

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

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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 6.0×10^{10} 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.

Infants: Prebiotics: Probiotics: Galacto-oligosaccharides: Fructo-oligosaccharides: Bifidobacteria: Short-chain fatty acids: Lactate: pH

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 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 (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.

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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 (Langhendries *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 (*n* 19) received a regular, non-supplemented infant formula (Nutrilon I; Nutricia, Zoetermeer, The Netherlands). The main compositional 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 (Raftiline HPFOS; Orafit Active Food Ingredients, Tienen, Belgium). The mixture comprised 90% GOS and 10% FOS in order to closely resemble the spectrum of molecular masses of the neutral oligosaccharide fraction in human milk (Stahl *et al.* 1994). The probiotic formula group (Bb-12; *n* 19) received the standard infant formula supplemented with 6.0×10^{10} 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

Table 1. Composition of the study formulas per litre

	Standard formula	GOS/FOS formula	Bb-12 formula
Energy (kJ)	2800	2800	2800
Protein (g)	14	14	14
Casein:whey	40:60	40:60	40:60
Fat (g)	35	35	35
Total carbohydrates (g)	75	75	75
GOS (g)	–	5.4	–
FOS (g)	–	0.6	–
Lactose (g)	75	67	75
Glucose (g)	–	2	–
Bb-12 (CFU)	–	–	6.0×10^{10}
Minerals			
Ca (mg)	540	540	540
P (mg)	270	270	270
Mg (mg)	50	50	50
Na (mg)	190	190	190
K (mg)	680	680	680
Cl [–] (mg)	430	430	430
Fe (mg)	5	5	5
Zn (mg)	5	5	5

GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; Bb-12, *Bifidobacterium animalis* strain Bb-12; CFU, colony-forming units.

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, 1.0×10^{10} (SEM 0.5×10^{10}) 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 Labortechnik, 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

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 $10\times$ in MilliQ and homogenised for 10 min using a stomacher (IUL Instruments, Barcelona, Spain). Then $350\ \mu\text{l}$ of homogenised faeces was mixed with $200\ \mu\text{l}$ formic acid (5%, by vol.), $100\ \mu\text{l}$ 2-ethylbutyric acid (1.25 g/l; Sigma-Aldrich, Zwijndrecht, The Netherlands) and $350\ \mu\text{l}$ 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 $10\times$ (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 ($1\ \text{cm}^2/\text{well}$) object slides; CBN lab suppliers, Drachten, The Netherlands) and air-dried. The dried samples were dehydrated in 96% ethanol for 10 min. Hybridisation buffer (20 mM-Tris-HCl, 0.9 M-NaCl, 0.1% SDS; pH 7.2) with Cy3-labelled *Bifidobacterium*-specific probe Bif164mod (5'-CAT CCG GYA TTA CCA CCC; $10\ \text{ng}/\mu\text{l}$), was preheated and added to the dried samples. Bif164mod is modified version of probe S-G-Bif-0164-a-A-18 (Langendijk *et al.* 1995), which detects the presence of bifidobacteria including the *B. animalis* species (Satokari *et al.* 2001). The slides were incubated overnight in a dark moist chamber at 50°C . After hybridisation the slides were washed for 30 min in 50 ml of preheated washing buffer (20 mM-Tris-HCl, 0.9 M-NaCl; pH 7.2) and briefly rinsed in MilliQ. For staining all bacteria, the samples were incubated with $0.25\ \text{ng}$ 4',6-diamidino-2-phenylindole (DAPI)/ μl in PBS for 5 min at room temperature. After DAPI staining the slides were briefly rinsed in MilliQ, dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) 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 twenty-five randomly chosen microscopic positions. At each position the percentage of bifidobacteria was determined by counting all cells with a DAPI filter set (SP100; Chroma Technology Corp., Brattleboro, VT, USA) and counting all bifidobacteria using a Cy3 filter set (41 007; Chroma Technology Corp.).

