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

Astrid M. Bakker-Zierikzee1*, Martine S. Alles2, Jan Kno1, Frans J. Kok1, Jules J. M. Tolboom3 and Jacques G. Bindels1,2

1Division of Human Nutrition, Wageningen University, PO Box 8129, NL-6700 EV Wageningen, The Netherlands
2Numico Research BV, Wageningen, The Netherlands
3Department of Paediatrics, University Medical Centre St Radboud, Nijmegen, The Netherlands

(Received 3 June 2004 – Revised 21 October 2004 – Accepted 2 February 2005)

Adding prebiotics or probiotics to infant formula to improve the intestinal flora of formula-fed infants is considered to be a major innovation. Several companies have brought relevant formulations onto the market. However, comparative data on the effects of pre- and probiotics on the intestinal microflora of infants are not available. The present study aimed 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. 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 galacto-oligosaccharides and fructo-oligosaccharides (6 g/l). The probiotic (Bb-12) group (n 19) received the same formula supplemented with 6·0 £ 1010 viable cells of *B. animalis* per litre. The standard group (n 19) received non-supplemented regular formula. A group of sixty-three breast-fed infants was included as a reference group. Faecal samples were taken at postnatal day 5 and 10, and week 4, 8, 12 and 16. Compared with the groups fed Bb-12 and standard formula, the GOS/FOS formula group showed higher faecal acetate ratio (69·7 % (SEM 2·7), 69·9 % (SEM 3·9) and 82·2 % (SEM 5·3); P<0·05) and lactate concentration (11·3 (SEM 7·9), 3·1 (SEM 2·3) and 34·7 (SEM 10·7) mmol/kg faeces) and lower pH (6·6 (SEM 0·2), 7·1 (SEM 0·2) and 5·6 (SEM 0·2); P<0·05) at 16 weeks. Differences in percentage of bifidobacteria between the GOS/FOS (59·2 % (SEM 7·7)), Bb-12 (52·7 % (SEM 8·0)) and the standard (51·8 % (SEM 6·4)) groups were not statistically significant at 16 weeks. 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 those of the standard group.


In breast-fed infants the intestinal microflora is dominated by bifidobacteria. In general, formula-fed infants have a more diverse flora (Bullen & Tearle, 1976; Benno et al. 1984; Balmer et al. 1989; Chierici et al. 1997; Harmsen et al. 2000). Fermentation by intestinal microflora results in the production of SCFA, which have different functions such as an energy source for colonocytes, regulating cell growth, lowering intestinal pH and inhibiting the growth of pathogens (Wang & Gibson, 1993). Branched SCFA, products of protein breakdown by intestinal bacteria, are potentially harmful. In breast-fed infants, 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* (Eklund, 1983; Wang & Gibson, 1993). 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 infant gut (Brand et al. 1998; Engfer et al. 2000). 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’ (Gibson & Roberfroid, 1995). 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 approached 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 (Boehm et al. 2002; Moro et al. 2002). It was found that feeding infants GOS/FOS formula significantly increased the number of bifidobacteria.

Besides prebiotics, another approach to improve the intestinal microflora is to add probiotics to infant formula (Langhendries et al. 1995). Probiotics were originally defined as ‘live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance’ (Fuller, 1989). The possible role of specific probiotic bacteria in recovery from atopic disease and treatment of rotavirus diarrhoea in children

Abbreviations: Bb-12, *Bifidobacterium animalis* strain Bb-12; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridisation; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

* Corresponding author: Dr A. M. Bakker-Zierikzee, fax +31 317 482782, email astrid.bakker@hetnet.nl
was elucidated in several studies (Isolauri et al. 2000; Kalliomaki et al. 2001). However, to date, only one study has focused on the effects of probiotics on the intestinal microflora of infants (Lanhendries et al. 1995).

Recognising the possible health benefits of a gut 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 formulas, the study was performed in one population under comparable environmental conditions. We hypothesised 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, similar to breast-fed infants, we expected to find a lower pH, higher ratios of acetate and higher amounts of lactate in the faeces of infants fed prebiotic and probiotic formulas, compared with infants fed standard formula.

