during daily ingestion of  $10^{10}$  viable Bb-12, the number of faecal bifidobacteria significantly increased (33,63,64). However, shortly after the feeding with Bb-12 ended, the numbers of faecal bifidobacterial decreased again below or close to the detection level, which indicates that the Bb-12 does not colonise the colon permanently (33).

### **Probiotics in infant nutrition**

Since a few years the number of infant formulas supplemented with probiotics is increasing. Recently, the Committee on Nutrition of the European Society of Paediatrics, Gastroenterology, Hepatology and Nutrition (ESPGHAN) published a review on the use of probiotics in infant formula, follow-on formula and special medical foods. The committee recognizes that there is evidence that some probiotic preparations have health benefits for infants but that the data on safety and clinical effects is only limited. It was recommended that further evaluation of the safety and efficacy of supplemental probiotic bacteria in infant nutrition is necessary (65). Until now, several studies on long-term consumption of Bb-12 showed that the probiotic was well tolerated and resulted in adequate growth of the infants (66-68).

### **Health effects of Bb-12 in infant nutrition**

Although several infant formulas containing Bb-12 are currently on the market, only a limited number of studies on the effect of these probiotics has been published.

In infants, most publications on Bb-12 studied their effects on incidence and duration of specific diseases like atopic eczema, gastrointestinal illness or immune parameters. Isolauri *et al.*, demonstrated that in infants

with atopic eczema, feeding a formula containing  $10^9$  cfu Bb-12/g reduced the extent, severity and subjective symptoms of atopic eczema. Skin condition significantly improved within two months and the severity of atopic eczema decreased. The probiotic group also showed a reduction in the concentration of soluble CD4 in serum, which is elevated in several diseases associated with chronic inflammation. Although it was stated that research is still in progress to determine the interaction between the intestinal microflora and immunology, in this study it was evaluated whether the documented immuno modulatory effects of probiotics provide clinical benefit. No information was given on mediating changes in microflora due to the probiotic intake (69). Kirjavainen *et al.*, showed that feeding an extensively hydrolyzed whey formula supplemented with  $10^9$  cfu Bb-12/g Bb-12 to infants with early onset atopic eczema protected them against an increase in *Bacteroides* and *E. coli* numbers during weaning. High numbers of *Bacteroides* and *E. coli* were associated with the extent of atopic sensitization (IgE), although it is unknown whether these bacteria actually promote atopic sensitization. No effect of Bb-12 supplementation on the number of bifidobacteria was found. The mechanism by which probiotic bifidobacteria may protect against atopic sensitization, without increasing the number of bifidobacteria in the intestine remains to be elucidated (70). Chouraqui *et al*, found that infants fed an acidified milk formula supplemented with *B. animalis* strain Bb-12 showed a trend for shorter episodes of diarrhoea compared with infants fed a standard formula. However, it should be noted that group sizes were very small (71).

## Effects of probiotics on faecal SIgA

Next to prebiotics, another method to increase intestinal bifidobacteria and thereby increase SIgA response is the use of probiotics. Several studies reported that supplementation of food with probiotics can increase SIgA response to several viruses and bacteria. However, as for prebiotics, most of these studies were performed in animals or *in vitro* and the mechanisms for this immune stimulation are largely unknown (55-

59). One study performed in infants, showed that daily ingestion of Bb-12 significantly increased total faecal SIgA and specific anti-poliovirus SIgA (72). Unfortunately, the study was not placebo controlled and the intervention group included only 7 children.

## Rationale for probiotics

Interest and knowledge on the use of probiotic in infant nutrition is increasing. However, knowledge about the mechanisms by which probiotics can positively affect the infants' health needs to be expanded.

First, although one of the requirements of a probiotic is the adherence and proliferation of the probiotic bacteria in the intestine, most studies focused on direct health effects of probiotics. To elucidate the mechanism by which probiotics influence health, it could be important to study whether probiotics act either directly on health parameters or indirectly by changing the composition and metabolic activity of the intestinal microflora.

Second, it is generally known that breast fed and formula fed infants have different composition of the intestinal microflora. Breast fed infants have a flora that is dominated by bifidobacteria, which is associated with positive health effects. The use of prebiotics and probiotics are both methods to positively change the composition of the intestinal microflora. By feeding formula fed infants an infant formula containing either prebiotics or probiotics, it might be possible to stimulate the intestinal microflora towards the composition and metabolic activity of the gut flora found in breast fed infant.

Additionally, it has been found that development of the intestinal microflora is closely correlated with the ability of the intestinal immune system to fully develop and protect the host against potential pathogens. Formula fed infants that cannot depend on the SIgA from the mother through her breast milk, might benefit from a formula that can increase intestinal SIgA secretion.

## Aim and outline of this thesis

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In multiple ways, breast feeding protects infants from infectious disease. Given the fact that different components, including human milk oligosaccharides, have a modulating effect on the intestinal flora towards a bifidobacterial flora, it is relevant to aim for infant formula exerting the same stimulating effect on bifidobacteria. We hypothesized that by adding either prebiotics or probiotics to infant formula it is possible to significantly increase the relative number and metabolic activity of bifidobacteria and lactobacilli in the intestinal microflora of infants. Additionally, we hypothesized that pre- and probiotics have a positive effect on developing appropriate immune response against pathogens, e.g. by increasing intestinal SIgA response. We conducted two studies with the objective to investigate the effects of infant formulas containing either prebiotics or probiotics on:

- The composition of the intestinal microflora e.g. % of bifidobacteria and % of lactobacilli,
- Metabolic activity of the intestinal microflora e.g. relative amounts of faecal SCFA, concentration of faecal lactate and faecal pH and
- Indicators of development of the secretory immune response e.g. faecal SIgA concentration

Chapter 2 focuses on the effects of infant formula containing a mixture of GOS/FOS, infant formula containing viable bifidobacteria (Bb-12) and standard non-supplemented infant formula on the composition and metabolic activity of the intestinal microflora. In chapter 3 we described the effects of infant formula containing GOS alone on the the flora. In chapter 4, by employing a new PCR method, we tested whether the infant formulas mentioned in chapter 2 and 3 also have a stimulating effect on lactobacilli. In chapter 5 we reported on the effect of the formulas described in chapter 2 on faecal SIgA secretion. Finally, the main results, critical methodological issues, safety aspects, suggestions for future research and a general conclusion are included in chapter 6.

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# 2

## Effects of infant formula containing a mixture of galacto- and fructo oligosaccharides or viable *Bifidobacterium animalis* on the intestinal flora during the first 4 months of life

Astrid Bakker-Zierikzee

Martine Alles

Jan Knol

Frans Kok

Jules Tolboom

Jacques Bindels

*Submitted for publication*

# Abstract

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## Objective

To compare the effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the composition and metabolic activity of the intestinal microflora.

## Design

Before birth, infants were randomised and double blindly allocated to one of three formulas. The prebiotic (GOS/FOS) group (n=19) received regular infant formula supplemented with a mixture of 0.6g/100ml galacto-oligosaccharides and fructo-oligosaccharides. The probiotic (Bb-12) group (n=19) received the same formula supplemented with  $3.5 \times 10^9$  viable cells of *Bifidobacterium animalis*/100ml. The standard group (n=19) received unsupplemented regular formula. A group of 63 breast fed infants was included as a reference group. Faecal samples were taken at postnatal day 5, 10, week 4, 8, 12 and 16.

## Results

The GOS/FOS formula group compared to the Bb-12 and standard group showed higher ratios acetate ( $82.2 \pm 5.3\%$ ,  $69.7 \pm 2.7\%$  and  $69.9 \pm 3.9\%$  mean  $\pm$  SEM at 16w,  $p < 0.05$ ), higher concentrations of lactate ( $34.7 \pm 10.7$ ,  $11.3 \pm 7.9$  and  $3.1 \pm 2.3$  mmol/kg faeces) and lower pH ( $5.6 \pm 0.2$ ,  $6.6 \pm 0.2$  and  $7.1 \pm 0.2$ ,  $p < 0.05$ ). Differences in % bifidobacteria between the GOS/FOS ( $59.2 \pm 7.7\%$ ; at 16w), Bb-12 ( $52.7 \pm 8.0\%$ ) and the standard group ( $51.8 \pm 6.4\%$ ) were not statistically significant.

## Conclusions

Feeding infants GOS/FOS formula resulted in a similar effect on metabolic activity of the flora as in breast fed infants. In the Bb-12 group, composition and metabolic activity of the flora were more similar to the standard formula group.

## Introduction

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In breast fed infants, intestinal microflora is dominated by bifidobacteria. In general, formula fed infants have a more diverse flora (1-5). Fermentation by intestinal microflora results in the production of short chain fatty acids (SCFA), which have different functions like energy source for colonocytes, regulation of cell growth, lowering intestinal pH and inhibition of the growth of pathogens (6). Branched SCFA, products of protein breakdown by intestinal bacteria are potentially harmful. In breast fed infant, the microflora produces high amounts of acetate and lactate, which in combination with a lower pH restricts the growth of potential pathogens like *Escherichia coli* and *Clostridium perfringens* (6,7). In formula fed infants, relatively high amounts of propionate and butyrate are found. Complex neutral oligosaccharides have been identified as the most likely prebiotic factor in human milk that stimulates the growth of bifidobacteria in the gut of infants (8,9). Prebiotics are defined as 'non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (10). Like human milk oligosaccharides, prebiotics in infant nutrition stimulate the growth of bacteria that are already present in the large intestine. Several investigators have reported on approaching the prebiotic effect of human milk oligosaccharides by using a mixture of 90% galacto-oligosaccharides (GOS) and 10% fructo-oligosaccharides (FOS) in regular infant formula (11,12). It was found that feeding infants GOS/FOS formula significantly increased the number of bifidobacteria (12). Besides prebiotics, another approach to improve the intestinal microflora is adding probiotics to infant formula (13). Probiotics are originally defined as 'live microbial food supplements, which beneficially affect the host animal by improving its intestinal microbial balance' (14). The possible role of specific probiotic bacteria in the recovery from atopic disease and treatment of rotavirus diarrhoea in children was elucidated in several studies (15,16). Until now only one study focused on the effects of probiotics on the

intestinal microflora of infants (13). Recognizing the possible health benefits of a intestinal flora dominated by bifidobacteria, the question is whether pre- or probiotics should be used to reach the best possible effect. In the present study, we investigated the effects of adding either prebiotic oligosaccharides or probiotic bacteria to the same standard infant formula, on the composition and metabolic activity of the intestinal microflora in infants. To make an optimal comparison of the prebiotic and probiotic formula, the study is performed in one population under comparable environmental conditions. We hypothesized that infants fed either prebiotics or probiotics will develop an intestinal microflora dominated by bifidobacteria. Bifidobacteria produce acetate and lactate, which have a lowering effect on intestinal pH. Therefore, we expected to find, similar to breast fed infants, a lower pH and higher ratios of acetate and higher amounts of lactate in the faeces of these infants, compared to infants fed standard formula.

## Subjects and methods

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### Subjects

63 Pregnant women who had decided to breast-feed 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 three days after delivery. The study was approved by the ethical committee of the Medical Centre St. Radboud, Nijmegen, the Netherlands. Written informed consent was obtained from the parents before enrolment in the study.

### Feeding groups

Infants of mothers who decided not to breast-feed, were randomly and double blindly allocated to one of three formula groups (GOS/FOS, Bb-12 or standard). Randomisation included a block size of three and was carried

out by a person not involved in the study. The formula tins containing the different products were coded using a number the infants received at inclusion. The standard formula group (n=19) received a regular, non-supplemented infant formula (Nutrilon I, Nutricia, the Netherlands). The main compositional data of the standard formula at standard dilution of 13.1 g/100ml are given in table 1. The prebiotic formula group (GOS/FOS; n=19) received the same standard infant formula supplemented with a mixture of 0.6 g/100ml transgalacto-oligosaccharides (GOS; Vivinal GOS, Borculo Domo Ingredients, Zwolle, the Netherlands) and fructo-oligosaccharides (FOS; Raftiline HP, Orafti 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 milk (17). The probiotic formula group (Bb-12; n=19) received the standard infant formula supplemented with  $6.0 \times 10^9$  viable cells *Bifidobacterium animalis* per 100ml (Bb-12; Christian Hansen Ltd., Hørsholm, Denmark). *B. animalis* strain Bb-12 (sometimes referred to as *B. lactis*) is a thoroughly investigated probiotic and has been found to survive passage through the gastrointestinal tract of adults and infants (13,18,19). Several studies demonstrated that during a period of daily ingestion of viable Bb-12 cells, the number of faecal bifidobacteria significantly increased (18,20,21).

The shelf life of the probiotic formula was tested during storage. After 12 months of storage  $1.0 \times 10^{10} \pm 0.5 \times 10^{10}$  cfu of *B. animalis* were recovered. The study formulas were fed *ad libitum* during the study period. Mothers were instructed to heat the water to a temperature of maximal 45°C before adding the milk powder. This was to avoid hot spots in the liquid milk during micro waving, possibly leading to killing of the bacteria. Mothers who decided to breast-feed were stimulated to continue breast feeding during the course of the study and were supported by a lactation consultant when needed. At termination of breast feeding their infants received one of the three formulas. Compliance was assessed by counting the number of unused formula tins during each visit and comparing the amount of consumed formula with the recorded food intake.

**Table 1. Composition of the study formulas per 100ml**

		Standard formula	GOS/FOS formula	Bb-12 formula
Energy	kcal	67	67	67
Protein	g	1.4	1.4	1.4
Casein/whey ratio		40/60	40/60	40/60
Fat	g	3.5	3.5	3.5
Total carbohydrates	g	7.5	7.5	7.5
GOS	g	-	0.54	-
FOS	g	-	0.06	-
Lactose	g	7.5	6.7	7.5
Glucose	g	-	0.2	-
<i>B. animalis</i> (Bb-12)	cfu	-	-	6.0x10 <sup>9</sup>
Calcium	mg	54	54	54
Phosphorus	mg	27	27	27
Magnesium	mg	5	5	5
Sodium	mg	19	19	19
Potassium	mg	68	68	68
Chloride	mg	43	43	43
Iron	mg	0.5	0.5	0.5
Zinc	mg	0.5	0.5	0.5

## Questionnaires

Demographic, clinical and anthropometrical data of the mother are 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 day 5, 10, 28 and once every 4 weeks thereafter until the end of the study.

## Faecal samples

Parents were asked to take faeces samples from their infants, at postnatal day 5, 10, 28 and once every 4 weeks thereafter. The samples were taken from the diaper, as soon as possible after defaecation, collected in faeces containers (Greiner Labortechnik, the Netherlands) and stored immediately at  $-20^{\circ}\text{C}$  by the parents. During the study period, participants were visited by the investigators regularly, to collect faeces samples and questionnaires. Infant formula was supplied on request. Faeces samples were transported in a portable freezer (minimal temperature  $-15^{\circ}\text{C}$ , MRFD-015, Veba Meditemp, the Netherlands) to the laboratory.

## Preparation of faecal samples

For the determination of SCFA, 1g of the samples was thawed in ice water, diluted 10x in MilliQ and homogenized for 10 minutes using a stomacher (IUL Instruments, Barcelona, Spain). 350  $\mu\text{l}$  Homogenized faeces was mixed with 200  $\mu\text{l}$  5% (v/v) formic acid, 100  $\mu\text{l}$  1.25 g/l 2-ethylbutyric acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 350  $\mu\text{l}$  MilliQ. The samples were centrifuged for 5 minutes at 15,000xg to remove large particles and the supernatant was stored at  $-20^{\circ}\text{C}$ . For the FISH analysis and lactic acid measurements, the 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  $-20^{\circ}\text{C}$ .

## Fluorescent In Situ Hybridisation

FISH analysis was performed as described (5,22,23) with some slight modifications. Paraformaldehyde fixed samples were applied to gelatine coated glass slides (PTFE coated 8-wells [1cm<sup>2</sup>/well] object slides, CBN lab suppliers, Drachten, The Netherlands) and air-dried. The dried samples were dehydrated in 96% ethanol for 10 minutes. Hybridisation buffer (20mM Tris-HCl, 0.9M NaCl, 0.1% SDS [pH 7.2]) with 10 ng/μl Cy3 labelled *Bifidobacterium* specific probe Bif164mod (5'-CAT CCG GYA TTA CCA CCC), was preheated and added to the dried samples. Bif164 mod is modified version of probe S-G-Bif-0164-a-A-18 (23), which detects the presence of bifidobacteria including the *B. animalis* species (24). The slides were incubated overnight in a dark moist chamber at 50°C. After hybridisation the slides were washed for 30 minutes in 50 ml preheated washing buffer (20mM Tris-HCl, 0.9M NaCl [pH 7.2]) and briefly rinsed in MilliQ. For staining all bacteria, the samples were incubated with 0.25 ng/μl 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at room temperature. After DAPI-staining the slides were briefly rinsed in MilliQ, dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and covered with a coverslip. The slides were automatically analysed using an Olympus AX70 epifluorescence microscope with automated 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, U.S.A.) and counting all bifidobacteria using a Cy3 filter set (41007, Chroma Technology Corp., Brattleboro, U.S.A.).

## Short chain fatty acid analysis

The SCFA acetic, propionic, *n*-butyric, iso-butyric and *n*-valeric acids were quantitatively determined by a Varian 3800 gas chromatograph (GC) (Varian, Inc., Walnut Creek, U.S.A.) equipped with a flame ionisation 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., USA) 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 10 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. SCFA concentrations were determined using 2-ethylbutyric acid as an internal standard. Faecal SCFA concentrations are dependent on the consistency of stools.

## Lactate

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

## pH

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

## Data analysis

Prior to the study, power calculations showed that to detect a difference in percentage of bifidobacteria between the intervention formula groups (GOS/FOS and Bb-12) and the standard formula group of 30% with a SD of

25%, 13 infants per group should be included. Because of an expected drop out of 30% in the formula groups, more infants than calculated were included in the study. Statistical package SPSS (version 11.0) was used for statistical analysis of the results. All values were checked for normality by visual inspection of the normal probability plots. Differences in percentage bifidobacteria, pH, relative amounts of SCFA and lactate between the groups were tested for significance using analysis of variance. In case of a significant difference ( $p < 0.05$ ), groups were compared by using the Bonferroni *post hoc* test.

Because it is not possible to double blindly assign breast- and bottle feeding and to ensure adequate randomisation, no statistical analyses were performed to compare the breast feeding group with any of the formula feeding groups. When an infant changed from breast- to formula feeding, it was considered a drop-out and only the samples taken during the period of complete breast feeding were included in the study. Samples taken after the switch from breast- to formula feeding were not included in the study.

## Results

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In total, 120 infants were included in the study between January 2000 and May 2003. 57 Infants started on formula directly after birth and were equally divided among the formula groups. Of the 63 infants that were breast fed after birth, 24 switched to formula feeding before the age of 16w and 5 infants dropped out. The characteristics of the participants are shown in table 2. In the formula groups, 13 infants dropped out within the first 16 weeks after birth: 4 in the standard group, 5 in the GOS/FOS group and 4 in the Bb-12 group. Reasons for drop out included: colic's, suspicion of cows' milk allergy, constipation and practical problems.