Short-chain fatty acid analysis

The SCFA acetic, propionic, n-butyric, isobutyric and n-valeric acids were quantitatively determined using a Varian 3800 gas chromatograph (Varian, Inc., Walnut Creek, CA, USA) equipped with a flame ionisation detector. The sample ($0.5\ \mu\text{l}$) was injected

at 80°C into the column (Stabilwax, $15\ \text{m}\times 0.53\ \text{mm}$, film thickness $1.00\ \mu\text{m}$; Restek Co., Bellefonte, PA, USA) using He as carrier gas ($20.7\ \text{kPa}$). New columns were conditioned overnight at 200°C . After injection of the sample, the oven was heated to 160°C at a rate of $16^{\circ}\text{C}/\text{min}$, followed by heating to 220°C at $20^{\circ}\text{C}/\text{min}$ and finally maintained at a temperature of 220°C for 1.5 min. The temperature of the injector and the detector was 200°C . After every ten samples the column was cleared by injection of $0.5\ \mu\text{l}$ formic acid (1%, by vol.) to avoid memory effects of the column, followed by injection of $0.5\ \mu\text{l}$ standard SCFA mix (1.77 mM-acetic acid, 1.15 mM-propionic acid, 0.72 mM-n-butyric acid, 0.72 mM-isobutyric acid, 0.62 mM-n-valeric acid; Sigma-Aldrich) 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

Homogenised faeces were thawed on ice and centrifuged for 5 min at $14\ 000\ \text{rpm}$, then $100\ \mu\text{l}$ of the supernatant was heated for 10 min at 100°C to inactivate all enzymes. Lactate was determined enzymatically using an 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 Handy-lab pH meter (Schott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler Toledo, Columbus, OH, USA).

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 SCFA analysis. There were no statistically significant differences in total SCFA concentration found between the formula groups. However, already after 10 d, differences in SCFA profiles could be seen between infants fed on GOS/FOS formula and infants

fed on standard or Bb-12 formula. Infants fed the GOS/FOS formula had higher percentages of acetate and lower percentages of propionate, butyrate and iC4-5 SCFA (isobutyrate, isovalerate and valerate) compared with infants fed the standard or the Bb-12 formula. There were no differences in the relative amounts of SCFA in the faeces of infants fed Bb-12 formula compared with infants fed the standard formula.

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 blindly 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 (Schmelzle *et al.* 2003), 69% after 6 weeks, 0.8 g/100 ml (Knol *et al.* 2003); and 65% after 6 weeks, 0.8 g/100 ml (Knol *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

Table 2. Characteristics of the study subjects

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

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

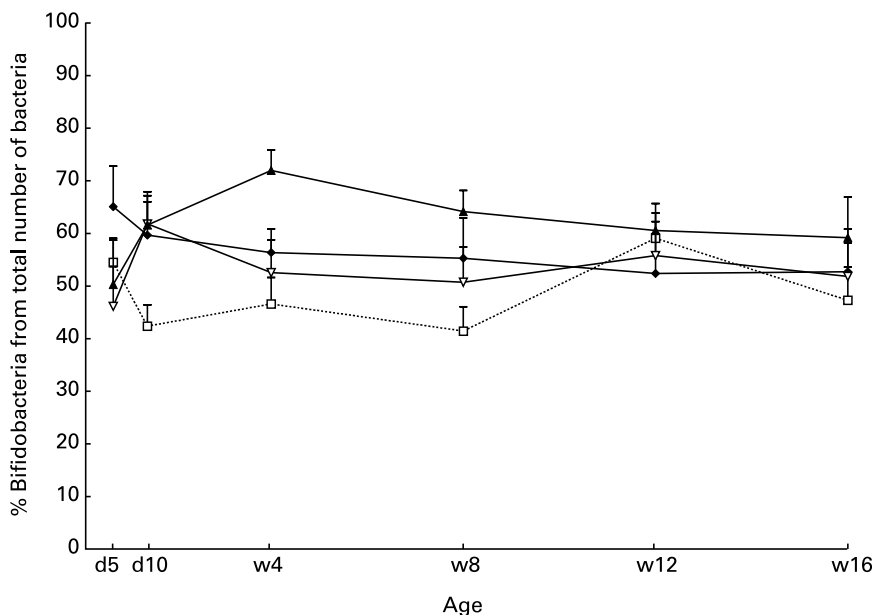


Fig. 1. Percentage of bifidobacteria from total number of bacterial cells per gram of faeces (wet weight) in infants fed breast milk (-□-), formula supplemented with a mixture of galacto-oligosaccharides and fructo-oligosaccharides (6 g/l; —▲—), formula supplemented with *Bifidobacterium animalis* strain Bb-12 (6.0×10^{10} viable cells/l; —◆—) and standard formula (—▽—), between birth and 16 weeks of age (d, day; w, week). Values are means with their standard error shown by vertical bars. No statistically significant differences were found between groups ($P > 0.05$).