Subjects and methods

Subjects

Sixty-three pregnant women who had decided to breast-feed, and fifty-seven who had chosen not to, were recruited during their last trimester of pregnancy. Infants with normal birth weight and no congenital abnormality, congenital disease or gastrointestinal disease were enrolled within 3 d 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 had 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 3 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 (GOS/FOS; n 19) received a regular, non-supplemented infant formula (Nutrilon I; Nutricia, Zoetermeer, The Netherlands). The main composition data of the standard formula at the standard dilution of 131 g/l are given in Table 1. The prebiotic formula group (GOS/FOS; n 19) received the same standard infant formula supplemented (6 g/l) with a mixture of trans-galacto-oligosaccharides (Vivinal GOS; Borculo Domo Ingredients, Zwolle, The Netherlands) and fructo-oligosaccharides (Raftilite HP FOS; 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 (Stahl et al. 1994). The probiotic formula group (Bb-12; n 19) received the standard infant formula supplemented with 6·0 × 10¹⁰ viable cells of Bifidobacterium animalis per litre (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 (Langhendries et al. 1995; Fukushima et al. 1998; Alander et al. 2001). Several studies have demonstrated that during a period of daily ingestion of viable Bb-12 cells, the number of faecal bifidobacteria increases significantly (Link-Amster et al. 1994; Schiffrin et al. 1995; Alander et al. 2001).

The shelf life of the probiotic formula was tested during storage. After 12 months of storage, 1·0 × 10¹⁰ (SEM 0·5 × 10¹⁰) colony-forming units 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 maximal temperature of 45 °C before adding the milk powder. This was to avoid hot spots in the liquid milk during microwaving, possibly leading to killing of the bacteria. Mothers who had 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 formula consumed with the recorded food intake.

Questionnaires

Demographic, clinical and anthropometric 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 and 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 defecation, collected in faeces containers (Greiner Labotechnik, Alphen a/d Rijn, The Netherlands) and stored immediately at −20 °C by the parents. During the study period, the investigators visited the participants regularly, to collect faeces samples and

---

### Table 1. Composition of the study formulas per litre

<table>
<thead>
<tr>
<th></th>
<th>Standard formula</th>
<th>GOS/FOS formula</th>
<th>Bb-12 formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>2800</td>
<td>2800</td>
<td>2800</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Casein:whey</td>
<td>40:60</td>
<td>40:60</td>
<td>40:60</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>GOS (g)</td>
<td>–</td>
<td>5·4</td>
<td>–</td>
</tr>
<tr>
<td>FOS (g)</td>
<td>–</td>
<td>0·6</td>
<td>–</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>75</td>
<td>67</td>
<td>75</td>
</tr>
<tr>
<td>Glucose (g)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Bb-12 (CFU)</td>
<td>–</td>
<td>6·0 × 10¹⁰</td>
<td>–</td>
</tr>
</tbody>
</table>

GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; Bb-12, Bifidobacterium animalis strain Bb-12; CFU, colony-forming units.
questionnaires. Infant formula was supplied on request. Faeces samples were transported to the laboratory in a portable freezer (minimal temperature –15°C, MRFD-015; Veba Meditemp, Uden, The Netherlands).

Preparation of faecal samples

For the determination of SCFA, 1 g of faeces was thawed in iced water, diluted 10 × in MilliQ and homogenised for 10 min using a stomacher (IUL Instruments, Barcelona, Spain). Then 350 μl of homogenised faeces was mixed with 200 μl formic acid (5%, by vol.), 100 μl 2-ethylbutyric acid (1·25 g/l; Sigma-Aldrich, Zwijndrecht, The Netherlands) and 350 μl MilliQ. The samples were centrifuged for 5 min at 15 000 g to remove large particles and the supernatant was stored at –20°C.

For the fluorescence in situ hybridisation (FISH) analysis and lactic acid measurements, the samples were thawed in iced water, diluted 10 × (w/v) in PBS, pH 7.4, and homogenised for 10 min using a stomacher. The homogenised faeces were stored at –20°C.

Fluorescence in situ hybridisation

FISH analysis was performed as described previously (Langendijk et al. 1995; Jansen et al. 1999; Harmsen et al. 2000) with some slight modifications. Paraformaldehyde-fixed samples were applied to gelatine-coated glass slides (polytetrafluoroethylene-coated eight-well (1 cm2/well) object slides; CBN lab suppliers, applied to gelatine-coated glass slides (polytetrafluoroethylene-coated some slight modifications. Paraformaldehyde-fixed samples were diluted 10 × in PBS for 5 min at room temperature. After DAPI staining the samples were centrifuged for 5 min at 15 000 g to remove large particles and the supernatant was stored at –20°C.