## Faecal bifidobacteria

The percentages of bifidobacteria in faeces of the feeding groups at the age of 5d, 10d, 4, 8, 12 and 16 weeks, are shown in figure 1. Although not statistically significant, the GOS/FOS group tends to have higher percentages of bifidobacteria from total bacteria at all ages compared to the standard and Bb-12 groups. Percentages of bifidobacteria in the formula groups are comparable to those found in the breast fed group.

**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 (n)	Male	5	12	10	33
	Female	14	7	9	30
Place of birth (n)	At home	7	8	10	40
	Hospital	12	11	9	23
Delivery (n)	Vaginal	14	16	18	59
	Caesarean	5	3	1	4
Birth Weight (g±SD)		3601± 501	3318± 602	3481± 524	3651± 601

## pH

The pH values measured in the faeces of the formula fed infants are shown in figure 2. Lowest pH was found in infants fed on breast milk. Faecal pH of faeces of infants fed the GOS/FOS formula was lower than the standard ( $p < 0.05$  at all ages except day 5)) and the Bb-12 formula group ( $p < 0.05$  from week 8 on). On age 10 days, the pH of infants fed on the Bb-12 formula was significantly lower compared to the standard formula group ( $p = 0.001$ ).

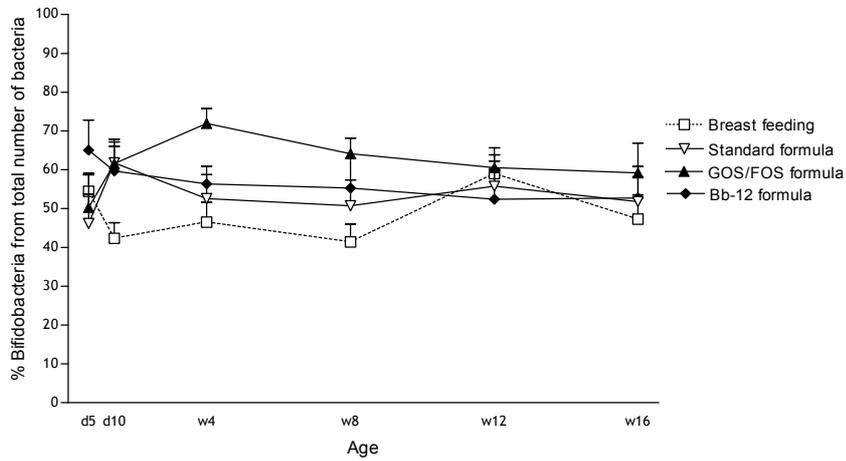


Figure 1: Percentage of bifidobacteria (mean (SEM)) per gram of wet weight faeces in infants fed on breast milk, GOS/FOS formula, Bb-12 formula and standard formula, between birth and 16 weeks of age. d=day, w=week. No statistically significant differences ( $p < 0.05$ ) were found.

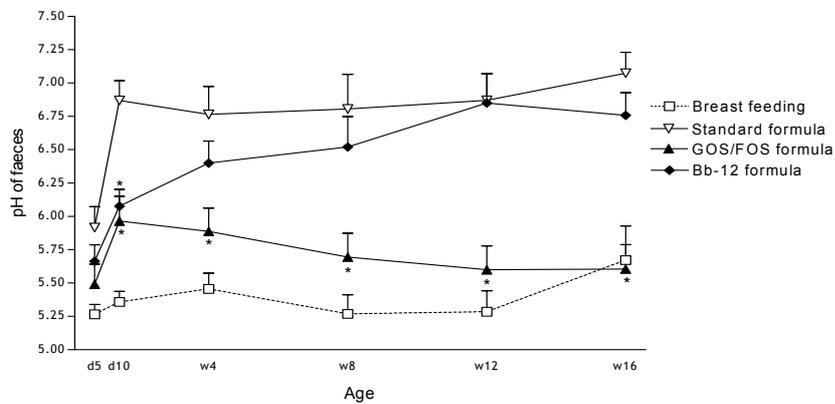


Figure 2: pH values of faeces (mean (SE)) from infants fed on breast milk, GOS/FOS formula, Bb-12 formula or standard formula, between birth and 16 weeks of age. D=day, w=week, \*statistically significant different from the standard formula group ( $p < 0.05$ ).

## Short chain fatty acids

The total amount of SCFA in the faeces is shown in table 3. The percentage of the different short chain fatty acids (SCFA) from total amount of SCFA, are shown in table 4. The table includes data from all available faeces samples that were large enough (0.5ml) to perform the SCFA and analysis. There are no statistically significant differences found in total SCFA concentration between the formula groups. However, already after 10 days, differences in the SCFA profiles can be seen between infants fed on GOS/FOS formula, compared to infants fed on standard or Bb-12 formula. Infants fed the GOS/FOS formula, have higher percentages of acetate and lower percentages of propionate, butyrate and iC4-5 SCFA (iso-butyrate, iso-valerate and valerate) when compared to infants fed the standard or the Bb-12 formula. There are no differences in the relative amounts of the SCFA in faeces of infants fed a Bb-12 formula compared to infants fed the standard formula.

## Lactate

The concentrations of lactate (mmol/kg faeces) of all groups are shown in table 3. Already from 5 days of age, the GOS/FOS formula group (not sign.) and the groups fed on breast milk have higher amounts of lactate compared to the standard and Bb-12 formula group. There are no differences found between the Bb-12 group and the standard formula group.

**Table 4.** Relative amount of short chain fatty acids (% of total SCFA) between birth and age 16w

	5 days	10 days	4 weeks	8 weeks	12 weeks	16 weeks	
Standard formula	Acetate	n=12 84.3 ± 3.4 <sup>‡</sup>	n=15 70.9 ± 2.0	n=12 71.8 ± 2.8	n=14 74.6 ± 2.9	n=14 73.9 ± 2.9	n=12 69.9 ± 3.9
	Propionate	12.9 ± 3.2	21.3 ± 2.6	17.8 ± 3.3	16.4 ± 2.0	17.8 ± 2.1	19.6 ± 2.7
	Butyrate	1.7 ± 0.5	4.6 ± 1.1	5.0 ± 1.1	6.1 ± 1.2	5.0 ± 0.9*	5.6 ± 0.9
	Sum iC4-5	1.1 ± 0.4	3.2 ± 0.5	5.5 ± 2.6	2.9 ± 0.7	3.2 ± 0.5	4.9 ± 0.8
GOS/FOS formula	Acetate	n=11 85.8 ± 5.1	n=16 84.0 ± 2.4	n=15 77.7 ± 2.2	n=16 83.5 ± 2.7 <sup>†</sup>	n=13 86.5 ± 2.1	n=14 82.2 ± 5.3
	Propionate	12.0 ± 4.7	13.5 ± 2.3	15.4 ± 2.0	11.4 ± 2.1 <sup>†</sup>	11.2 ± 1.8	14.3 ± 4.9
	Butyrate	0.5 ± 0.3	1.4 ± 0.4	5.8 ± 2.2	3.7 ± 1.2	1.2 ± 0.3	2.1 ± 0.5
	Sum iC4-5	1.7 ± 0.7	1.1 ± 0.4	1.1 ± 0.3	1.4 ± 0.4 <sup>†</sup>	1.0 ± 0.4	1.5 ± 0.4
Bb-12 formula	Acetate	n=16 80.0 ± 3.8	n=12 73.7 ± 4.0	n=14 75.6 ± 3.4	n=16 71.9 ± 2.5	n=13 66.1 ± 3.4	n=15 69.7 ± 2.7
	Propionate	16.3 ± 3.6	22.3 ± 3.3	18.7 ± 3.3	21.6 ± 1.9	24.6 ± 2.3	21.6 ± 1.9
	Butyrate	1.4 ± 0.4	2.4 ± 1.0	2.9 ± 0.7	3.2 ± 0.6	5.1 ± 0.8	5.9 ± 1.0
	Sum iC4-5	2.3 ± 0.8	1.7 ± 0.7	2.8 ± 1.2	3.3 ± 0.5	4.2 ± 1.0	2.8 ± 0.6
Breast milk	Acetate	n=32 89.5 ± 1.8	n=33 89.3 ± 1.9	n=28 91.0 ± 1.8	n=22 91.2 ± 1.6	n=24 86.1 ± 3.3	n=17 89.7 ± 2.7
	Propionate	7.0 ± 1.5	5.8 ± 1.3	4.3 ± 1.2	5.4 ± 1.4	7.5 ± 2.2	6.4 ± 2.1
	Butyrate	1.6 ± 0.4	2.3 ± 0.5	2.6 ± 0.6	1.9 ± 0.5	3.0 ± 0.7	1.6 ± 0.4
	Sum iC4-5	2.0 ± 0.4	2.6 ± 0.4	2.1 ± 0.4	1.6 ± 0.3	3.5 ± 0.8	2.2 ± 0.5

**Table 3.** Concentrations (mmol/kg faeces) of lactate and total SCFA<sup>1</sup> between birth and age 16w

	5 days	10 days	4 weeks	8 weeks	12 weeks	16 weeks	
Standard formula	Lactate	14.0 ± 7.7 (13) <sup>1</sup>	4.7 ± 3.0 (19)	5.5 ± 3.0 (16)	7.5 ± 6.4 (15)	13.3 ± 8.9 (15)	2.7 ± 1.3 (14)
	Total SCFA	54.7 ± 12.6 (12)	62.0 ± 7.9 (15)	68.3 ± 10.3 (13)	76.5 ± 13.2 (14)	73.9 ± 11.9 (14)	68.6 ± 14.0 (12)
GOS/FOS formula	Lactate	10.7 ± 4.3 (14)	12.5 ± 3.7 (18)	14.6 ± 4.4 (17)	27.8 ± 5.3 (18)	27.3 ± 7.7 (13)	40.9 ± 10.7 (14)
	Total SCFA	56.5 ± 7.7 (11)	62.3 ± 7.4 (16)	83.1 ± 8.8 (15)	76.0 ± 8.4 (16)	76.1 ± 12.2 (13)	67.7 ± 11.7 (14)
Bb-12 formula	Lactate	13.9 ± 4.8 (19)	8.2 ± 3.8 (17)	5.3 ± 1.8 (17)	14.9 ± 6.6 (17)	3.6 ± 2.1 (16)	6.1 ± 4.2 (15)
	Total SCFA	60.7 ± 6.2 (16)	58.3 ± 9.2 (12)	88.3 ± 11.5 (14)	91.9 ± 15.8 (16)	76.7 ± 11.9 (13)	98.2 ± 16.7 (15)
Breast milk	Lactate	13.7 ± 2.8 (43)	15.5 ± 3.3 (44)	22.9 ± 4.4 (35)	31.6 ± 5.3 (31)	42.5 ± 7.2 (26)	45.2 ± 9.0 (22)
	Total SCFA	48.7 ± 4.4 (32)	54.7 ± 4.9 (33)	59.8 ± 4.8 (28)	62.8 ± 5.4 (22)	60.4 ± 4.9 (24)	59.2 ± 6.9 (17)

<sup>1</sup>Sum of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate<sup>2</sup>mean ± SEM (n)

## Discussion

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In the aim to stimulate the typical intestinal microbial ecology of breast fed infants in formula fed infants, our study compares the effects of adding either prebiotics or probiotics to a standard infant formula. We found that infant formula containing a mixture of GOS and long chain FOS induced a metabolic activity of the intestinal microflora similar to that in breast fed infants (high acetate, lactate and low pH). We also observed that infant formula containing viable bifidobacteria induced a metabolic activity comparable to that in infants fed standard infant formula (SCFA pattern typical for mixed type flora, neutral pH).

To investigate whether infant formula containing pre- or probiotics can induce an intestinal microflora comparable to that in breast fed infants, one should ideally compare results of both formula groups to those observed in the breast fed group. However, we did not statistically compare the breast feeding group to the formula feeding groups because it is not possible to randomise and double blindly assign infants to breast feeding and further because of the obvious selection bias due to social and educational differences between breast- and formula feeding mothers (25). By limiting statistical analysis to the formula groups, we are still able to compare the effect of the prebiotic and probiotic component on gut flora.

Although all formulas were well accepted and tolerated, 13 of the 57 formula fed infants dropped out before age 16 weeks. The number of dropouts was not significantly different ( $p=0.334$ ) between groups. The reasons given for dropping out were similar between the feeding groups, which therefore did not give cause to selection bias.

Despite different product compositions and a the somewhat lower dosage of GOS/FOS in our study (0.6 g/100ml), the percentages of bifidobacteria found in the GOS/FOS group (64% at 8 weeks of age) are similar to the percentages observed by Schmelzle *et al.* (76% after 6 weeks 0.8 g/100ml)(26), Knol *et al.*, (69% after 6 weeks 0.8 g/100ml) (27), and Knol

*et al.*, (65% after 6 weeks 0.8 g/100ml) (28). For unknown reasons, the percentage of bifidobacteria in the standard formula group found in our study is higher than that observed in previous studies. Differences in composition of the formula (other than the carbohydrates) may play a role.

Results of the probiotic group are difficult to compare to other studies, because until now, only one study reported the effect of a probiotic infant formula on the intestinal microflora but it did not give any quantitative data on the stimulation of bifidobacteria (13).

In the present study, we found that feeding infants infant formula supplemented with a GOS/FOS mixture has a marked effect on the metabolic activity of the intestinal microflora. In general, the pH and relative amounts of SCFA in faeces of our breast-fed and the standard formula group are similar to the findings of others (29-31).

The relative amounts of SCFA and lactate concentration and pH in the faeces of the GOS/FOS group are comparable to the concentrations seen in breast fed infants. High relative amounts of acetate, high concentration of lactate, together with the low faecal pH, creates conditions that are less favourable for *Enterobacteriaceae* and other potential opportunistic pathogens that can be present in low numbers or obtained from the environment (colonisation resistance) (32,33). As in breast fed infants, the relative amounts of faecal butyrate and propionate in the GOS/FOS group are lower compared to the standard and Bb-12 groups. This indicates that in the breast milk and GOS/FOS fed groups, the intestinal microflora contains lower numbers of butyrate- and propionate- producing bacteria like *Clostridium* and *Bacteroides*. Butyrate is an important fuel for the colonic mucosa in adults, where it stimulates intestinal mucosal cell proliferation, but because of low faecal levels in breast fed infants, it is considered less important for infants. The low faecal concentration of iC4-5 SCFA (iso-butyrate, iso-valerate and valerate) and low faecal pH that was seen in the GOS/FOS and breast fed group indicate that protein fermentation by, for instance, *E. coli* is low.

The major difference to other studies using the GOS/FOS mixture is the relatively high percentage of bifidobacteria in the standard formula group, which prevented us from finding statistically significant differences in bacterial composition. This finding was rather unexpected and warrants further discussion. The clear difference between the standard formula groups and the prebiotics group with respect to all parameters of intestinal microbial metabolic activity including pH, and the similarity with the findings in the breast fed group, indicates a distinct effect of the GOS/FOS mixture. A possible explanation for the discrepancy between the findings in metabolic activity and those of microbial analysis may be found in the presumption that the mixture of GOS/FOS used in our study predominantly stimulated the growth of other lactic acid-producing bacteria like *lactobacillus* (3,29). In fact the prebiotic mixture, containing low as well as high molecular mass oligosaccharides, was designed to create optimal growth conditions for both bifidobacteria and lactobacilli (11). For the group of lactobacilli no specific FISH probe is available and for the accurate quantification of Lactobacilli new methods need to be developed. Another possibility is that although high numbers of bifidobacteria were present in the standard and Bb-12 formula groups, the metabolic activity of the bacteria was low due to limiting substrate availability. Small amounts of lactose that escaped digestion in the small intestine might have stimulated bifidobacterial growth in the standard and Bb-12 formula groups without providing sufficient amounts of substrate for full-blown metabolic activity (34). This could have led to less acetate and lactate production and subsequent higher pH. Different from traditional plating methods, the FISH method used in this study does not make a distinction between metabolically active and non-active bacteria (5).

In the Bb-12 group the percentage of bifidobacteria was already very high after 5 days (65%), but declined during the first 16 weeks of life to 53% and no significant differences with the other groups were found. This initial rapid colonization might be expected because space and nutrients are not limiting. However, based on the metabolic activity parameters showing a closer proximity of the Bb-12 group to the standard formula

group, we conclude that a strong bifidogenic effect of the Bb-12 formula is unlikely.

In our study adding viable *B. animalis* strain Bb-12 to a standard infant formula did not have a distinct effect on the number of bifidobacteria and metabolic activity of the intestinal microflora. Nevertheless, several studies showed that specific probiotic bacteria including strain Bb-12 may have a role in prevention and treatment of different diseases (15,35,36). According to the original definition by Fuller (14), probiotics should change microbial balance to have a health effect. Our results do not support such an effect on microflora of a widely used probiotic strain (*B. animalis*, Bb-12). It is possible that health effects of the probiotic strain Bb-12 already occur in the small intestine or do not require major changes in the intestinal microflora more distally.

In conclusion, feeding infants a formula containing the prebiotic GOS/FOS mixture resulted in high relative amounts of faecal acetate and concentration of faecal lactate and a low faecal pH. In the infants who received the standard formula or the formula with added viable cells *B. animalis* strain Bb-12, a similar microflora and metabolic activity were found. Comparison of the results of the formula groups with the breast fed group reveals a similar effect on metabolic activity of the intestinal flora in the prebiotic formula group only. The observed shift from a more proteolytic (putrefaction) to a more saccharolytic colon physiology could be considered a health benefit for the infant.

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Effects of an infant formula  
supplemented with galacto-  
oligosaccharides on the  
composition and metabolic activity  
of the intestinal microflora

Astrid Bakker-Zierikzee

Martine Alles

Frans Kok

Jan Knol

Jules Tolboom

Jacques Bindels

*Submitted for publication*

# Abstract

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## Objectives

To study the effects a standard infant formula containing galacto-oligosaccharides (GOS) on the composition and metabolic activity of the intestinal microflora.

## Methods

At birth, infants of whom the mother had decided not to breast-feed, were at random and double blindly allocated to one of two formula groups. The GOS formula group (n=17) received an infant formula supplemented with 0.6g/100ml GOS. The standard group (n=17) received the same infant formula without oligosaccharides. Faecal samples were taken at postnatal days 5 and 10 and week 4, 8, 12 and 16. Percentage of bifidobacteria, relative amounts of short-chain-fatty acids (SCFA), lactate concentration and pH were measured in faeces.

## Results

Differences in percentage of bifidobacteria that are found between the GOS ( $59.2 \pm 7.7\%$ ; mean  $\pm$  SEM at 16 weeks of age) and the standard group ( $51.8 \pm 6.4\%$ ) were not statistically significant. Only at 4w the relative amounts of acetate and butyrate were slightly but significantly different between the formula groups. No significant differences were found in lactate concentration and faecal pH.

## Conclusions

We conclude that infant formula containing (0.6 g/100ml) GOS as the only source of non-digestible oligosaccharides does not have a marked effect on the composition or the metabolic activity of the intestinal flora compared to formula without GOS. In contrast, the reference breast fed group showed a lower pH and higher ratios of acetate and lactate as compared to the cow's milk based infant formulas that were used in the study.

## Introduction

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Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides that can be fermented by bifidobacteria (1,2). Major end products of the fermentation of GOS by bifidobacteria are acetate and lactate, which can have a lowering effect on the faecal pH (3,4). Although not confirmed by all studies (5,6), several investigators found that in adults a daily dose of GOS significantly increased the number of faecal bifidobacteria (7,8) and faecal acetate concentrations (9).

In the last 4 years, a number of studies reported the effects of infant milk formulas supplemented with oligosaccharides on the faecal flora of infants. Addition of several bifidus factors to infant formula was found to stimulate the presence of bifidobacteria in the intestinal flora. Nagendra found that incorporation of 0.5% lactulose in milk formula was adequate to stimulate a bifidobacteria in the intestinal microflora of infants (10). Rueda studied the influence of supplementing an adapted milk formula with gangliosides and found this milk to significantly modify faecal flora (11). Guesry *et al.*, were not able to find any bifidogenic effect of an infant feeding containing 200, 400 or 600 mg fructo-oligosaccharides (FOS) (12). Others found that infant formula containing a mix of 90% GOS and 10% FOS resulted in an increase of bifidobacteria (13-15).

No studies were published so far on the bifidogenic effects of infant formula containing GOS alone. Available studies on GOS were performed *in vitro*, in animals or in adults and were not conclusive concerning its prebiotic effects (5,8,9,16-18).

In this study, we aimed to investigate the effect of an infant formula containing GOS on the composition and metabolic activity of the intestinal micro flora in term infants. We expected that GOS added to infant formula would be fermented rapidly and stimulate the growth of bifidobacteria. In infants fed the GOS formula, fermentation of galacto-oligosaccharides by bifidobacteria was thought to lead to higher acetate

and lactate production and a lower faecal pH compared with infants fed a standard formula.

## Materials and methods

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### Subjects

71 Pregnant women in their last trimester of pregnancy were recruited in the study. After the birth of their infant, 37 mothers started breast feeding and 34 decided to start formula feeding. Infants with normal birth weight, no congenital abnormality, congenital disease or gastrointestinal disease were enrolled within 5 days after delivery. The study was approved by the ethical committee of the University Medical Centre St. Radboud, Nijmegen, the Netherlands. Written informed consent was obtained from the parents before enrolment.

### Feeding groups

Infants of mothers who decided not to breast-feed, were at random and double blindly allocated to one of two formula groups (GOS or standard). The standard formula group (n=17) received a regular, non-supplemented infant formula (Nutrilon, Nutricia, the Netherlands). The GOS formula group (n=17) received the same basic infant formula (Nutrilon, Nutricia, the Netherlands) supplemented with 0.6 g/100ml galacto-oligosaccharides (Vivinal GOS, Borculo Domo Ingredients, Zwolle, the Netherlands). The main composition of the study formulas at standard dilution of 13.1 g/100ml is given in table 1. The study formulas were fed *ad libitum*. Mothers who decided to breast-feed were stimulated to continue breast feeding during the course of the study and were supported by a lactation consultant when needed. At termination of breast feeding their infants received one of the two intervention formulas. Compliance was assessed by counting the number of unused formula tins during each visit and

comparing this to the amount of consumed formula as recorded in the 'food-intake diary'.

**Table 1.** Composition of the study formulas per 100ml

		Standard formula	GOS formula
Energy	Kcal	67	67
Protein	g	1.4	1.4
Casein/whey ratio		40/60	40/60
Fat	g	3.5	3.5
Total carbohydrates	g	7.5	7.5
Lactose	g	6.7	7.5
Glucose	g	0.2	-
GOS		0.6	-
Calcium	mg	54	54
Phosphorus	mg	27	27
Magnesium	mg	5	5
Sodium	mg	19	19
Potassium	mg	64	64
Chloride	mg	43	43
Iron	mg	0.5	0.5
Zinc	mg	0.5	0.5

## Questionnaires

Demographic, clinical and anthropometrical data on the parents and information on the pregnancy 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 and 10, and weeks 4, 8, 12 and 16.

## Faecal samples

Parents were asked to take faecal samples from their infants, on the 6 consecutive time points, as described in the previous paragraph. The samples were taken from the diaper, as soon as possible after defecation, by using a faeces container (Greiner Labortechnik, the Netherlands) and stored immediately at -20°C.

During the study period, investigators visited the participants regularly to collect faecal samples and questionnaires. Infant formula was supplied on request. Faecal samples were transported in a portable freezer (minimal temperature -15°C, MRFD-015, Veba Meditemp, the Netherlands) to the laboratory.

## Percentage of bifidobacteria

Fluorescent in situ Hybridisation (FISH) was used to determine the relative amounts of bifidobacteria from total bacterial count and was described previously (chapter 2)

## Short chain fatty acid (SCFA), lactate and pH

The relative amounts of faecal acetic, propionic, n-butyric and n-valeric acids, the concentration of faecal lactate and the faecal pH were determined as described previously in chapter 2.

## Data analysis

Prior to the study, the required sample size was calculated based on the findings by Harmsen *et al.* (19). Assuming a difference in the number of bifidobacteria relative to the total number of bacteria of 30% with a standard deviation of 25%, a two-sided Alfa of 0.05 and a Beta of 0.2, a

group size of 13 infants was needed. Statistical analysis of the results was done using the statistical computer package SPSS (version 11.0).

All values were checked for normality by visual inspection of the normal probability plots. Differences in percentage bifidobacteria, the percentages of short chain fatty acids, lactate concentrations and pH between the groups were assessed for significance using analysis of variance. In case of a significant difference ( $p < 0.05$ ), groups were compared with the Bonferroni *post hoc* test. To be able to study the effect of the prebiotic GOS in a double blind, randomised manner, we decided to exclude the breast fed group from the statistical analysis and to only compare the GOS formula group to the placebo formula group. Data from the breast fed group are included in the tables and figures, but only given when the infant was solely fed breast milk at that time point.

## Results

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Of the 71 infants that were included in the study, 34 started on formula feeding directly after birth and these infants were equally divided among the formula groups. Mean birth weight, number of infants born at home or in the hospital, number of vaginal and caesarean deliveries and sex did not differ between the feeding groups and are shown in table 2. Although all formulas were well tolerated and accepted by the participating infants, 2 of the infants fed on standard formula dropped out before the age of 16 weeks. No infants in the GOS formula group dropped out. In the breast feeding group, 3 of the 37 infants dropped out and 22 switched to complete or partial formula feeding before the age of 16 weeks. Reasons for drop out included: colic, suspicion of cows' milk allergy, constipation and practical problems such as moving to another region or the mother resuming work.

**Table 2. Characteristics of study subjects**

		GOS Formula n=17	Standard Formula n=17	Breast milk n=37
Sex (n)	Male	9	9	17
	Female	8	8	21
Place of birth (n)	At home	6	8	23
	Hospital	11	9	15
Mode of delivery (n)	Vaginal	16	14	35
	Caesarean	1	3	3
Birth Weight (g±SD)		3531 ± 673	3487 ± 628	3626 ± 377

## Bifidobacteria

The percentages of bifidobacteria in faeces produced from birth until the age of 16 weeks are shown in figure 1. Although infants fed on GOS formula had slightly higher relative amounts of bifidobacteria compared to infants fed on standard formula from week 16 on, these differences were statistically not significant. Percentages of bifidobacteria in both formula groups were comparable with the results in the breast fed group.

## PH

The faecal pH, measured at the 6 time points between birth and 16 weeks of age is shown in figure 2. Between the formula groups, no statistically significant differences were found. Although not statistically tested, pH of infants fed on breast milk seems lower than that of infants fed on GOS formula and standard formula.

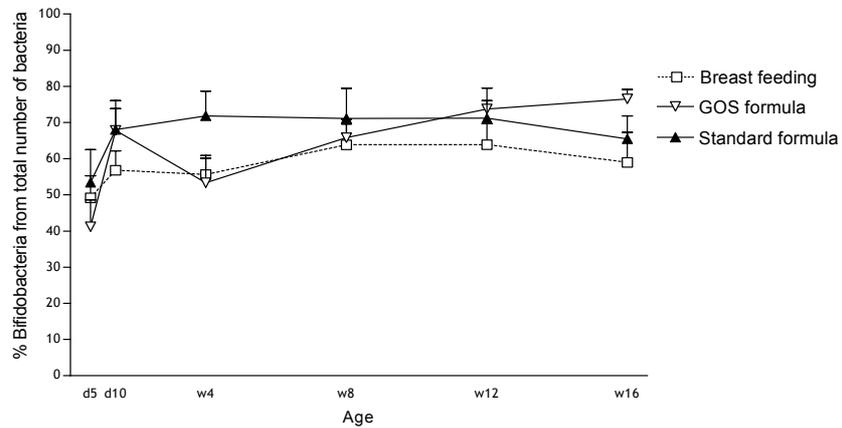


Figure 1. Percentage of bifidobacteria (mean (SEM)) per gram of wet faeces in infants fed on breast milk, GOS formula or standard formula, between birth and 16 weeks of age. d= day, w=week. No significant differences were found.

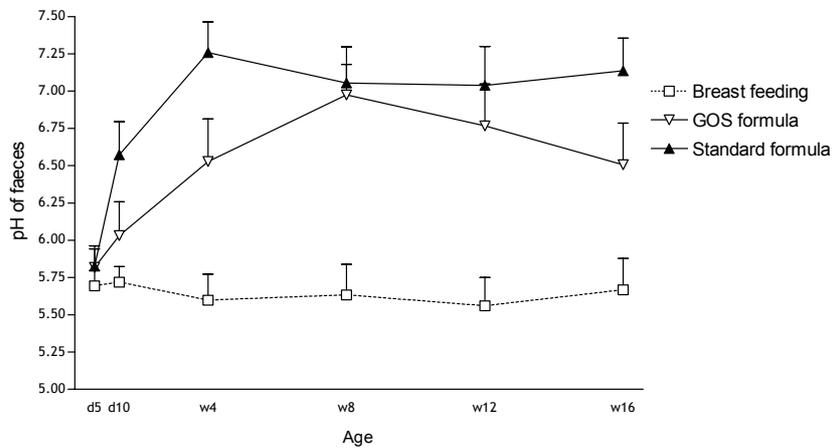


Figure 2. pH of faeces (mean (SEM)) from infants fed on breast milk, GOS formula or standard formula, between birth and 16 weeks of age. d= day, w=week. No statistically significant differences ( $p < 0.05$ ) were found.

## Short chain fatty acids

Relative amounts of acetate, propionate, butyrate and iC4-5 SCFA (sum of iso-butyrate (4-carbon), valerate (5-carbon) and iso-valerate (5-carbon)), are shown in table 4. Total concentrations of SCFA are shown in table 3. At 4 weeks of age percentage of faecal acetate in the GOS formula group was statistically significant higher ( $p < 0.05$ ) and the percentage of butyrate was statistically significant lower ( $p < 0.05$ ) compared to the standard formula group. No statistically significant differences were found between the formula groups in the relative amounts of the faecal SCFA at the other time points. Although not statistically tested, the relative amounts of SCFA of the formula groups were not comparable to the breast fed group.

## Lactate

The concentrations of faecal lactate in the three feeding groups are shown in table 3. At all time points, the concentration of total faecal lactate of the GOS group, was not significantly higher than in the standard group and did not seem comparable to the lactate concentrations in the breast fed group.

**Table 3.** Concentrations (mmol/kg faeces) of lactate and total SCFA<sup>1</sup>, between birth and age 16w

	5 days	10 days	4 weeks	8 weeks	12 weeks	16 weeks	
Standard formula	Lactate	14.0 ± 7.1 (14)	3.0 ± 1.8 (16)	0.9 ± 0.7 (16)	4.1 ± 3.7 (15)	5.0 ± 4.4 (15)	0.8 ± 0.7 (15)
	Total SCFA	68.5 ± 9.8 (16)	75.5 ± 7.7 (17)	78.1 ± 8.3 (17)	81.4 ± 5.8 (16)	70.4 ± 5.0 (15)	72.7 ± 8.6 (15)
GOS formula	Lactate	6.2 ± 3.9 (17) <sup>2</sup>	11.6 ± 6.0 (15)	20.5 ± 8.6 (15)	4.2 ± 2.6 (16)	12.2 ± 5.1 (17)	12.2 ± 5.1 (16)
	Total SCFA	63.3 ± 8.5 (16)	79.6 ± 8.9 (16)	87.9 ± 7.5 (17)	85.6 ± 8.2 (17)	86.1 ± 7.8 (17)	87.7 ± 8.0 (16)
Breast milk	Lactate	4.0 ± 1.7 (22)	4.0 ± 1.7 (22)	8.5 ± 4.1 (24)	11.2 ± 5.2 (17)	26.2 ± 6.8 (14)	21.5 ± 6.4 (12)
	Total SCFA	38.1 ± 5.1 (27)	38.1 ± 5.1 (27)	52.4 ± 8.0 (27)	56.7 ± 9.6 (19)	60.8 ± 8.7 (15)	54.1 ± 6.0 (12)

<sup>1</sup>Sum of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate<sup>2</sup>mean ± SEM (n)

**Table 4.** Relative amount of faecal SCFA (% of total SCFA<sup>1</sup> concentration) between birth and age 16w

	5 days	10 days	4 weeks	8 weeks	12 weeks	16 weeks
	n=15	n=16	n=16	n=16	n=15	n=14
Standard formula						
Acetate	81.6 ± 3.0	71.8 ± 3.5	68.4 ± 3.1	73.6 ± 2.9	74.1 ± 1.7	72.5 ± 2.6
Propionate	14.7 ± 2.7	20.5 ± 2.7	19.5 ± 2.2	16.4 ± 2.1	17.8 ± 1.7	20.4 ± 2.3
Butyrate	2.2 ± 0.7	5.4 ± 1.2	8.6 ± 2.6	6.7 ± 1.1	5.4 ± 0.8	5.0 ± 0.8
Sum of iC4-5	1.5 ± 0.6	2.3 ± 0.6	3.5 ± 0.7	3.3 ± 0.7	2.7 ± 0.5	3.3 ± 0.6
GOS formula						
	n=16	n=16	n=17	n=17	n=17	n=16
Acetate	82.0 ± 3.2 <sup>2</sup>	80.1 ± 3.0	81.8 ± 2.6 <sup>*</sup>	74.3 ± 2.0	79.2 ± 2.5	78.3 ± 2.8
Propionate	12.9 ± 2.9	16.1 ± 2.5	13.9 ± 2.0	20.4 ± 1.6	15.7 ± 1.8	16.3 ± 2.0
Butyrate	4.3 ± 2.0	2.5 ± 1.5	2.4 ± 0.7	3.0 ± 0.7 <sup>*</sup>	2.8 ± 0.7	3.3 ± 0.7
Sum of iC4-5	0.8 ± 0.3	1.3 ± 0.4	1.9 ± 0.5	2.3 ± 0.5	2.4 ± 0.6	2.1 ± 0.6
Breast milk						
	n=27	n=27	n=19	n=17	n=15	N=13
Acetate	91.1 ± 1.5	90.5 ± 1.7	90.8 ± 1.5	91.0 ± 1.9	90.4 ± 3.2	89.5 ± 3.0
Propionate	4.0 ± 0.6	3.5 ± 0.8	5.2 ± 1.2	5.1 ± 1.2	4.3 ± 1.4	6.1 ± 1.9
Butyrate	2.8 ± 0.7	2.6 ± 1.0	1.3 ± 0.3	2.3 ± 0.7	2.6 ± 1.2	3.3 ± 1.3
Sum of iC4-5	2.2 ± 0.6	3.4 ± 0.9	2.7 ± 0.7	1.6 ± 0.7	2.7 ± 1.0	1.1 ± 0.4

<sup>1</sup>Sum of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate

<sup>2</sup>mean ± SEM

<sup>3</sup>Sum of iso-butyrate, iso-valerate and valerate

<sup>\*</sup>Statistically significantly different compared to standard formula (p<0.05)

## Discussion

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In this study, we have shown that infant formula containing GOS, as the only source of non-digestible oligosaccharides, does not have the same effect on the composition and metabolic activity of the intestinal microflora as breast milk (high acetate, lactate and low pH).

Breast fed infants usually have an intestinal microflora dominated by bifidobacteria (20,21). Human milk contains complex oligosaccharides, which in combination with the nutrient composition and poor buffering capacity of human milk, are thought to create optimal conditions in the colon for the growth and activity of bifidobacteria. The high numbers of bifidobacteria in faeces of breast fed infants produce high relative amounts of faecal acetate and lactate, which in combination with the low faecal pH, creates conditions that are less favourable for *Enterobacteriaceae* and other potential opportunistic pathogens that can be present in low numbers or obtained from the environment (colonization resistance) (22,23). Galacto-oligosaccharides are short chain oligosaccharides that are not digested in the small intestine and are available as a substrate for bacteria living in the proximal colon (5,7). *In vitro* experiments show that GOS is quickly fermented by the enzymatic activity of  $\beta$ -galactosidase, which is produced by bifidobacteria but also by several other species like *Lactobacillus*, *Bacteroides*, and *Clostridium* (24). In our study, we did not find any distinct effect of GOS on the number of bifidobacteria. Possibly, the GOS entering the large intestine of the infants was not only used by bifidobacteria but also by other species (25). This was confirmed by the fact that, although the total amount of SCFA of the GOS group was little higher than the standard group, SCFA patterns was only significantly different at 4w. This indicates that although substrate availability in the GOS group might have been higher, it did not result in large differences in the flora composition.

To investigate whether an infant formula containing prebiotics can induce an intestinal microflora comparable to that in breast fed infants, one

should ideally compare results of the prebiotic formula group to those of the breast fed group. However, since it is not possible to randomise and double blindly assign breast feeding, the breast fed group cannot be compared statistically to the formula groups. Selection is likely to occur due to differences in social and educational status between breast feeding and formula feeding mothers (26). By limiting the statistical analysis to the formula groups, we were still able to compare the effect of the prebiotic component on intestinal microflora and predict the proximity to breast feeding.