The fluorescence in situ hybridisation (FISH) analysis and lactic acid measurements, the samples were thawed in iced water, diluted 10 × (w/v) in PBS, pH 7.4, and homogenised for 10 min using a stomacher. The homogenised faeces were stored at –20°C.

Data analysis

Prior to the study, power calculations showed that to detect a difference in the percentage of bifidobacteria between the intervention formula groups (GOS/FOS and Bb-12) and the standard formula group of 30% with SD of 25%, thirteen infants should be included per group. Because of an expected drop-out of 30% in the formula groups, more infants than calculated were included in the study. The SPSS statistical package (version 11.0; SPSS Inc., Chicago, IL, USA) was used for analysis of the results. All values were checked for normality by visual inspection of the normal probability plots. Differences in percentage of bifidobacteria, pH, relative amounts of SCFA and lactate between the groups were tested for significance using ANOVA. In the case of a significant difference (P<0.05), groups were compared by using the Bonferroni post hoc test.

Because it is not possible to assign breast- and bottle-feeding double blindly 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

In total, 120 infants were included in the study between January 2000 and May 2003. Fifty-seven infants started on
formula feeding directly after birth and were divided equally among the formula groups. Of the sixty-three infants who were fed breast milk directly after birth, twenty-four switched to formula feeding before the age of 16 weeks and five infants dropped out. The characteristics of the study subjects are shown in Table 2. In the formula groups, thirteen infants dropped out of the study within the first 16 weeks after birth: four in the standard group, five in the GOS/FOS group and four in the Bb-12 group. Reasons for dropping out included colic, suspicion of cows’ milk allergy, constipation and practical problems.

Faecal bifidobacteria

The percentage of bifidobacteria in the faeces of infants at the age of 5 d, 10 d, and 4, 8, 12 and 16 weeks is shown in Fig. 1 for the different feeding groups. Although not statistically significant, the GOS/FOS group tends to have a higher percentage of bifidobacteria in the total bacterial count at all ages compared with the standard and Bb-12 formula groups. Percentages of bifidobacteria in the formula groups are comparable to that found in the breast-fed group.

**pH**

The pH values measured in the faeces of the formula-fed infants are shown in Fig. 2. The lowest pH was found in infants fed on breast milk. Faecal pH of infants fed the GOS/FOS formula was lower than in the standard (P<0.05 at all ages except 5 d) and the Bb-12 formula groups (P<0.05 from week 8 on). At age 10 d, the faecal pH of infants fed the Bb-12 formula was significantly lower than that in the standard formula group (P=0.001).

Short-chain fatty acids

The total amount of SCFA in the faeces is shown in Table 3. The percentages of the different SCFA from the total amount of SCFA are shown in Table 4. The table includes data from all available faeces samples that were large enough (0.5 ml) to perform the bacterial count at all ages compared with the standard and Bb-12 formula groups. Percentages of bifidobacteria in the formula groups are comparable to that found in the breast-fed group.

**Lactate**

The concentrations of lactate (mmol/kg faeces) in all groups are shown in Table 3. Already from 5 d of age, the GOS/FOS formula group (NS) and the group fed on breast milk had higher amounts of faecal lactate than did the standard and Bb-12 formula groups. No differences were found between the Bb-12 group and the standard formula group.

**Discussion**

With the aim of stimulating the typical intestinal microbial ecology of breast-fed infants in formula-fed infants, the present study compared 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 the results of both formula groups with those observed in the breast-fed group. However, we did not statistically compare the breast-feeding group with the formula feeding groups because it is not possible to randomise and double blind assign infants to breast-feeding and also because of the obvious selection bias due to social and educational differences between breast- and formula feeding mothers (Ford & Labbok, 1990). 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, thirteen of the fifty-seven formula-fed infants dropped out before the age of 16 weeks. The number of drop-outs 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 rise to selection bias.

Despite different product compositions and the somewhat lower dosage of GOS/FOS in the present study (0.6 g/100 ml), the percentage of bifidobacteria found in the GOS/FOS group (64 % at 8 weeks of age) is similar to that observed by other investigators: 76 % after 6 weeks, 0.8 g/100 ml (Schmelzl et al. 2003), 69 % after 6 weeks, 0.8 g/100 ml (Kno et al. 2003); and 65 % after 6 weeks, 0.8 g/100 ml (Kno et al. 2002). For unknown reasons, the percentage of bifidobacteria in the standard formula group in the present study is higher than that observed previously. Differences in composition of the formula (other than the carbohydrates) may play a role.