In contrast to other studies, we were not able to find a higher percentage of bifidobacteria in infants fed on breast milk compared to those fed on standard formula (14,19). However, consistent with other studies were the high relative amounts of acetate and lactate, low relative amounts of propionate and butyrate and low faecal pH of our breast fed group (27,28). It is possible that other lactic acid producing bacteria like lactobacilli are responsible for the high amounts of acetate and lactate in our breast fed group. The high percentage of bifidobacteria in our standard formula could be explained by the somewhat higher lactose content of our formula. Part of the lactose might have escaped digestion and stimulated the growth of bifidobacteria in the large intestine. Another possible explanation could include geographical differences in the composition of the intestinal microflora between infants in our study and the infants in other studies that investigated intestinal microflora. Several studies showed geographic variation in the intestinal microflora throughout different parts of the world (29-31). Factors like diet and aseptic cleaning procedures during delivery are thought to play a role in these regional differences. In contrast to most other European countries, most Dutch children are born at home. The home environment compared to the aseptic environment of a hospital might affect the early colonization of intestine.

In this study, with group sizes of 15 and 16 and standard deviations of 24.7% and 10.5% at age 16 weeks for the percentage of faecal bifidobacteria, in the standard and GOS formula groups respectively, we

had an 80% chance of detecting a difference in percentage of bifidobacteria of 15%. The actual difference that we found between the formula groups was 7.4%, and our study thus lacked the power to detect this difference. The question is whether a difference of 7.4% is enough to be biologically relevant, i.e. has a profound effect on the infant's health. Earlier studies using FISH to determine the percentage of bifidobacteria in faeces of breast- and formula fed infants showed differences between 18 and 38% (14,19)(chapter 2).

Boehm *et al.* reported on the microbial effects of an infant formula containing a mixture of short chain GOS and long chain FOS (DP>10), which was selected to mimic the molecular size distribution of human milk oligosaccharides and to provide substrate for bacteria in all parts of the colon (32). It was found that the GOS/FOS formula increases faecal bifidobacteria (13). In a study by Knol *et al.*, it was shown that addition of this mix of GOS and long chain FOS not only stimulates the number of bifidobacteria in the intestinal flora, but also significantly increased the relative amount of faecal acetate and that it lowered faecal pH (in press JPGN). Several *in vitro* and *in vivo* studies showed that molecules with longer chain lengths (like inulin) are fermented more slowly and with less hydrogen excretion, compared to molecules with shorter chains (like GOS)(33-35). Slow fermentation increases metabolic activity of the microflora in the distal as well as the proximal colon. Favourable SCFA are then produced throughout the whole colon and together with the low pH can be measured in the faeces.

We conclude that an infant formula containing galacto-oligosaccharides as the only source of non-digestible oligosaccharides in a concentration of 0.6 g/100ml does not have a marked effect on the composition or the metabolic activity of the intestinal flora compared to the same formula without GOS. In contrast the reference breast fed group showed a lower pH and higher ratios of acetate and lactate as compared to the cow's milk based infant formulas that were used in the study.

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The effects of infant formulas  
containing either prebiotics or  
probiotics on the intestinal  
lactobacilli in formula fed infants

Astrid Bakker-Zierikzee

Monique Haarman

Martine Alles

Frans Kok

Jacques Bindels

Jan Knol

*Submitted for publication*

# Abstract

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## Objectives

Lactobacilli are considered to be an important group of micro-organisms in the intestinal microflora of breast fed infants. We studied the effects of infant formulas containing either prebiotics or probiotics on the percentage of faecal lactobacilli.

## Methods

At birth, infants of whom the mother had decided not to breast-feed, were at random and double blindly allocated to one of four formula groups. In total 36 infants received standard infant formula; 19 received a prebiotic formula containing a specific mixture of 0.6 g/100ml GOS/FOS (ratio 9/1), 17 received a prebiotic formula containing 0.6g/100ml GOS and 19 received a probiotic formula containing  $6.0 \times 10^9$  viable cells/100ml *Bifidobacterium animalis* Bb-12 (Bb-12). A group of 101 breast fed infants was included as a reference group. Faecal samples taken on age d5 and w12 were analysed, by using a newly developed duplex 5'nuclease PCR assay to determine the percentage of lactobacilli.

## Results

In the breast fed group, the percentages of lactobacilli increased significantly between d5 and w12 from 1.1% to 6.4%. At w12, the GOS/FOS group showed a percentage of lactobacilli that was significantly higher than in the standard formula group ( $6.1 \pm 2.6\%$  mean  $\pm$  SEM vs.  $0.9 \pm 0.4\%$ ;  $p=0.007$ ). No significant differences were found between the GOS ( $1.1 \pm 0.4\%$ ) and Bb-12 ( $2.4 \pm 1.7\%$ ) versus the standard formula group.

## Conclusions

We conclude that adding GOS/FOS to infant formula increases the percentage of faecal lactobacilli similar to the percentages found in breast fed infants, whereas standard infant formula and standard formula containing only GOS or viable bifidobacteria do not.

## Introduction

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In healthy breast fed infants, the intestinal microflora is dominated by bifidobacteria and lactobacilli, whereas formula fed infants have a more adult flora with higher counts of *Enterobacteriaceae*, clostridia and *Bacteroides* (1-3). This difference intestinal microflora between breast- and formula fed infants may have considerable health effects. Fermentation by lactobacilli and bifidobacteria in the colon leads to production of acetate and lactate (4,5). High amounts of acetate and lactate in combination with a low faecal pH, create conditions that are less favourable for *Enterobacteriaceae* and other potential opportunistic pathogens (6,7). Lactobacilli and bifidobacteria also directly interfere with potentially pathogenic micro-organisms by competing for nutrients and epithelial adhesion sites. Several studies showed that addition of specific lactobacilli may prevent or reduce the severity of diarrhoea (8,9). In addition, it is hypothesised that lactobacilli and bifidobacteria added as a probiotic supplement to infant formula may positively interfere with the development or severity of atopic diseases (10).

Because the intestinal microflora of breast fed infants is considered provide a major health benefit to infants, attempts have been made to change the composition of flora of formula fed infants towards that of breast fed infants. By supplementing infant formulas with either probiotics or prebiotics it might be possible to increase the relative number of bifidobacteria and lactobacilli in the flora of formula fed infants.

Probiotics are originally defined as '*live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance*' (11). Until now, many studies on probiotics focused on their suppressive effects on the growth of potential pathogens (12-16). The effects of certain probiotics on the growth of lactobacilli and bifidobacteria that are already present in the intestinal microflora are largely unknown. Production of organic acids creates conditions that are

favourable for the growth of bifidobacteria and lactobacilli. Our previous report did not show distinct effects of an infant formula containing viable bifidobacteria on the faecal SCFA profile, lactate concentrations and pH (17, chapter 2). However, it is possible that the probiotics established some changes in the milieu of the proximal colon that create optimal conditions for the growth of lactobacilli.

Prebiotics are defined as 'non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (18). Studies on prebiotics showed that infants fed an infant formula containing a mixture of galacto-oligosaccharides (GOS) and long chain fructo-oligosaccharides (FOS) have an intestinal microflora dominated by bifidobacteria and a metabolic activity of the flora similar to those found in breast fed infants (17,19,20). Two studies have been performed with infant formula containing GOS or FOS alone and were not able to show a distinct effect on composition and metabolic activity of the intestinal microflora (21)(chapter 3).

In infants, the effects of pre- and probiotics on the number of bifidobacteria have been studied extensively, but far less is known about their effects on the number of intestinal lactobacilli. Although the numbers of lactobacilli in the breast fed infants are much lower than the numbers of bifidobacteria, stimulation of growth of lactobacilli might have significant health effects. By using traditional plating methods, Moro *et al.*, already demonstrated that feeding infants an infant formula containing GOS/FOS resulted in a significantly higher numbers of faecal lactobacilli compared to unsupplemented formula. To study the flora in more detail, a newly developed duplex 5' nuclease assay was used in this study to specifically quantify the genus *Lactobacillus* (submitted for publication).

In this report, we present the results of two studies on the effects of pre- and probiotic infant formulas with almost identical design, and described the effects of three newly designed infant formulas containing either

prebiotics (GOS/FOS mixture or GOS) or probiotics (*B. animalis*, Bb-12) on the percentages of faecal lactobacilli. In parallel, a group of breast fed infants and a group of infants fed a standard unsupplemented infant formula were included as reference groups. We hypothesized that after 12 weeks of age, infants fed on either one of the prebiotic or the probiotic formula have higher numbers of lactobacilli in their faeces compared to infants fed the same unsupplemented infant formula.

## Materials and methods

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In this study, we analysed faecal samples of two separate but almost identical double blind, randomised intervention studies on the effects of pre- and probiotic infant formulas on the composition and metabolic activity of the intestinal microflora. Because the method and region of recruitment, composition of the standard infant formula, study design, logistics and baseline characteristics of the two studies were similar, we combined the breast fed groups, as well as standard formula groups of both studies.

### Subjects

In both studies, infants with normal birth weight, no congenital abnormality, congenital disease or gastrointestinal disease were enrolled within 3 days after delivery. Both studies were approved by the medical ethical committee of the region Arnhem-Nijmegen (Committee on Research Involving Human Subjects, located in the University Medical Centre St. Radboud in Nijmegen). Written informed consent was obtained from the parents before enrolment in the studies.

### Feeding groups

Infants of mothers who decided not to breast-feed, were randomly and double blindly allocated to one of the formula groups. Randomisation was

performed per study, included a block size of 3 (study A) or 2 (study B) and was carried out by the logistics manager of Numico Research BV, who was not involved in the study in any other way.

In the first study (chapter 2), 63 infants started on breast-feeding directly after birth; 19 infants received the regular infant formula (Standard; Nutrilon I, Nutricia, the Netherlands); 19 infants received the standard infant formula (GOS/FOS) supplemented with a mixture of 0.6 g/100ml galacto-oligosaccharides (GOS; Vivinal GOS, Borculo Domo Ingredients, Zwolle, the Netherlands) and fructo-oligosaccharides (FOS; Raftiline HP, Orafiti active food ingredients, Tienen, Belgium) in a ratio of 9 to 1 (GOS/FOS); 19 infants received the standard infant formula supplemented with  $6.0 \times 10^9$  viable cells *B. animalis* Bb-12 per 100ml (Bb-12; Christian Hansen Ltd., Hørsholm, Denmark). In the second study (chapter 3), 38 infants started on breast feeding directly after birth, 17 infants received the regular infant formula (Standard; Nutrilon I, Nutricia, the Netherlands); 17 infants received the standard infant formula supplemented with 0.6 g/100ml galacto-oligosaccharides (GOS; Vivinal GOS, Borculo Domo Ingredients, Zwolle, the Netherlands). The main compositional data of the study formulas are given in table 1.

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. Mothers were instructed not to microwave the formula or to heat the water to a temperature higher than 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 breast-feed were stimulated to continue breast feeding during the course of the study and were supported by a lactation consultant when needed.

## Questionnaires

Questionnaires on 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 day 5, 10, 28 and once every 4 weeks thereafter until the end of the study.

**Table 1. Composition of the study formulas per 100ml**

		Standard formula	GOS/FOS formula	GOS formula	Bb-12 formula
Energy	kcal	67	67	67	67
Protein		1.4	1.4	1.4	1.4
Casein/whey ratio	g	40/60	40/60	40/60	40/60
Fat	g	3.5	3.5	3.5	3.5
Carbohydrates total	g	7.5	7.5	7.5	7.5
Lactose	g	7.5	6.7	6.7	7.5
Glucose	g	-	0.2	0.2	-
GOS	g	-	0.54	0.6	-
FOS	g	-	0.06	-	-
<i>B. animalis</i> Bb-12	cfu	-	-	-	6.0x10 <sup>9</sup>
Calcium	mg	54	54	54	54
Phosphorus	mg	27	27	27	27
Magnesium	mg	5	5	5	5
Sodium	mg	19	19	19	19
Potassium	mg	64	64	64	64
Chloride	mg	43	43	43	43
Iron	mg	0.5	0.5	0.5	0.5
Zinc	mg	0.5	0.5	0.5	0.5

## Faecal samples

Parents were asked to take faecal samples from their infants, at postnatal day 5, 10, 28 and once every 4 weeks thereafter. For the analysis of the percentage of lactobacilli in the intestinal microflora, we used faecal samples taken at age 5 days (first sample taken during intervention) and 12 weeks (infants at this age have a relatively stable microflora and are exclusively fed on breast milk or infant formula). The samples were taken from the diaper, as soon as possible after defecation, collected in faeces containers (Greiner Labortechnik, 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, the Netherlands) to the laboratory.

## Duplex 5' nuclease assay

for 20 minutes at 3300 g to remove debris and large particles. DNA was extracted from the supernatant, by binding of DNA to silica particles after lyses with the NucliSense Isolation Kit (BioMerieux, Boxtel, The Netherlands) (22). Finally, DNA was eluted from the silica particles with sterile milliQ and stored at -20 °C until further processing. For the quantification of the genus *Lactobacillus* as percentage of the total bacterial load, a newly developed duplex 5'nuclease assay was used (manuscript in preparation).

## Data analysis

Statistical package SPSS for Windows (version 11.0) was used for statistical analysis of the results. All values were checked for normality by visual inspection of the normal probability plots. Differences in percentage of lactobacilli between the feeding groups were tested for significance using analysis of variance. In case of a significant difference ( $p < 0.05$ ), the groups were compared by using the Dunnett *post hoc* test with the standard formula group as reference. Within a feeding group, differences

between age d5 and w12 were tested using a paired samples t-test. Because it is not possible to double blindly assign breast and formula feeding and ensure adequate randomisation, no statistical analyses were performed comparing the breast to the formula fed groups. Data from the breast fed group are only used when the infant was exclusively fed on breast milk at that time point

## Results

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### Feeding groups

Subject characteristics of those infants that provided at least one faecal sample (d5 and/or w12) for analysis are shown in table 2.

Between age 5 days and 12 weeks, group sizes of most feeding groups decreased. Before the age of 12 weeks 6 breast fed, 5 standard-, 4 GOS/FOS-, no GOS- and 3 Bb-12 formula fed infants dropped out. Reasons for drop out included: colics, suspicion of cows' milk allergy, constipation and practical problems such as moving, increase of work hours etc. Further, many infants that were breast fed, switched to formula feeding before the age of 12 weeks. In our studies, 23 breast fed infants switched to formula feeding during the study. Of these infants, only samples taken during complete breast feeding were included in the analyses. Finally, because all samples were previously used to analyse the percentage of bifidobacteria, pH and SCFA concentration (chapter 2 and 3), 22 of all available samples did not contain enough faeces to perform additional PCR analyses.

**Table 2.** Baseline characteristics of the study participants that provided at least one faecal sample

		Standard formula n=34	GOS/FOS formula n=16	GOS formula n=17	Bb-12 formula n=17	Breast milk n=87
Sex	Male	22	6	9	10	41
	Female	12	10	8	7	46
Place of birth	At home	13	8	6	10	54
	Hospital	21	8	11	7	33
Mode of delivery	Vaginal	27	13	16	16	80
	Caesarean	7	3	1	1	7
Birth Weight (g) ± SD		3547± 574	3361±644	3531± 673	3472± 554	3743± 657

## Percentage of lactobacilli

The percentages of *Lactobacillus* from the total bacterial count in faecal samples of the infants fed on breast milk, GOS/FOS formula, GOS formula, Bb-12 formula and standard formula at age 5 days and 12 weeks are shown in figure 1. The GOS/FOS showed a marked increase in the percentages of faecal lactobacilli ( $p=0.253$ ) from age 5 days ( $2.3 \pm 1.1\%$ ) to 12 weeks ( $6.1 \pm 2.6\%$ ), which was comparable to the increase found in breast fed infants. A much smaller increase was found in the BB-12 formula group ( $p=0.997$ ) between d5 ( $2.0 \pm 0.9\%$ ) and w12 ( $2.4 \pm 1.7\%$ ). In the GOS and standard formula groups, the percentage of lactobacilli showed a small decrease between day 5 and week 12 of age (from  $1.6 \pm 1.1\%$  to  $1.1 \pm 0.4\%$ ;  $p=0.580$  and from  $1.6 \pm 0.8$  to  $0.9 \pm 0.4\%$ ;  $p=0.532$  respectively).

At age 12 weeks, the GOS/FOS fed group showed a mean percentage of *Lactobacillus* ( $6.1 \pm 2.6$  mean  $\% \pm$  SEM), which was significantly higher compared to the standard formula group ( $0.9 \pm 0.4\%$ ;  $p=0.007$ ). No

significant differences in the percentage of lactobacilli were found between the GOS ( $1.1\% \pm 0.4\%$ ,  $p=0.998$ ) and Bb-12 formula groups ( $2.4\% \pm 1.7\%$ ,  $p=0.677$ ) compared to the standard formula group.

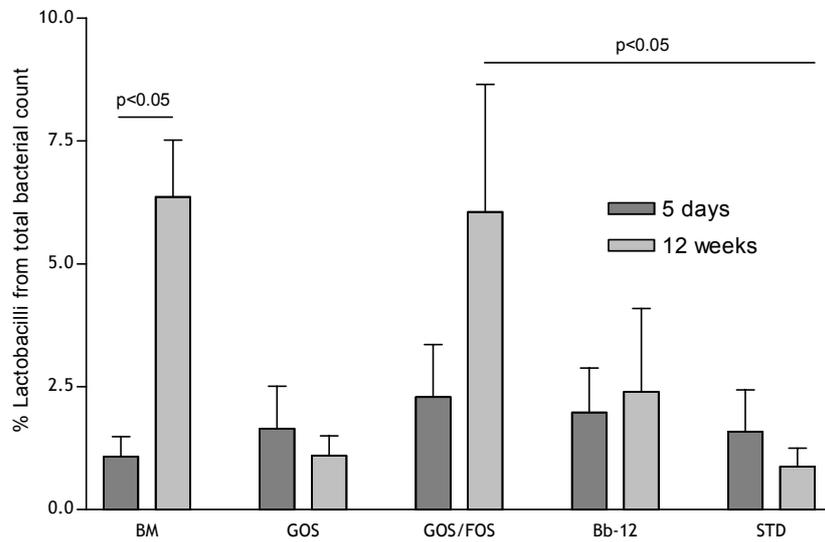


Figure 1. Percentage of lactobacilli (mean  $\pm$  SEM) in infants fed on standard-, GOS/FOS-, GOS- or Bb-12 infant formula or BF at age 5 days and 12 weeks. Numbers in the bars represent the number of infants of which samples were analysed

## Discussion

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In this study, we showed that feeding infant formula containing the prebiotic mixture of GOS and long chain FOS resulted in a percentage of faecal lactobacilli at age 12 weeks similar to that found in breast fed infants. It was the first study that used a highly specific and sensitive real time PCR to quantify the relatively small amounts of lactobacilli in faeces of breast- and formula fed infants. To investigate whether infant formula containing pre- or probiotics can induce an intestinal microflora comparable to that in breast fed infants, one should ideally compare results of the formula groups to those of the BF group. However, since it is not possible to randomize and double blindly assign breast feeding, selection bias is likely to occur due to differences in social and educational status between breast feeding and formula feeding mothers (23). Yet, by limiting the statistical analyses to the formula groups, we were still able to compare the effects of the prebiotic and probiotic components on the intestinal microflora and predict the proximity to breast feeding.

During the study several participating infants dropped out, switched from breast- to formula feeding or were not able to provide faecal samples on d5 and w12. This group of infants did not differ from the others with respect to sex, place of birth, mode of delivery and birth weight and therefore did not give cause to any major selection bias.

Earlier studies showed that next to nutrition other factors could have significant effects on the composition of the intestinal microflora. Several studies showed that infants born by caesarean section (CS) often show a delay in the development of the intestinal microflora, which is mainly shown by lower numbers of infants colonised by specific bacteria (24-26). In our study, despite the randomisation of the formula groups, the GOS/FOS includes a somewhat higher number of infants born by CS, than the other groups. Although several studies showed that in CS infants the less infants were colonised with lactobacilli, but no effect could be

demonstrated on the actual number of lactobacilli. In our study, excluding infants born by CS, did not show a distinct effect on the mean percentages of lactobacilli at d5 (2.5% without CS vs. 2.3% in all infants) and w12 (6.8% vs. 6.1%). Treatment with antibiotics early in life may cause short-term (lactobacilli and bifidobacteria) and long-term disturbances of the intestinal microflora (25,26). In the present study, 2 infants in the standard and 2 in the GOS/FOS formula group received antibiotics before w12. However, analysis after exclusion of infants that used antibiotics did not show any distinct changes in the mean percentages of faecal lactobacilli of either the standard formula group (d5: 1.6% without antibiotics vs. 1.6% in all infants, w12: 0.6% vs. 0.9%) or the GOS/FOS formula group (d5: 2.3% vs. 2.3% and w12: 7.3% vs. 6.1%).

Although several studies confirmed that infant formulas supplemented with GOS/FOS increased the number of faecal bifidobacteria, only one study investigated the effects on the number of lactobacilli with classical plating techniques. Moro *et al.*, showed that feeding infants an infant formula containing 0.4 and 0.8 g/100ml GOS/FOS for 28 days, resulted in a statistically significant higher number of faecal lactobacilli compared to unsupplemented infant formula (5.7, 5.0 and 3.0 log<sub>10</sub> of CFU/g wet weight faeces respectively) The current study confirms this finding.

Earlier we showed that infant formula containing a mixture of GOS and long chain FOS induced a faecal SCFA profile, pH and lactate concentration similar to that in breast fed infants (high acetate, lactate and low pH, chapter 2). In infants fed on infant formula containing GOS or viable *B. animalis* we found faecal SCFA profiles, pH and lactate concentrations more similar to those found in standard formula fed infants (more divers SCFA profile, high pH and low lactate, chapter 2 and 3). Although we expected that differences in metabolic end-products are caused by the stimulation of bifidobacteria by the GOS/FOS mixture, we did not find statistically significant differences in the percentage of bifidobacteria between the feeding groups (17). We suggested that other bacteria that produce acetate and lactate, like lactobacilli, might be partly responsible for the high amounts of acetate and lactate in the

GOS/FOS group. In this report, we confirmed our hypothesis that GOS/FOS has a stimulating effect on the growth of lactobacilli in the intestinal microflora. Probably, in contrast to GOS, adding GOS/FOS created optimal conditions for the growth of bifidobacteria and lactobacilli in the infant's intestine. The mixture consisting of 90% GOS and 10% long-chain FOS is composed to mimic the size distribution of human milk oligosaccharides and to synergistically promote the growth of beneficial bacteria like bifidobacteria and lactobacilli in all parts of the colon (27). Our previous (17) and current data show that feeding infants a formula containing viable *B. animalis* Bb-12 did not have a significant effect on the percentage of total bifidobacteria and lactobacilli. Apparently, infant formula containing viable *B. animalis* does not change the intestinal microflora by increasing the total number of bifidobacteria and lactobacilli.

We conclude that adding GOS/FOS (in a ratio of 9 to1) to infant formula increases the percentage of faecal lactobacilli to the levels found in breast fed infants, whereas standard infant formula and formula containing GOS or viable *B. animalis* do not.

## Acknowledgments

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Faecal SIgA secretion  
in infants fed on  
prebiotic or probiotic  
infant formula

Astrid Bakker-Zierikzee

Ric van Tol

Hilde Kroes

Martine Alles

Frans Kok

Jacques Bindels

*Submitted for publication*

# Abstract

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## Introduction and aim

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, that 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. Therefore, we aimed to study the effects of prebiotic and probiotic infant formulas on the faecal SIgA levels.

## Methods

At birth, infants of whom the mother had decided not to breast feed, allocated to one of three formula groups in a randomised, double blind fashion. In total, 19 infants received standard infant formula; 19 received prebiotic formula containing a specific mixture of 0.6 g GOS/FOS/100ml formula and 19 received probiotic formula containing  $6.0 \times 10^9$  cfu *Bifidobacterium animalis*/100ml formula. Faecal samples were taken on postnatal d5, d10, w4 and every 4 weeks thereafter. SIgA in faeces was determined by using a specific ELISA.

## Results

During the intervention, infants fed on prebiotic formula showed a trend towards higher faecal SIgA levels compared to the standard formula fed infants with a significantly higher ( $p=0.015$ ) SIgA concentration at the age of 16 weeks (Median ( $P_{25}$ - $P_{75}$ ) of 0.84 (0.6-1.8) vs. 0.39 (0.1-0.9) mg/g faeces). In contrast, infants fed on the probiotic formula showed a highly variable faecal SIgA concentration with no statistically significant differences compared to the standard formula group.

## Conclusion

Formula fed infants may benefit from infant formulas containing a prebiotic mixture of GOS and FOS because they have been shown to increase faecal SIgA secretion. Adding viable *B. animalis strain* Bb-12 to infant formula did not elicit such a beneficial effect.

## Introduction

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Secretory immunoglobulin A (SIgA) is one of the most abundant immunoglobulins in the human body. It is the predominant immunoglobulin in mucosal surfaces and the main constituent of the humoral immune response. 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). Secretory IgA deficiency in humans is one of the most common immunodeficiency's 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 plasmacell) 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 has not matured yet and passive immunity in this phase is provided by breast milk, which contains high levels of SIgA and anti-microbial peptides. SIgA in breast milk are mainly directed against the mother's previous and recent 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 values of 0.5-1mg/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 weeks 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 weeks and 6 months of life, faecal IgA concentrations in both breast fed and formula fed infants converge towards similar levels. At age 1-2 years, 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 (GALT), including SIgA secretion in the intestine (10,11). Moreau *et al.*, 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.

Prebiotics and probiotics are both methods to change the intestinal microflora towards a healthier flora mainly by increasing the number of bifidobacteria and/or lactobacilli. 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 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).

Since 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 to infants fed on a standard, unsupplemented infant formula.

## Materials and methods

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### Subjects

63 Pregnant women who had decided to breast-feed 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 ethical committee of the region Arnhem-Nijmegen (Committee on Research Involving Human Subjects, located in the University Medical Centre St. Radboud in Nijmegen). Written informed consent was obtained from the parents before enrolment in the study.

### Feeding groups

Infants of mothers who decided not to breast-feed were allocated to one of three formula groups (GOS/FOS, Bb-12 or standard) in a randomised, double blinded fashion. Randomisation included a block size of 3 and was carried out by the logistics manager of Numico Research BV, 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, the Netherlands). The composition of the standard formula at a standard dilution of 3 scoops per 100ml formula

is given in table 1. The prebiotic formula group (GOS/FOS; n=19) received the same standard infant formula supplemented with a mixture of 0.6 g/100ml galacto-oligosaccharides (GOS; Vivinal GOS, Borculo Domo Ingredients, Zwolle, the Netherlands) and fructo-oligosaccharides (FOS; Raftiline HP, Orafti 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  $6.0 \times 10^9$  cfu *Bifidobacterium animalis* per 100ml formula (Bb-12; Christian Hansen Ltd., Hørsholm, Denmark). Shelf life of the probiotic formula was tested and reported previously (chapter 2). Mothers were instructed to heat the water to a temperature of maximal 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 breast-feed were stimulated to continue breast feeding 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 day 5, 10, 28 and once every 4 weeks thereafter.

**Table 1. Composition of the formulas per 100ml**

		Standard formula	GOS/FOS formula	Bb-12 formula
Energy	kcal	67	67	67
Protein	g	1.4	1.4	1.4
Casein/whey ratio		40/60	40/60	40/60
Fat	g	3.5	3.5	3.5
Carbohydrates total	g	7.5	7.5	7.5
Lactose	g	7.5	6.7	7.5
Glucose	g	-	0.2	-
GOS	g	-	0.54	-
FOS	g	-	0.06	-
B. animalis	cfu	-	-	6.0x10 <sup>9</sup>
Calcium	mg	54	54	54
Phosphorus	mg	27	27	27
Magnesium	mg	5	5	5
Sodium	mg	19	19	19
Potassium	mg	64	64	64
Chloride	mg	43	43	43
Iron	mg	0.5	0.5	0.5
Zinc	mg	0.5	0.5	0.5

## Faecal samples

Parents were asked to take faecal samples from their infants, at postnatal day 5, 10, 28 and once every 4 weeks thereafter. The samples were taken from the diaper, as soon as possible after defecation, collected in faeces containers (Greiner Labortechnik, 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, the Netherlands) to the laboratory.

### Faecal homogenates

For the determination of the SIgA concentration by ELISA, 10% W/v faecal homogenates were prepared according to standard procedures. In short, the frozen (-20°C) faecal samples were defrosted on ice. Suspensions were made by adding 1 gram faeces to 9 ml of PBS and homogenizing for 10 minutes using a stomacher. The homogenates were stored at -80°C until further processing.

### Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA plates (NUNC 384 well Immuno-Maxisorp) were coated overnight at 4 °C with Mouse  $\alpha$  Human Secretory Component (Sigma, clone GA-1), 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 hour at room temperature with PBS containing 1% of 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 13.000 rpm for 5 minutes at 4 °C) were serial diluted 10 times in PBS/BSA. Purified human IgA isolated from colostrum (Sigma, I-1010) was used as a positive standard. The samples and standards were incubated for 2 hours at room temperature. Plates were then washed five times and Biotin conjugated Mouse  $\alpha$  Human IgA<sub>1/2</sub> monoclonal antibody (Pharmingen, clone G20-359) was added to the plates at a concentration of 0.2 $\mu$ g/ml PBS/BSA. After 1 hour of incubation the plates were washed five times and incubated with Streptavidin conjugated HRP (CLB, M2051) 1:50.000 diluted in PBS/BSA for 30 minutes at room temperature. The wells were then washed and incubated for 5-10 minutes with 50  $\mu$ l of a TMB substrate solution. The enzymatic colour

development was stopped by adding 1.8 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm using a plate reader. SIgA concentrations were calculated from the standard curve.

## Data analysis

Because the concentration SIgA was not normally distributed, results are expressed as median and the range between the 25<sup>th</sup> (P<sub>25</sub>) and 75<sup>th</sup> (P<sub>75</sub>) percentile. SIgA concentrations of the Bb-12 and GOS/FOS groups at all ages separately were compared to the standard formula group using the Mann Whitney Test. Because it is not possible to randomize and double blindly assign breast feeding, statistical analysis were performed only on the formula fed groups.

## Results

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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 were published previously (20, chapter 2). Of the 63 infants that were fed on breast milk directly after birth 40 switched to complete or partial formula feeding, 12 were completely breast fed and 11 dropped out during the intervention period. A number of 57 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: 5 in the standard group, 5 in the GOS/FOS group and 6 in the Bb-12 group. Reasons for drop out included: colic's, suspicion of cows' milk allergy, constipation and practical problems. The median and the range between P<sub>25</sub> and P<sub>75</sub> of the faecal SIgA concentration found in the infants between birth and 32 weeks 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 to 12 weeks, faecal SIgA levels show a fast decrease towards

levels that are slightly higher compared to those in formula fed infants. In the GOS/FOS formula group, integrated over the complete intervention period, faecal SIgA concentration was higher compared to infants fed standard formula, mounting to a statistically significant difference at w16. In the Bb-12 formula group no marked differences compared to the other formula groups could be observed.

**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 (n)	Male	5	12	10	33
	Female	14	7	9	30
Place of Birth (n)	At home	7	8	10	40
	Hospital	12	11	9	23
Mode of Delivery (n)	Vaginal	14	16	18	59
	Caesarean	5	3	1	4
Birth Weight (g)		3601 ± 501	3318 ± 602	3481 ± 524	3651 ± 601

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

Age	Standard formula			GOS/FOS formula			Bb-12 formula			Breast milk		
	N	Med	P <sub>25</sub> -P <sub>75</sub>	N	Med	P <sub>25</sub> -P <sub>75</sub>	N	Med	P <sub>25</sub> -P <sub>75</sub>	N	Med	P <sub>25</sub> -P <sub>75</sub>
D5	13	0	0.0-0.0	15	0	0.0-0.0	19	0	0.0-0.0	43	10.9 2	6.2-15.0
D10	19	0	0.0-0.0	18	0	0.0-0.0	17	0	0.0-0.0	44	7.72	4.0-13.9
W4	16	0.23	0.1-1.0	17	0.74	0.3-1.1	17	0.35	0.1-1.6	36	4.24	1.6-7.6
W8	15	0.70	0.3-2.7	18	0.78	0.4-1.6	17	0.62	0.3-1.1	30	3.18	1.7-5.2
W12	15	0.75	0.4-1.8	13	1.14	0.7-1.3	16	0.52	0.9-1.7	24	1.58	0.8-3.7
W16	14	0.39	0.1-0.9	14	0.84 <sup>*</sup>	0.6-1.8	15	0.36	0.1-1.5	22	1.10	0.5-1.6
W20	14	0.45	0.0-0.7	14	0.52	0.3-1.1	12	0.44	0.2-1.5	18	0.94	0.4-2.4
W24	13	0.16	0.1-0.7	13	0.65	0.3-1.7	14	0.57	0.0-1.3	12	0.77	0.3-2.7
W28	13	0.16	0.0-0.7	12	0.33	0.1-1.4	12	0.17	0.0-0.3	15	0.29	0.1-0.5
W32	12	0.17	0.0-0.4	12	0.14	0.1-0.6	13	0.26	0.1-0.6	12	0.26	0.2-0.9

<sup>\*</sup>p<0.05 compared to the standard formula group

## Discussion

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In this study, we found indications that adding non-digestible oligosaccharides and to a lesser extent viable *B. animalis* to infant formula results in higher levels of faecal SIgA when compared to unsupplemented infant formula.

Although our results are very promising, due to the very high variation of the faecal SIgA concentrations found in the formula groups, a statistically significant difference was demonstrated only at 16 weeks of age. We believe that this high variation might have masked the effect of GOS/FOS on faecal SIgA secretion. Nevertheless, we feel this to be an important observation since this additional effect of prebiotics on endogenous SIgA production occurs in a critical episode of maturation and increasing SIgA production by plasma cells in the intestinal mucosa (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, did however not indicate major differences between the groups concerning 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 according to the National Vaccination Programme (National Institute of Public Health and the Environment, RIVM). 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. In general we conclude that 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 are 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 ( $1.8 \pm 1.0$ ,  $1.7 \pm 1.0$  and  $1.6 \pm 1.2$  stools per day  $\pm$  SD in respectively. the GOS/FOS, Bb-12 and standard formula group) and reported stool consistency  $\pm$  SD ( $2.6 \pm 0.8$ ,  $2.8 \pm 0.9$  and  $2.7 \pm 0.9$  in respectively. the GOS/FOS, Bb-12 and standard formula group, based on a 5 point Likert scale: 1-watery, 2-soft-puddinglike, 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 therefore 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 might 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, *E. coli* and *Eubacterium*. Theoretically, lower numbers of Clostridia 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 if particular flora components may have added to the large variation of the faecal SIgA concentrations (25). Generally, an increase of SIgA levels 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 clear trend of higher SIgA concentrations in the GOS/FOS formula group compared to 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 group to 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 randomise and double blindly assign breast feeding. Due to differences in social and educational status between breast feeding and formula feeding mothers, selection bias is likely to occur. By limiting statistical analysis to the formula groups, we are still able to assess the effects of the prebiotic and probiotic component on faecal SIgA concentrations.

Several studies on pre- and probiotics have been performed to support luminal SIgA production in both animals and humans with the aim to improve 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 *Bifidobacterium animalis* Bb-12) fed to rats for four weeks increased the local SIgA production in the ileum (27). In a recent study 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 (pIgR) expression compared to control mice (26).

The results from our study confirm the observed differences in faecal SIgA levels between breast- and formula fed infants. A comparison of total faecal SIgA secretion between several infant formulas has not been done 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 very interesting to study the effect of pre- and probiotics on the levels of 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 used probiotic strain Bb-12 was found to be less effective.

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6

**General discussion**

This thesis is based on the results of two intervention studies in, which we investigated the effects of prebiotics and probiotics on the composition and metabolic activity of the intestinal microflora and the intestinal secretory immune globulin A (SIgA) response in infants. In this general discussion, the main findings are summarized and put into perspective. Additionally, the methodology of the studies and safety issues of the use of pre- and probiotics in infant nutrition discussed. Finally, recommendations for further research and an overall conclusion are given at the end of this chapter.

Table 1 summarises the main findings of both studies. The table shows the composition and metabolic activity of the intestinal microflora and faecal SIgA concentration of all feeding groups. Results on the percentage of bifidobacteria, SCFA profile, lactate concentration and pH are given at age 16 weeks, percentage of lactobacilli at age 12 weeks. At these ages, composition and metabolic activity of the flora is considered to be fairly stable and the results give a good representation of the findings described in this thesis.

**Table 1** Main findings on the effects of breast milk, standard infant formula, 2 prebiotic infant formulas (GOS/FOS and GOS) and a probiotic infant formula (Bb-12) on the composition (percentages of bifidobacteria and lactobacilli) and metabolic activity of the intestinal microflora (relative amounts of SCFA, pH and faecal lactate concentration) and faecal SigA concentration at 12 or 16 weeks of age.

Feeding group <sup>1,2</sup>	Bifidobacteria and lactobacilli % from total bacteria <sup>3,4</sup>	pH	SCFA % from total amount of SCFA	Lactate Mmol/kg faeces	SigA Median mg/g faeces
Standard formula	52% bifidobacteria 1% lactobacilli	7.1	70% acetate 20% propionate 6% butyrate	3	0.9
GOS/FOS formula	59% bifidobacteria 6% lactobacilli	5.6	82% acetate, 14% propionate 2% butyrate	41	1.4
GOS formula	77% bifidobacteria 1% lactobacilli	6.5	78 % acetate 16 % propionate 3% butyrate	12	-
Bb-12 formula	53% bifidobacteria 2% lactobacilli	6.8	70% acetate 22% propionate 6% butyrate	6	0.9
Breast feeding	47% bifidobacteria 6 % lactobacilli	5.7	90% acetate 6% propionate 2% butyrate	45	1.7

<sup>1</sup> data given on breast milk, standard-, GOS/FOS- and Probiotic formula-fed infants originate from chapter 2.