Results of the probiotic group are difficult to compare with other studies because, to date, only one study has reported the
effect of a probiotic infant formula on the intestinal microflora but did not give any quantitative data on the stimulation of bifidobacteria (Langhendries et al. 1995).

In the present study, we found that feeding infants an infant formula supplemented with a mixture of GOS and long-chain FOS 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 group and the standard formula group are similar to the findings of others (Ogawa et al. 1992; Siigur et al. 1993; Edwards et al. 1994). However, the relative amounts of SCFA, the lactate concentration and the pH in the faeces of the GOS/FOS group are comparable to values seen in breast-fed infants. High relative amounts of acetate and high concentrations of lactate, together with the low faecal pH, create conditions that are less favourable (colonisation resistance) for Enterobacteriaceae and other potential opportunistic pathogens that can be
Concentration of lactate (mmol/kg faeces) and total short-chain fatty acids (mmol/kg faeces) in the faeces of infants, between birth and 16 weeks of age, of the different study groups*

Table 3. (Mean values with their standard error and number of determinations)

|                  | 5 days |          | 10 days |          | 4 weeks |          | 8 weeks |          | 12 weeks |          | 16 weeks |          |
|------------------|--------|----------|---------|----------|---------|----------|---------|----------|---------|----------|---------|----------|----------|
|                  | Lactate| SCFA     | Lactate| SCFA     | Lactate| SCFA     | Lactate| SCFA     | Lactate| SCFA     | Lactate| SCFA     | Lactate| SCFA     |
| Standard formula | 14.0   | 7.7      | n       | 13       | 4.7    | 3.0      | 19      | 5.5      | 3.0    | 16       | 7.5    | 6.4      | 15      | 13.3     | 8.9      | 14.0     | 2.7      | 1.3      | 14       |
| GOS/FOS formula  | 60.7   | 4.4      | n       | 32       | 5.4    | 4.9      | 33      | 22.9     | 4.4    | 35       | 5.3    | 4.3      | 3.3    | 44       | 5.5      | 3.2      | 35       | 5.5      | 3.2      | 35       |
| Breast milk      | 13.6   | 4.4      | n       | 32       | 5.4    | 4.9      | 33      | 22.9     | 4.4    | 35       | 5.3    | 4.3      | 3.3    | 44       | 5.5      | 3.2      | 35       | 5.5      | 3.2      | 35       |

GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; Bb-12, Bifidobacterium animalis strain Bb-12.

*No statistically significant differences were found between groups (P > 0.05).

A. M. Bakker-Zierikzee et al. 1994; Isolauri et al. 1994; Isolauri, 2001). According to the present study, adding viable B. animalis strain Bb-12 may have a role in the prevention of diarrhoea. Nevertheless, several studies have shown that specific probiotic bacteria, including B. animalis strain Bb-12, are beneficial for the treatment of diarrhoea. In the present study, adding viable B. animalis strain Bb-12 may have a role in the prevention of diarrhoea. Nevertheless, several studies have shown that specific probiotic bacteria, including B. animalis strain Bb-12, are beneficial for the treatment of diarrhoea.

The major difference with other studies using the GOS/FOS mixture is the relatively high percentage of bifidobacteria in the breast-fed group, indicating a distinct effect of the GOS/FOS mixture. The breast-fed group to the standard formula group, we conclude that a strong bifidogenic effect of the Bb-12 formula is unlikely. On the other hand, the GOS/FOS mixture may have a role in the treatment of diarrhoea. In the present study, adding viable B. animalis strain Bb-12 may have a role in the prevention of diarrhoea. Nevertheless, several studies have shown that specific probiotic bacteria, including B. animalis strain Bb-12, are beneficial for the treatment of diarrhoea.
original definition by Fuller (1989), 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, high concentration of faecal lactate and a low faecal pH. In the infants who received the standard formula or the formula with added viable cells of 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.

Acknowledgements

We would like to thank the parents and other caregivers for their participation in this study; Esmeralda van der Linde, Rob Slump and Jochem Steenbakkers for analysis of the faecal samples; and Bertha Stallinga and Martine van de Brink for data collection. Klaartje de Jong is acknowledged for her efforts in the initial phase of designing the study.

References