<sup>2</sup> data on GOS formula fed infants originate from chapter 3

<sup>3</sup> results of % bifidobacteria, pH, SCFA, lactate and SigA are given at age 16 weeks

<sup>4</sup> results of % lactobacilli are given at age 12 weeks.

# Prebiotics

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## Prebiotics and the composition of the intestinal microflora

Prebiotic infant formulas containing either GOS alone (chapter 3) or a mixture of 90% GOS and 10% FOS (chapter 2) both resulted in an intestinal microflora dominated by bifidobacteria (>50% of intestinal microflora consist of bifidobacteria). However, due to an unexpected high percentage of bifidobacteria in the control group (infants fed the standard unsupplemented formula), no statistically significant effect of the prebiotics could be demonstrated. On the other hand, we did find a statistically significant effect of the GOS/FOS formula on the percentage of lactobacilli, which was not demonstrated in the GOS- or standard formula fed infants.

The percentage of bifidobacteria in our standard formula group was much higher compared to that of the standard formula group of other studies (56% in our study at w12 vs. 35-40% in other studies)(1). We speculate that differences in the composition of the infant formulas might have caused these differences. Although small differences in the amount of macro- and micronutrients exist between the standard formulas, it is possible that the relatively high lactose content of our standard formula (10.9g/100kcal) might escaped digestion and absorption in the small intestine and stimulated the growth of bifidobacteria in our control group (2). Maclean *et al.* showed that in infants an average of 1.8% of lactose intake is not digested in the small intestine (3). This is therefore possible that in our standard formula group, a small amount of lactose escaped digestion and became available for fermentation by the colonic microflora. Several bacteria that are present in the large intestine of infants (including bifidobacteria) are able to ferment lactose (4). Therefore, if 0.9g/100kcal undigested lactose can stimulate the growth of bifidobacteria to the same extend as GOS and FOS can, the high lactose content of our formula might

explain the high percentage of bifidobacteria in the faeces of our standard formula group.

In the comparison of the two prebiotic formulas, we showed that infant formula containing 0.6g/100ml GOS/FOS (chapter 2) has a similar effect on the percentages of bifidobacteria as formula containing 0.6g/100ml GOS (chapter 3). The mixture of 90% short chain GOS and 10% long chain FOS was designed to mimic the molecular size distribution of human milk oligosaccharides. It is postulated by the investigators that the combination of both compounds might have a synergistic stimulating effect on the growth of bifidobacteria and lactobacilli (5-7). In our study we were not able to show different effects on the percentage of bifidobacteria between GOS and GOS/FOS fed groups. Therefore a synergistic effect of GOS and FOS on the percentage of bifidobacteria in the intestinal microflora cannot be inferred.

In contrast to the effects on bifidobacteria, we did find that the GOS/FOS formula has a significantly stimulating effect on the percentage of faecal lactobacilli. We could not find this effect in the infants fed on GOS alone, which leads us to the question whether the effect in the GOS/FOS group is an effect of the FOS, which comprised only 10% of the added non-digestible oligosaccharides, or an actual synergistic effect of GOS and FOS. Several investigators have shown in adults that, although FOS did significantly increase the number of bifidobacteria, no effect was found on the number of lactobacilli (8-14). Unfortunately, no studies on the effects of FOS alone on lactobacilli in infants are available, which makes it difficult to answer this question. Nevertheless, based on the findings that either GOS alone (in infants, chapter 3) or FOS alone (in adults) does not significantly affect the number of lactobacilli, we hypothesise that GOS and FOS together could create an optimal environment for the growth of lactobacilli.

## Prebiotics and the metabolic activity of the intestinal microflora

In chapter 2 we showed that the faecal profile of fermentation products (SCFA, lactate and pH) of the GOS/FOS fed infants is consistent with the high percentage of faecal bifidobacteria and lactobacilli. In contrast, this is not the case in the GOS group, in which the high percentages of bifidobacteria are not reflected in the faecal pattern of fermentation products (chapter 3). We suggest two possible explanations for this apparent inconsistency.

First, we suggest that the higher percentages of lactobacilli demonstrated in the intestinal microflora of the GOS/FOS fed infants are responsible for the differences in the percentage of acetate (from total SCFA concentration), lactate concentration and pH found in their faeces. However, it could be questioned whether a 7% difference of lactobacilli between the GOS/FOS and the standard formula group, can account for the observed differences in faecal SCFA, lactate and pH. The high amount of available substrate, provided by the prebiotic GOS/FOS mixture might have stimulated other endogenous, acetate- and lactate-producing bacteria like *Bacteroides*, and clostridia (15). In infants under the age of 20 weeks, *Bacteroides* can account for up to 40%, clostridia for up to 20% and Eubacteria for up to 20% of the intestinal microflora (16,17). However, several studies showed that FOS and GOS are selectively utilized by most strains of bifidobacteria and not by *Bacteroides* and clostridia (18,19). Additionally, Knol *et al.*, demonstrated that infant formula containing the GOS/FOS mixture, significantly decreased the percentage of clostridia and Eubacteria (16,17). The percentage of *Bacteroides* also decreased, but this was not significantly different. In our study we did not analyse the percentage of other bacteria than bifidobacteria and lactobacilli in the faecal samples. Therefore, based on the information by Knol *et al*, we conclude that it is unlikely that in our GOS/FOS group, other bacteria than bifidobacteria and lactobacilli are responsible for the differences in SCFA profile compared to the standard- and GOS formula group.

Second, the differences in the pattern of faecal metabolic end products between the groups may reflect only differences in the flora composition in the distal colon, whereas the faecal flora (measured by FISH) reflects cumulative composition differences in all parts of the colon. GOS are short chain oligosaccharides that are rapidly fermented (20,21). As a result, fermentation would be complete in the very proximal colon and might only stimulate the growth of bifidobacteria and lactobacilli in that part of the large intestine. SCFA produced in the proximal colon are most likely absorbed before leaving the body in the faeces (22). The GOS/FOS mixture is designed to mimic the molecular size distribution of human milk and contains short chain oligosaccharides that are rapidly fermented in the proximal colon and long chain oligosaccharides that are more slowly fermented more distally in the colon {Jenkins, Kendall, et al. 1999 ID: 38}. Consequently, bifidobacteria and lactobacilli are stimulated in all parts of the colon. The SCFA profile of the GOS/FOS fed group reflects higher numbers of bifidobacteria and lactobacilli present in the distal colon.

Therefore we conclude that most likely, GOS/FOS formula stimulated the growth of bifidobacteria and lactobacilli in all parts of the colon, whereas in the GOS and standard formula groups only bifidobacteria in the proximal colon are stimulated.

## Prebiotics and the intestinal secretory immune response

Several studies *in vitro* or in animals showed that prebiotics significantly increased the levels of faecal SIgA. Unfortunately, our study appeared to lack statistical power to confirm these findings in infants. Several suggestions could be made to explain these findings.

Although our results are very promising, due to the very high variation of the faecal SIgA concentrations found in the formula groups, only one statistically significant difference was demonstrated. Several reasons for the high variation of the faecal SIgA concentrations can be suggested. Disease frequency and vaccination of the infant can have an effect on the

levels of faecal SIgA. The health questionnaire, primarily designed to report formula tolerance, did however not indicate major differences between the groups concerning frequency of vomiting (2-13% of all questionnaires reported vomiting), fever (4-8%) and diarrhoea (3-8%). In the Netherlands, infants are vaccinated according to the National Vaccination Programme (National Institute of Public Health and the Environment, RIVM). 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. Further, SIgA secretion was measured in wet weight faeces and might therefore be influenced by factors like defaecation rate and consistency of the stool. However, several studies showed that measuring SIgA levels in faeces gives a good representation of the amount of SIgA available in the colon (23,24). Based on our health questionnaires, no differences were found in defaecation rate ( $1.8 \pm 1.0$ ,  $1.7 \pm 1.0$  and  $1.6 \pm 1.2$  stools per day  $\pm$ SD in resp. the GOS/FOS, Bb-12 and standard formula group) and stool consistency  $\pm$ SD ( $2.6 \pm 0.8$ ,  $2.8 \pm 0.9$  and  $2.7 \pm 0.9$  in resp. the GOS/FOS, Bb-12 and standard formula group, based on a 5 point Likert scale: 1-watery, 2-soft-puddinglike, 3-soft formed, 4-dry formed, 5-dry hard pellets) between the formula groups. Generally, an increase of SIgA levels 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 clear trend of higher SIgA concentrations in the GOS/FOS formula group compared to the standard formula group. For future research we recommend that, more infants per group should be included to reach statistical significance.

The contradictory results between our findings in humans and findings in animals might also be partially explained by differences between ileal and faecal levels of SIgA. Although in animals it was shown several times that pre- and probiotics do induce higher intestinal SIgA production, this effect is mostly found in ileal samples. Effects on faecal samples were either not found or were much smaller. It was suggested that bacterial species with

IgA degrading capability (e.g. *Clostridium*) are responsible for the difference in SIgA concentrations between ileal and faecal samples (25-27). Although previous findings show that in infants fed on GOS/FOS formula, the intestinal microflora contains between 70 and 90% lactic acid bacteria with significantly lower percentages of *Clostridium*, *E. coli* and *Eubacterium* compared to the standard formula group (1,17,28,29), variations in composition of the flora may add to the large variations found in faecal SIgA concentrations recovered in the faeces of our study participants.

## Probiotics

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### Probiotics and the composition of the intestinal microflora

Feeding infants a probiotic infant formula containing viable *Bifidobacterium animalis* strain Bb-12 resulted in an intestinal microflora dominated by bifidobacteria. However, due to an unexpectedly high percentage of bifidobacteria in infants fed unsupplemented formula, no statistically significant effect of the probiotics could be shown. In contrast to the prebiotic formulas, the lactose content of the probiotic formula is identical to that of the standard infant formula. Therefore, we conclude that there actually is no marked effect of viable *B. animalis* in the formula on the composition of the intestinal microflora. We suggest two possible explanations for these findings.

First, it is possible that the viable bifidobacteria did not survive transit through the upper gastrointestinal tract. Ingested (exogenous) probiotic bacteria are confronted by many factors that may negatively affect their viability. These factors include gastric acid and secretions of the small intestine such as bile salts and pancreatic enzymes. Moreover, in the large intestine, the bacteria must compete effectively with a complex and metabolically active indigenous flora. Several *in vivo* studies showed that

at least a part of the Bb-12 ingested (by adults or infants) survives passage through the gastrointestinal tract and can be recovered from the faeces. Generally, during intervention strain Bb-12 was found in the faeces of 44 to 100% of the subjects (30-33). In our study, we found that Bb-12 was recovered (Bb-12 counts of  $>10^6$ ) in 80% of the probiotic formula group (unpublished results). Based on these findings, we conclude that in the majority of our study participants, significant amounts of Bb-12 ingested with the formula survived passage and reached the colon alive.

Second, it is possible that ingestion of (relatively low numbers) *B. animalis* Bb-12 could not induce an extra significant increase upon the already very high numbers of bifidobacteria found in our study participants. In some studies on probiotics in infants it was demonstrated that ingestion of viable bifidobacteria did increase the rate of colonization (e.g. more infants colonised), but did not significantly affect the total number of bifidobacteria (34,35). Another study in infants showed that ingestion of Bb-12 did increase the number of Bb-12 found in the faeces (from 0 to  $8.8 \log_{10}$ ), but did not significantly increase the total number of bifidobacteria ( $10.2 \log_{10}$ ) (36). In adults, ingestion of Bb-12 did have a significant effect on the numbers of bifidobacteria (37) (38) but this increase was higher when the initial bifidobacterial levels were lower (39). These results suggest that when initial bifidobacterial numbers are already high, it is difficult to further increase the size of the bifidobacterial population by ingesting exogenous bifidobacteria. In our study, infant fed on standard unsupplemented infant formula have rather high percentages of bifidobacteria in their intestinal microflora, which might explain why no effect of probiotic intake could be observed.

Our findings indicate that healthy infants harbouring rather high numbers of bifidobacteria in their flora possibly do not benefit from a probiotic infant formula to change the composition of the intestinal microflora. Infants suffering from physiological disorders and infectious disease (e.g. allergy and antibiotic associated diarrhoea) usually have a disturbed intestinal microflora characterized by low numbers of bifidobacteria and lactobacilli (40-44). It might therefore be possible that when initial

bifidobacterial numbers are low, they could be increased to normal levels by feeding probiotics. Also in infants treated with antibiotics, which consequently had very low levels of bifidobacteria, it was found that by feeding probiotics it was possible to increase the number of bifidobacteria and thereby restore normal microbiological balance (45).

### **Probiotics and the metabolic activity of the intestinal microflora**

Our findings on the effects of probiotic Bb-12 in infant formula on the metabolic activity of the intestinal microflora are in line with our findings in the GOS and standard infant formula. The SCFA profile of the faeces indicates that the probiotic infant formula does not have a marked change on the metabolic activity of the intestinal microflora as was found in breast fed and GOS/FOS formula fed infants. It has been stated that SCFA profiles changes through different regions in the colon. Consequently, SCFA profile in the faeces mostly represents the SCFA production in the distal colon. The faecal SCFA profile from the probiotic formula group indicate that in the distal colon, the composition of the intestinal flora is more diverse than in breast fed or GOS/FOS fed infants and contains less acetate and lactate producing bacteria (bifidobacteria and lactobacilli). The high percentages of bifidobacteria found in this group, most likely originate from the proximal colon. SCFA produced in this region are mostly absorbed during transit through the colon.

In general we found that feeding infant formula containing viable bifidobacteria does not increase the metabolic activity of acetate and lactate producing bacteria (especially bifidobacteria and lactobacilli) in the distal colon.

## Probiotics and the intestinal secretory immune response

We demonstrated that adding viable bifidobacteria to infant formula did not show a clear trend toward higher faecal SIgA levels. Moreau *et al.*, indicated that especially the presence of *Bifidobacterium* in the infant's intestine is important for the synthesis of IgA against viral enteropathogens. They suggested that foods promoting bifidobacteria in the intestine could be instrumental in promoting a beneficial effect on health (46). Fukushima *et al.*, showed that mice fed Bb-12 for 12 days showed significantly high levels of faecal total IgA compared to that of the control group. The fact that all bacteria recovered in the faeces consist of the administered strain, indicated that the probiotic bifidobacteria were responsible for the increased SIgA production (47). Therefore, the fact that we were not able to demonstrate a significant effect of probiotic Bb-12 in infant formula on the percentages of bifidobacteria might be indicative for the lack of a distinct stimulating effect on SIgA.

Total SIgA concentration in the faeces consists of many different SIgA specific against a wide array of bacterial and viral pathogens. If Bb-12 has a stimulating effect only on a few specific SIgA, this increase is most likely not large enough to increase total SIgA levels. Fukushima conducted a probiotic feeding trial in infants and found a significant stimulating of Bb-12 effect on faecal levels of anti-polio SIgA. However, a more pronounced effect was found on total SIgA indicating that anti-polio SIgA was not the only specific SIgA that was stimulated. Although, we did not find an effect on total SIgA, it is possible that an effect could still be seen on specific SIgA. Effects of probiotics on specific SIgA should be evaluated further, for example to see whether immune response to a vaccine can be increased.

Another possibility is that by ingesting Bb-12, specific SIgA directed against these exogenous probiotic bacteria is produced. As a consequence, the SIgA prevented the Bb-12 to adhere and colonise. Several studies focussed on the stimulating effects of Bb-12 on for instance anti-polio SIgA or anti-rotavirus SIgA, but no studies have been published that evaluated

the production of anti-Bb-12 SIgA. In a study by Marini *et al.*, prolonged administration of probiotic LGG in preterm infants induced a rise of total SIgA and SIgA specific against the probiotic (48,49). It is unknown whether any Bb-12 specific SIgA was formed in our study participants.

## Methodological issues

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### Selection bias

To investigate whether an infant formula containing pre- or probiotics can induce an intestinal microflora comparable to that in breast fed infants, one should ideally compare results of the formula groups to those of the breast fed group. However, since it is not possible to randomise and double blindly assign breast feeding; the breast fed group cannot be compared statistically to the formula groups. Selection bias is likely to occur due to differences in social and educational status between breast feeding and formula feeding mothers (50). Yet, by limiting the statistical analysis to the formula groups, we were still able to compare the effect of the prebiotic component on intestinal microflora and predict the proximity to breast feeding.

Another possible selection bias could occur from the decline of the group sizes, due to different reasons. First, between 6% and 21% of the infants in the different feeding groups dropped out. Reasons for drop out included: colic's, suspicion of cows' milk allergy, constipation and practical problems. Additionally, 36% of infants that were breast fed directly after birth, switched to formula feeding during the study. Of these infants, only samples taken during complete breast feeding were included in the analysis. Finally, because samples were used for multiple analyses (chapter 2 and 3), 16% of the available samples did not contain enough faeces to perform additional PCR analyses. Although during the study several infants dropped out, switched from breast- to formula feeding or were not able to provide faecal samples on d5 and w12, this group of

infants did not differ from the rest of the feeding group with respect to sex, place of birth, mode of delivery and birth weight and therefore did not give cause to any major selection bias (table 2).

**Table 2.** Baseline characteristics of study dropouts

		Standard formula n=7	GOS/FOS formula n=5	GOS formula n=0	Bb-12 formula n=4	Breast milk n=55
Sex (n)	Male	3	2	-	3	27
	Female	4	3	-	1	28
Place of birth (n)	At home	5	2	-	3	31
	Hospital	2	3	-	1	24
Mode of delivery (n)	Vaginal	5	4	-	4	48
	Caesarean	2	1	-	0	7
Birth Weight (g)		3653± 532	3345± 211	-	3231± 335	3614± 532

## Information bias

A potential source of information bias could be the method used to analyse the faecal samples. In this study, we used Fluorescent In Situ Hybridisation (FISH) to determine the percentage of faecal bifidobacteria. FISH is a molecular method that has been further developed and automated during the last years(51,52). During the first year of our studies, most remarkable development of the FISH method was the transition of a semi automated to a fully automated counting procedure. To analyse all samples with the same method, all samples that were already analysed by the semi-automated procedure, were retested with the fully automated procedure. Therefore, we conclude that the development of FISH method did not cause major information bias.

Additionally, the fact that the study was completely double blind and that during each analysis included samples from all groups; we expected no systematic errors in the faecal analysis in one of the study groups.

Another source of potential information bias could be the collection of faecal samples for the analysis. In all feeding groups, samples were collected according to the same guidelines, were frozen in a home freezer and all equally transported to the lab. Although samples were analysed within 1 year of sampling, some samples remained longer in the freezer than others. In our studies, allocation of feeding was carefully randomised, which prevented that samples of one feeding group remained longer in the freezer than the other groups. It is possible that consistency of the faeces might influence outcome measures that are expressed per gram wet weight faeces. In general, stool consistency of breast fed infants is lower than in formula fed infants. This is also the case in our study. Between the formula groups on the other hand, no differences were found in stool consistency. Because the breast milk group was excluded from the statistical analysis, differences in stool consistency most likely did not influence our results.

## Confounding factors

Earlier studies showed that next to nutrition, factors like mode of delivery or the use of antibiotics can have effects on the composition of the intestinal microflora. Infants born by the caesarean section are less often colonised or have lower number of bifidobacteria, lactobacilli and *Bacteroides* compared to infants born via the vaginal route (42,53). In our study, despite the randomisation of the formula groups, the GOS/FOS and standard formula group include a percentage of birth by caesarean section (16 and 26% respectively) that is somewhat higher compared to the other groups (6%, 5% and 6% in the GOS, Bb-12 and breast fed groups respectively). If birth by caesarean section leads to lower percentages of bifidobacteria and lactobacilli in these groups, this would mean an underestimation of our effect during the first weeks of birth. However,

omitting infants born by caesarean section from statistical analysis did not affect our results.

Bennet *et al.* also showed that treatment with antibiotics has a significant decreasing effect on the number of anaerobic bacteria in newborn infants and is therefore considered to be a major confounding factor in studies on the composition of the intestinal microflora (40). In our studies, the use of antibiotics before the age of 16 weeks was reported for eight infants, but these infants were divided equally over the study groups (3 in standard formula group, 2 in Bb-12 formula group and 3 in breast fed group). Omitting infants treated with antibiotics from statistical analysis did not affect our results.

## Safety issues of prebiotics and probiotics

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In recent years, two approaches have been proposed to achieve an intestinal flora of formula infants more similar to that of breast fed infants: First, the addition of non-digestible carbohydrates as prebiotics and second, the addition of live bacteria as probiotics to infant formula.

### Prebiotics

In adults, safety of prebiotics has been studied extensively and showed no serious side effects. Some minor effects were reported including the occurrence of soft stools, diarrhoea or flatulence after ingestion of large amounts prebiotics (10,12,48).

In infants, data on prebiotic supplementation of infant formulas do not indicate any serious adverse effects. In a recent study by Schmelzle *et al.*, it was shown that infant formula containing 0.8 g/100ml GOS/FOS, partially hydrolysed whey protein, modified vegetable oil with a high  $\beta$ -palmitic acid content and starch was well accepted and resulted in normal growth of the infants (29). The softer stool, which is sometimes found in

infants that consumed the GOS/FOS formula, was more similar to the stools of breast fed infants and has been considered positive, because hard stools are more common in formula fed infants (54). No other side effects were observed in any of the GOS/FOS studies (28,29,54,55). In 2001, the Scientific Committee on food of the European commission has concluded, based on the available scientific data, that it has no major concerns about the use of up to 0.8 g/100ml of a combination of 90% GOS and 10% FOS in infant formula (56).

Very recently, three papers of the same group on rat model studies on salmonella infection resistance suggested potential untoward effect of FOS and/or its colonic fermentation products (58-60). In rats fed on low calcium (20mmol calcium per kg diet), ingestion of FOS resulted in an increased rate of translocation in a salmonella challenged model. An increase in the cytotoxicity of faecal water and an increased faecal mucin excretion were found, suggesting that FOS or rather its fermentation product have an irritating effect on the colonic mucosa, thus inducing increased permeability. Interestingly, administration of calcium phosphate to the rat's diets counteracted this effect, indicating that with a normal to high calcium intake, adverse effects are absent. We take these findings very serious and strongly advise that the effect of FOS is carefully investigated. However, at this moment it is not clear whether these results are relevant to humans in general and to infant in particular, or whether they are experimental artefacts reproducible only under rather non-physiological conditions. We need properly designed studies in at least one other animal species and establish dose-response relationships over the full physiological range and also research in humans. According to our current insights, major concern for infant formulas supplemented with amount between 0.04 and 0.087 g FOS/100ml in the presence of 45-55 mg Ca/100ml is not justified. The prebiotic concept for infant formulas is based on analogy with human milk oligosaccharides available for colonic fermentation at similar amounts. Induction of a faecal SCFA profile and pH close to those observed in breast fed infants argue against untoward and irritating fermentation products. If the level of colonic calcium would be a

major determinant in the effect of colonic fermentation of non-digestible oligosaccharides, breast fed infants would be at increased risk compared to formula fed ones because of the lower amount of calcium in breast milk (approximately 25-30 mg/100ml) and the superior calcium absorption in the small intestine. Finally, although our study was not designed for studying this type of side effects, we did not encounter any signs of untoward intestinal effects (i.e. incidence of diarrhoea) of the infants in the GOS/FOS group and all infants showed normal growth.

## Probiotics

Although the "Infant Formulae Directive" does not specify that the addition of live bacteria to infant and follow-on formula is permitted as technological additive for other purposes than acidified milks, in recent years several formulas with added live bacteria have been introduced onto the European market. Since then, several working groups reviewed and evaluated the criteria, health effects and safety issues of several pre- and probiotics (60-62) (BgVV Working Group, 1999). It was recommended that nutritional, physiological and therapeutic effects of all individual strains be demonstrated by appropriate clinical studies. It also emphasised the need to fully evaluate the safety of probiotics, in particular with respect to infection risk in humans with a compromised immune system or at risk for endocarditis.

In general, there are only few reports on the effects of feeding large amounts of live bacteria for any extended period of time to infants. With respect to *B. animalis* strain Bb-12, several studies on tolerance and safety are available. In a prospective, double-blind, randomised, placebo controlled study with healthy infants aged 3-24 months, it was found that long-term consumption of yoghurt containing  $10^6$  to  $10^7$  CFU of Bb-12 and *Streptococcus thermophilus* was well tolerated and resulted in adequate growth (63). A double blind, placebo controlled study by Langhendries *et al.*, showed that a 2 month intervention with infant formula containing  $10^6$  viable Bb-12 per gram of milk powder, was well tolerated and promoted normal growth (34). In a safety study by Abi-Hanna *et al.*, 119 healthy

free-living infants attending day care centres received standard formula containing either  $10^8$  cfu Bb-12/g,  $10^7$  cfu Bb-12/g or no Bb-12. It was shown that long-term consumption of these live probiotic containing formula by these infants as their sole nutrition was well tolerated, safe and resulted in adequate growth (64). Cases of infection with Bifidobacterium during supplementation with this organism have not been reported.

## Recommendations for future research

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Based on the results of many *in vitro* and *in vivo* studies, it is generally recognized that an intestinal microflora dominated by metabolically active bifidobacteria and lactobacilli and an adequate SIgA production against pathogens are beneficial for health. Studies have shown that even in industrialised countries like the Netherlands, infants fed on breast-milk still suffer less from infection than infants fed on infant formula. Breast-milk contains numerous humoral and cellular anti-infective factors that are not (yet) incorporated in infant formula. Important health effects of breast feeding are attributed to the distinct intestinal microflora most likely caused by the high amounts of non-digestible oligosaccharides in the milk. In the aim to mimic the intestinal microflora of breast fed infants, we showed very positive results of infant formula containing a mixture of non-digestible oligosaccharides GOS and FOS. We demonstrated that feeding infants the GOS/FOS formula resulted in an intestinal microflora, which is in composition and metabolic activity very similar to that of breast fed infants. Therefore, after showing effects of infant formula containing a mixture of GOS and FOS on intermediate endpoints like composition and metabolic activity of the intestinal microflora and faecal secretion of SIgA, the next step should be to focus on hard clinical endpoints. It is therefore important to show a clear effect of the prebiotic components by including a true control group that contains no prebiotic

components at all. It is realistic to expect effects of the GOS/FOS mixture on the frequency and duration of gastrointestinal infection and/or SIgA specific against a certain disease. This would complete the scientific basis for this formulation.

For the infant formula containing viable bifidobacteria, we showed that these bacteria did not colonise the intestine of the infants and thereby did not increase the relative number of bifidobacteria in the intestinal microflora. However, some studies showed that ingestion of Bb-12 by infants has profound effects on hard clinical endpoints sometimes without colonizing the intestine. Therefore, concerning probiotics, we recommend that research should focus on expanding the knowledge on the effects of probiotics on infants' health, especially on atopic disease, allergy and the incidence and duration of rotavirus diarrhoea. However, based on our results we suggest that most likely, it is not always necessary for the probiotic bacteria to colonise the GI tract.

## General conclusion

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We conclude that infant formula containing a mixture of 90% GOS and 10% FOS induces an intestinal microflora, which is in composition (percentage of bifidobacteria and lactobacilli) and metabolic activity (SCFA profile, pH and lactate) comparable to those found in breast fed infants. Additionally, we showed that infant formula containing GOS or viable *B. animalis* strain Bb-12 resulted in an intestinal microflora more similar to that found in standard formula fed infants. As for faecal SIgA secretion we concluded that the GOS/FOS infant formula demonstrated a trend toward higher faecal SIgA concentrations, which were much less or absent in the infants fed Bb-12 or GOS.

Although, more research is needed to elucidate the effects of GOS/FOS formula on hard clinical endpoints, it can be reasonably assumed that

infants fed on GOS/FOS will have a health benefit compared to infants fed on standard infant formula or infant formula containing GOS.

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## Summary

# Introduction

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In breast fed infants, the intestinal microflora usually consists of 80 to 90% bifidobacteria. It is generally agreed that an intestinal microflora dominated by bifidobacteria is very beneficial for health and might partly explain why breast fed infants suffer less often from gastrointestinal illnesses. During the last two decades many attempts have been made to mimic the intestinal flora of breast fed infants in formula fed infants. In recent years, the concept of prebiotics and probiotics has been developed to beneficially change the intestinal microflora and thus induce positive health effects. The mechanisms of their action need to be elucidated, but the effects on the immune response probably represent a key factor in our understanding.

We conducted two infant nutrition studies with the objective to compare the effects of infant formulas containing either prebiotics or probiotics in infants on:

- The composition of the intestinal microflora e.g. the percentage of bifidobacteria (chapter 2 and 3) and lactobacilli (chapter 4) in the total intestinal microflora.
- Metabolic activity of the intestinal microflora e.g. relative amounts of short chain fatty acids, lactate concentration and pH of faeces (chapter 2 and 3).
- Indicators of development of the secretory immune response e.g. faecal SIgA concentration (chapter 5).

We hypothesised that by adding either prebiotics or probiotics to infant formula it is possible to increase the relative number and metabolic activity of bifidobacteria and lactobacilli in the intestinal microflora and stimulate the development of the immune response.

## Study design

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Between September 2000 and August 2004, we performed two intervention studies with pre- and probiotic infant formula with infants born in the region of Arnhem-Nijmegen. In both studies we measured the composition and metabolic activity of the intestinal microflora and the SIgA secretion in infants from birth until 16 weeks of age (composition and activity of the flora) or 32 weeks (SIgA). Prior to the studies, power calculations showed that to detect a difference in percentage of bifidobacteria between the intervention formula groups and the standard formula group of 30% with a SD of 25%, 13 infants per group should be included. Because of an expected drop out of 30% in the formula groups, more infants than calculated were included in the study.

For the first study, we recruited 63 women in their last trimester of pregnancy who had decided to breast-feed and 57 who chose not to. At birth, infants of whom the mother had decided not to breast-feed, were at random and double blindly allocated to one of the following formula groups. The standard formula group (n=19) received a regular, non-supplemented infant formula. The GOS/FOS formula group (n=19) received standard infant formula supplemented with a mixture of 0.6 g/100ml galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). The probiotic formula group (n=19) received standard infant formula supplemented with  $6.0 \times 10^9$  viable cells/100ml *Bifidobacterium animalis* strain Bb-12.

For the second study, 72 pregnant women were recruited of whom 38 decided to breast-feed their infant and 34 who decided not to. At birth, infants of whom the mother had decided not to breast-feed, were at random and double blindly allocated to one of the following formula groups. The standard formula group (n=17) received a regular, non-supplemented infant formula. The GOS formula group (n=17) received standard infant formula supplemented with 0.6 g/100ml GOS. In both studies, infants with normal birth weight, no congenital abnormality,

congenital disease or gastrointestinal disease were enrolled within 3 days after delivery. Except for the intervention, the design of both studies was identical.

During intervention parents were asked to take faeces samples from the diaper of their infants. Information on delivery, baseline characteristics, intake of the formula and health were obtained from questionnaires at postnatal day 5, 10, 28 and once every 4 weeks thereafter. During the study period, the investigators visited the participants regularly to collect faeces samples and questionnaires.

Because it is not possible to double blindly assign breast and bottle-feeding and to ensure adequate randomization, no statistical analyses were performed to compare the breast feeding group with any of the formula feeding groups. Data from the breast fed group are only given when the infant was only fed breast milk at that time point.

## Prebiotics

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In chapter 2 and 3 we showed that both the GOS/FOS and GOS formula induced an intestinal microflora dominated by bifidobacteria ( $59.2 \pm 7.7\%$  and  $76.5 \pm 2.6\%$  mean  $\pm$  SEM percentage of bifidobacteria from total bacterial respectively at 16w). However, compared to the standard infant formula ( $56 \pm 6.4\%$ ), no statistically significant effect of the 0.6g/100ml GOS/FOS mixture or the 0.6g/100ml GOS could be demonstrated. The relatively high bifidogenicity found in the infants fed on standard formula was unexpected and is extensively discussed. In chapter 4 we did show a significant effect of GOS/FOS on the percentage of lactobacilli ( $6 \pm 2.6\%$  lactobacilli at 12w,  $p=0.007$ ) compared to the standard formula group ( $1 \pm 0.4\%$ ). No significant effect was found for the GOS formula ( $1 \pm 0.4\%$  at 12w) compared to the standard formula group.

In chapter 2 we showed that, next to the intestinal microflora, infant formula containing the GOS/FOS mix induced a faecal SCFA profile

(acetate/propionate/ butyrate/others) which is comparable to that found in breast fed infants (82/14/2/2% vs. 90/6/2/2% at age 16w). In contrast, chapter 3 shows that the faecal SCFA pattern of GOS fed infants is more like that in standard formula fed infants (78/16/3/2 vs. 73/20/5/3 at age 16w). Additionally, we demonstrated that the faecal lactate concentration of the GOS/FOS group was comparable to breast fed infants ( $40.9 \pm 10.7$  vs.  $45.2 \pm 9.0$  mmol lactate/kg wet faeces), whereas that of GOS fed infants was more like standard formula fed infants ( $12.2 \pm 5.1$  vs.  $0.8 \pm 0.7$ ). Most profound was the faecal pH of the GOS/FOS group, which was highly comparable to that in breast-fed infants ( $5.6 \pm 0.2$  vs.  $5.7 \pm 0.3$ ). As with SCFA profile and lactate concentration, the faecal pH of GOS fed infants was more comparable to that of standard formula fed infants ( $6.5 \pm 0.3$  vs.  $7.1 \pm 0.2$ )

In chapter 5 we showed that in infants fed on GOS/FOS infant formula, the faecal SigA levels during the course of the study were higher compared to infants fed on standard infant formula. This difference was however only statistically significant ( $p=0.015$ ) at age 16 weeks ( $0.84$  ( $0.6-1.8$ ) vs.  $0.39$  ( $0.1-0.9$ ), median ( $P_{25}-P_{75}$ )).

## Probiotics

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In chapter 2 we showed that infant formula containing viable *B. animalis* strain Bb-12, resulted in an intestinal microflora dominated by bifidobacteria ( $69.7 \pm 2.7\%$  at age 16 weeks). However, as for the prebiotic formulas, compared to standard unsupplemented formula ( $69.9 \pm 3.9\%$  at 16w) no significant effect of the probiotics could be demonstrated. Additionally, chapter 4 shows that no marked effect of the Bb-12 could be seen on the percentage of lactobacilli from total bacterial count compared to the standard infant formula ( $2.4 \pm 1.7\%$  versus  $0.9 \pm 0.4\%$  at 12w).

In chapter 2 we showed that adding viable *B. animalis* strain Bb-12 did not have a marked effect on metabolic activity of the intestinal microflora. The SCFA profile (%acetate/propionate/butyrate/others) of the probiotic formula group was comparable to standard formula fed infants (70/22/6/3 versus 70/20/6/5 at 16w). Additionally, we demonstrated that the mean pH and faecal lactate concentration of the probiotic group was comparable to that of the standard formula group ( $6.1 \pm 4.2$  mmol/kg lactate and pH  $6.6 \pm 0.2$  vs.  $2.7 \pm 1.3$  mmol/kg lactate and pH  $7.1 \pm 0.2$  age 16w).

Chapter 5 showed that there is no clear trend of the probiotic infant formula on the faecal SIgA secretion in infants compared to the standard formula group (0.36 (0.1-1.5) vs. 0.39 (0.1-0.9), median (P<sub>25</sub>-P<sub>75</sub>) at 16w). The SIgA was highly variable and no statistically significant differences were found compared to the standard formula.

## Discussion and conclusion

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In chapter 6, we discussed our main findings concerning the effects of prebiotic and probiotic infant formulas. At first we speculated about an explanation for the very high percentages of bifidobacteria in the intestinal flora of the control group and suggested that the relatively high lactose content of our formula might be responsible. Second, we discussed the differences in the composition and metabolic activity of the intestinal flora of the GOS/FOS- versus the GOS formula group. The differences in SCFA profile, lactate concentration and pH are most likely caused by differences in fermentation rate of the long chain FOS and short chain GOS. Additionally, we addressed the possibility of a synergistic effect of GOS and FOS on the growth of lactobacilli. We also evaluated the promising results of GOS/FOS formula on SIgA concentration, but that this was only statistically significant at age 16 weeks. We speculated on the reason for the very high variation in SIgA concentration in the formula groups. Further, we discussed why in the probiotic infant formula groups,

no marked effects on the composition and metabolic activity and the SIgA secretion were demonstrated. We suggest that probably, due to the very high percentage of bifidobacteria in our standard formula group, it is not possible to increase bifidobacteria any further.

In the evaluation of the methodology of our studies we discussed whether social economical differences between breast- and formula fed infants and the high dropout rate in our breast fed group caused any major selection bias. We demonstrated that the groups of dropouts did not differ from the rest of the participants with respect to baseline characteristics. Nevertheless, we decided to exclude breast fed infants from statistical analysis. Concerning information bias we concluded that no major bias is expected from the collection and lab analysis of faecal samples to determine intestinal microflora. As confounding factors we discussed mode of delivery and use of antibiotics. Although these factors can influence the intestinal microflora, in our study this would mean that we underestimated the percentage of bifidobacteria and lactobacilli of our GOS/FOS group. Next, we reflect on several issues concerning safety issues of the use of pre- and probiotics in infant nutrition. In the last years, several studies evaluated the safety of infant formulas containing GOS and FOS as prebiotics or viable *B. animalis* strain Bb-12 as probiotics in infant nutrition. It was concluded that all formulas were well tolerated, safe and resulted in an adequate growth. Finally, we gave several recommendations for future research. We recommended that for prebiotic GOS/FOS formula future research should focus mainly on hard clinical endpoints especially on the frequency and duration of gastrointestinal infections. Concerning probiotics, until now the results on probiotics and atopic disease and diarrhoeal disease are promising. In the future, we recommend that research focus on expanding the knowledge on the effects of probiotics on infants' health. However, based on our results we concluded that most likely it is not always necessary for the probiotic bacteria to colonise the gastro intestinal tract.

We conclude that infant formula containing the GOS/FOS mixture induces an intestinal microflora, which is in composition and metabolic activity

comparable to those found in breast fed infants. In contrast, we showed that infant formula containing GOS or viable *B. animalis* strain Bb-12 resulted in an intestinal microflora more similar to that found in standard formula fed infants. Additionally we demonstrated the GOS/FOS fed infants demonstrated a trend toward higher faecal SIgA concentrations, which were much less or absent in the infants fed Bb-12 or GOS.

Although, more research is needed to elucidate the effects of GOS/FOS formula on hard clinical endpoints, it can be reasonably assumed that infants fed on GOS/FOS will have a health benefit compared to infants fed on standard infant formula, infant formula containing GOS or an infant formula containing viable Bb-12.

## Samenvatting

## Inleiding

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Bij borstgevoede zuigelingen bestaat de darmflora voor 80 tot 90% uit bifidobacteriën. Het wordt algemeen aangenomen dat een darmflora die wordt gedomineerd door bifidobacteriën, positief is voor de gezondheid. Het zou zelfs deels kunnen verklaren waarom borstgevoede kinderen minder vaak lijden aan darminfecties. Gedurende de laatste 20 jaar, zijn er veel pogingen gedaan om de darmflora van borstgevoede zuigelingen meer te laten lijken op die van flesgevoede zuigelingen. Een aantal jaren geleden zijn de concepten prebiotica en probiotica ontwikkeld. Door het toevoegen van substraat, dat selectief de groei van "gezonde" bacterien stimuleert (prebiotica), of het toevoegen van levende bacteriën (probiotica), zouden belangrijke gezondheids effecten kunnen worden bereikt. Hoewel het mechanisme waarmee pre- en probiotica werken nog niet helemaal duidelijk is, is het waarschijnlijk dat hun effect op het immuunsysteem een belangrijke rol speelt.

In de afgelopen 4 jaar hebben we twee zuigelingenstudies uitgevoerd met het doel te onderzoeken wat de effecten van zuigelingenvoeding met prebiotica of probiotica zijn op:

- De samenstelling van de darmflora zoals het percentage bifidobacteriën (hoofdstuk 2 en 3) en lactobacillen (hoofdstuk 4) in de darmflora.
- De metabole activiteit van de darmflora zoals de relatieve hoeveelheden van korte keten vetzuren, lactaat concentratie en pH van de ontlasting (hoofdstuk 2 en 3)
- Indicatoren voor de ontwikkeling van de secretoire immuun respons zoals SIgA concentratie in ontlasting (hoofdstuk 5).

## Opzet van de studies

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In de periode van september 2000 tot augustus 2004 hebben we in de regio Arnhem-Nijmegen twee interventiestudies met zuigelingen uitgevoerd. In beide studies hebben we gekeken naar de effecten van prebiotica en probiotica op de ontwikkeling van de darmflora vanaf de geboorte tot de leeftijd van 16 weken. Daarnaast hebben we gedurende de eerste 32 weken de effecten van de prebiotische en probiotische voedingen op de faecale SIgA concentratie bestudeerd.

Voorafgaand aan de studies hebben powerberekeningen aangetoond dat per groep minstens 13 kinderen geïnccludeerd moesten worden. In verband met een verwachte uitval van ongeveer 30% zijn uiteindelijk meer kinderen geïnccludeerd. Voor de eerste studie, hebben we 120 zwangere vrouwen geworven. Van deze zwangeren hadden er 63 besloten om borstvoeding te geven en hadden er 57 besloten om geen borstvoeding te geven. Direct na de geboorte zijn de moeders die geen borstvoeding wilden of konden geven, gerandomiseerd en dubbel blind verdeeld over één van de flesvoedingsgroepen: de standaard flesvoedingsgroep (n=19) kreeg een standaard, ongesupplementeerde zuigelingenvoeding, de GOS/FOS flesvoedingsgroep (n=19) kreeg dezelfde standaard voeding gesupplementeerd met een mix van 0.6g/100ml galacto-oligosacchariden (GOS) en fructo-oligosacchariden (FOS) en de probiotische flesvoedingsgroep (n=19) kreeg de standaard voeding met daaraan toegevoegd  $6.0 \times 10^9$ /100ml levende *Bifidobacterium animalis* stam Bb-12.

Voor de tweede studie hebben we 72 zwangeren geworven, waarvan 38 hadden besloten borstvoeding te geven en 34 hadden besloten om dit niet te doen. Na de geboorte werden de zuigelingen van de moeders die niet wilden borstvoeden, gerandomiseerd en dubbel blind verdeeld over de volgende groepen: de standaard flesvoedingsgroep (n=17) kreeg standaard, ongesupplementeerde zuigelingenvoeding en de GOS groep (n=17) kreeg de standaard flesvoeding gesupplementeerd met 0.6g/100ml GOS.

In beide studies werden de kinderen binnen 3 dagen na de bevalling geïncubeerd indien ze voldeden aan de inclusiecriteria: normaal geboortegewicht, geen aangeboren afwijking en geen maag-darm ziekte. Behalve de interventie, was de opzet van beide studies identiek. De flesvoedingen werden verstrekt vanaf de geboorte tot de leeftijd van 32 weken.

Tijdens de interventieperiode werd de ouders gevraagd om op vaste tijden (dag 5, 10, 28 en elke 4 weken daarna) ontlastingmonsters te nemen uit de luier van hun kind. Aanvullend werd d.m.v vragenlijsten, informatie verzameld over de gezondheid en voeding van het kind. Gedurende het onderzoek werden de ouders regelmatig bezocht door de onderzoekers om de ontlastingmonsters en vragenlijsten op te halen.

Omdat het niet mogelijk is om borstvoeding gerandomiseerd en dubbel blind toe te wijzen, is de borstvoedingsgroep niet statistisch vergeleken met de flesvoedingsgroepen. De resultaten van de borstvoedingsgroep zijn beschouwd als referentiegroep. Alleen data van kinderen die op een tijdstip volledig borstvoeding hebben gekregen zijn weergegeven.

## Prebiotica

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In hoofdstuk 2 en 3 hebben we laten zien dat zowel de GOS/FOS als de GOS flesvoeding een darmflora induceerden die, net als in borstgevoede zuigelingen, werd gedomineerd door bifidobacteriën (respectievelijk  $59.2 \pm 7.7$  en  $76.5 \pm 2.6\%$  gemiddelde  $\pm$  SEM %bifidobacterien van het totale aantal bacterien op leeftijd 16w). Echter, door de onverwacht hoge percentages bifidobacteriën in de standaard flesvoedingsgroep ( $56 \pm 6.4\%$  bif.) konden er geen significante effecten van de 0.6g/100ml GOS/FOS en GOS aangetoond worden. Met betrekking tot het percentage lactobacillen, hebben we in hoofdstuk 4 getoond dat de GOS/FOS voeding echter wel een significant stimulerend effect t.o.v de standaard flesvoeding ( $6 \pm 2.6\%$  lactobacillen op 12w voor GOS/FOS vs.  $1 \pm 0.4\%$ , voor standaard,  $p=0.007$ ).

Er is geen significant effect gevonden van de GOS voeding ( $1\pm 0.4\%$  at 12w) vergeleken met de standaard voeding.

Met betrekking tot de metabole activiteit van de darmflora, hebben we in hoofdstuk 2 laten zien dat de GOS/FOS voeding een korte-keten-vetzuren patroon (relatieve hoeveelheden (%) van acetaat/propionaat /butyraat/overig) induceert dat vergelijkbaar is met die van borstgevoede zuigelingen (82/14/2/2 voor GOS/FOS vs. 90/6/2/2% voor borstvoeding, op leeftijd 16w). Zuigelingenvoeding met alleen GOS resulteerde echter in een korte-keten-vetzuren patroon dat meer overeenkomt met dat van de standaard flesvoedingsgroep (78/16/3/2 voor GOS vs. 73/20/5/3% voor standaard, op 16w). Ook de lactaatconcentratie (mmol lactaat per kg faeces) en de pH van de ontlasting van de GOS/FOS groep, zijn duidelijk meer vergelijkbaar met die van borstgevoede kinderen ( $40.9\pm 10.7$  lactaat en pH  $5.6\pm 0.2$  voor GOS/FOS vs.  $45.2\pm 9.0$  lactaat en pH  $5.7\pm 0.2$  voor borstvoeding). Net als het korte-keten-vetzuren patroon, lijkt de lactaat concentratie en pH in GOS gevoede kinderen ( $12.2\pm 5.1$  lactaat en pH  $6.5\pm 0.3$ ) meer op dat van de standaard flesgevoede kinderen ( $0.8\pm 0.7$  lactaat en pH  $7.1\pm 0.2$ ).

In hoofdstuk 5 hebben we laten zien dat zuigelingen gevoed met GOS/FOS flesvoeding gedurende de gehele interventieperiode een hogere faecale SIgA concentratie hebben dan de kinderen gevoed met GOS en standaard flesvoeding. Echter, dit verschil was alleen statistisch significant ( $p=0.015$ ) op leeftijd 16 weken  $0.84$  ( $0.6-1.8$ ) vs.  $0.39$  ( $0.1-0.9$ ), mediaan ( $P_{25}-P_{75}$ ).

## Probiotica

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In hoofdstuk 2 hebben we getoond dat zuigelingenvoeding met levende *B.animalis* (Bb-12) een darmflora induceerde die gedomineerd werd door bifidobacteriën ( $69.7\pm 2.7\%$  bif. op 16w). Echter, zoals in de prebiotische flesvoeding, hebben we ook voor de Bb-12 flesvoedingsgroep geen significant effect kunnen aantonen t.o.v de standaard flesvoedingsgroep

( $69.9 \pm 3.9\%$  bif). Tevens is ook geen significant verschil aangetoond in het percentage lactobacillen tussen de Bb-12 en de standaard flesvoedingsgroep ( $2.4 \pm 1.7\%$  voor Bb-12 versus  $0.9 \pm 0.4\%$  voor standaard op 12w).

Toevoegen van levende *B. animalis* stam Bb-12 had geen opvallend effect op de metabole activiteit van de darmflora (hoofdstuk 2). Het korte-keten-vetzuren profiel (%acetaat/propionaat/butyraat/overig) van de Bb-12 voeding was vergelijkbaar met die van de standaard flesvoedingsgroep (70/22/6/3 voor Bb-12 versus 70/20/6/5 voor standaard op 16w). Ook de pH en lactaat concentratie in de ontlasting van de Bb-12 groep was vergelijkbaar met die van de standaard flesvoedingsgroep ( $6.1 \pm 4.2$  mmol/kg lactaat en pH  $6.6 \pm 0.2$  voor Bb-12 vs.  $2.7 \pm 1.3$  lactaat en pH  $7.1 \pm 0.2$  voor standaard op 16w).

Op de concentratie SIgA in de ontlasting (hoofdstuk 5) kon geen duidelijke trend van de Bb-12 voeding worden aangetoond vergeleken met standaard ongesupplementeerde flesvoeding (0.36 (0.1-1.5) vs. 0.39 (0.1-0.9), mediaan ( $P_{25}$ - $P_{75}$ ) op 16w). De SIgA concentratie was zeer variabel over de tijd en er zijn geen significante verschillen gevonden ten opzichte van de standaard flesvoedingsgroep.

## Discussie en conclusie

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In hoofdstuk 6, zijn de belangrijkste bevindingen over de effecten van pre- en probiotische zuigelingenvoedingen bediscussieerd. Allereerst hebben we gespeculeerd over een verklaring voor de hoge percentages bifidobacteriën in de darmflora van de controle groep. We hebben hierbij gesuggereerd dat het relatief hoge lactose gehalte in de standaard voeding hier verantwoordelijk voor zou kunnen zijn. Vervolgens hebben we de verschillen in samenstelling en metabole activiteit van de darmflora tussen de GOS/FOS en de GOS groep bediscussieerden. We hebben gesuggereerd dat de verschillen in korte-keten-vetzuren profiel, lactaat

concentratie en pH veroorzaakt kunnen zijn door de verschillen in fermentatie snelheid van het lange-keten FOS en het korte-keten GOS. Tevens hebben we de mogelijkheid van een synergistisch effect van GOS en FOS op de groei van lactobacillen besproken en zijn de veelbelovende effecten van GOS/FOS op de faecale SIgA concentratie geëvalueerd. We hebben gespeculeerd over de reden voor de hoge variatie in SIgA concentratie binnen de groepen. Ook hebben we gediscussieerd over waarom er in de Bb-12 groep geen duidelijk effect op de samenstelling en metabole activiteit van de darmflora en faecal SIgA concentratie is gevonden. We suggereren dat waarschijnlijk, door het hoge percentage bifidobacteriën dat wordt veroorzaakt door de standaard flesvoeding, het niet mogelijk is om met het toevoegen van levende Bb-12, het percentage bifidobacteriën nog verder te verhogen.

In de evaluatie van de studie methodologie hebben we bediscussieerd in hoeverre sociale economische verschillen tussen borst- en flesgevoede zuigelingen en de hoge uitval in de borstvoedingsgroep een grote selectiebias hebben veroorzaakt. We hebben laten zien dat de groep van uitvallers niet verschilde van de overige deelnemers met betrekking tot basis karakteristieken. Ondanks alles hebben we toch besloten om de borstgevoedings groep uit te sluiten van de statistische analyses. Betreffende informatiebias hebben we geconcludeerd dat er geen grote bias te verwachten is door de verzameling en laboratorium analyse van de ontlastingmonsters. Als mogelijke confounding factoren in onze studie zijn naar voren gekomen: de wijze van geboorte (vaginaal vs. keizersnede) en het gebruik van antibiotica. Hoewel beide factoren de darmflora kunnen beïnvloeden, zou het ietwat hogere percentage keizernedes in de GOS/FOS groep een onderschatting van het percentage bifidobacteriën en lactobacillen kunnen betekenen en dus onze conclusies eerder versterken dan verzwakken.

Vervolgens hebben we de veiligheidsaspecten van het gebruik van pre- en probiotica in zuigelingen voeding besproken. In de laatste jaren, hebben verschillende studies de veiligheid van zuigelingenvoedingen met GOS en FOS of levende *B. animalis* stam Bb-12 onderzocht. Over het algemeen is

geconcludeerd dat de voedingen met GOS, FOS of Bb-12 goed worden getolereerd, veilig zijn en resulteren in een goede groei.

Voor toekomstig onderzoek naar de effecten van prebiotische zuigelingenvoeding hebben we aanbevolen deze meer te focussen op harde klinische eindpunten, zoals de frequentie en duur van infecties. Wat betreft de probiotische zuigelingenvoeding, zijn de resultaten van eerdere studies op atopische ziekte en diarree zeer veelbelovend. Voor de toekomst, willen wij adviseren dat onderzoek naar de mechanismen en effecten van Bb-12 op de gezondheid van het kind wordt gecontinueerd. Echter, gebaseerd op onze resultaten, voegen wij toe dat het hoogstwaarschijnlijk niet per se nodig is voor de probiotische bacterie om het darmstelsel van het kind te koloniseren.

We concluderen dat zuigelingenvoeding met GOS/FOS een darmflora induceert die qua samenstelling en metabole activiteit vergelijkbaar is met die van borstgevoede zuigelingen. In contrast, hebben we laten zien dat zuigelingen voeding met alleen GOS of levende *B. animalis* Bb-12 resulteert in een darmflora die meer lijkt op die van zuigelingen gevoed met standaard ongesupplementeerde flesvoeding. Tevens, hebben we gedemonstreerd dat in tegenstelling tot de GOS en Bb-12 voeding, de GOS/FOS flesvoeding resulteerde in een duidelijke trend naar een hogere faecal SIgA concentratie.

Hoewel meer onderzoek nodig is om de effecten van GOS/FOS flesvoeding op harde klinische eindpunten op te helderen, kan met alle redelijkheid worden aangenomen dat kinderen gevoed met GOS/FOS voeding kunnen profiteren van een gezondheidsbevorderend effect vergeleken met kinderen gevoed met een standaard voeding, een voeding met 0.6g/100ml GOS voeding of een voeding met  $6.0 \times 10^9$ /100ml levende Bb-12.

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Arnhem, 13 november 2004

*Astrid*

## Curriculum Vitae

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Astrid Maria Bakker-Zierikzee werd geboren op zondag 19 oktober 1975 in Katwijk aan Zee. In 1994 behaalde zij haar VWO diploma aan het St. Adelbert college te Wassenaar en in datzelfde jaar begon zij met de studie Voeding van de Mens aan de Wageningen Universiteit. In juni 1998 studeerde zij af in de richting "Voeding en Fysiologie". Gedurende haar studie verrichtte zij onderzoeken op de vakgroep Fysiologie van Mens en Dier onder leiding van Victor Schreurs en op de afdeling Gastroenterologie van het Universitair Medisch Centrum Utrecht onder leiding van Prof. Louis Akkermans. In het kader van een stage verbleef zij gedurende 5 maanden op de McMaster University, Hamilton in Canada, onder leiding van Dr. Stefanie Atkinson. Aansluitend op haar studie begon zij, in nauwe samenwerking met Numico Research BV, een promotieonderzoek aan de afdeling Humane Voeding van Wageningen Universiteit. De resultaten van haar onderzoek staan beschreven in dit proefschrift. Van september 2000 tot september 2001 heeft zij deel genomen aan de organisatie van een 10-daagse studiereis voor de AIO's van de afdeling, naar Italië, Zwitserland en Duitsland. Sinds 1 november 2004 is zij werkzaam als onderzoeker bij TNO Voeding, afdeling "Food and Chemical Risk Analysis", te Zeist.

# Opleidingstraject

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## Cursussen

Ontwerp en analyse van experimenten	2000
Begeleiden en organiseren van een afstudeervak	2000
"Ecophysiology of the gastrointestinal tract"	2001
Systematisch literatuuronderzoek	2001
"Scientific writing"	2002
Tutoretraining	2003
"Nutrition and lifestyle epidemiology"	2003
Good Clinical Practice -ICH Training en advies	2003
Time planning and project management-DLV Communicatie	2004

## Symposia and congressen

Darmendag	2001, 2003
Congres "European Society of Pediatrics, Gastroenterology, Hepathology and Nutrition	2001, 2004
Symposium "New Developments in infant nutrition"	2002
Symposium: "Gut flora in health and disease-potential role of probiotics"	2004

## Algemeen

VLG aio-dagen en uitstroomcursus	2000,2004
AIO studiereis, Italie-Zwitserland-Duitsland	2001
NWO talentendag	2003



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