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

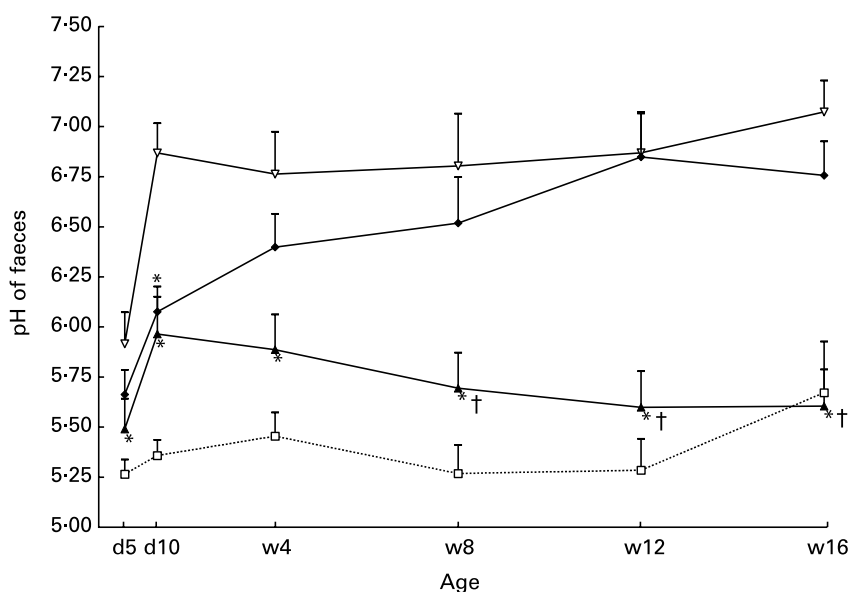


Fig. 2. pH values of faeces in infants fed breast milk (-□-), formula supplemented with a mixture of galacto-oligosaccharides and fructo-oligosaccharides (6 g/l; —▲—), formula supplemented with *Bifidobacterium animalis* strain Bb-12 (6.0×10^{10} viable cells/l; —◆—) and standard formula (—▽—), between birth and 16 weeks of age (d, day; w, week). Values are means with their standard error shown by vertical bars. Mean values were significantly different from those of the standard formula group: * $P < 0.05$. Mean values were significantly different from those of the Bb-12 formula group: † $P < 0.05$.

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

	5 days			10 days			4 weeks			8 weeks			12 weeks			16 weeks			
	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	
Standard formula	Lactate	14.0	7.7	13	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
	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
	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	14.0	4.8	19	8.2	3.9	17	5.3	1.8	17	14.9	6.6	17	3.6	2.1	16	6.1	4.2	15
	SCFA	60.7	6.2	16	59.3	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	13.6	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
	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

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

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

present in low numbers or obtained from the environment (Moon, 1983; Adams & Hall, 1988). As in breast-fed infants, the relative amounts of faecal butyrate and propionate in the GOS/FOS group are lower than in the standard and Bb-12 groups. This indicates that in the groups fed breast milk and GOS/FOS the intestinal microflora contains lower numbers of butyrate- and propionate-producing bacteria such as *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 (isobutyrate, isovalerate and valerate) and low faecal pH that were seen in the GOS/FOS and breast-fed groups indicate that protein fermentation by e.g. *E. coli* is low.

The major difference with 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 group 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 might be found in the presumption that the GOS/FOS mixture used in the present study predominantly stimulated the growth of other lactic acid-producing bacteria like *Lactobacillus* (Bullen & Tearle, 1976; Edwards *et al.* 1994). In fact, the prebiotic mixture, which contains low- as well as high-molecular-mass oligosaccharides, was designed to create optimal growth conditions for both bifidobacteria and lactobacilli (Boehm *et al.* 2002). No specific FISH probe is available for lactobacilli, and new methods need to be developed for their accurate quantification. 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 (MacLean *et al.* 1983). This could have led to less acetate and lactate production and subsequently higher pH. Different from traditional plating methods, the FISH method used in the present study does not make a distinction between metabolically active and inactive bacteria (Harmsen *et al.* 2000).

In the Bb-12 group the percentage of bifidobacteria was already very high after 5 d (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 colonisation 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 the present 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 have shown that specific probiotic bacteria including strain Bb-12 may have a role in the prevention and treatment of different diseases (Saavedra *et al.* 1994; Isolauri *et al.* 2000; Isolauri, 2001). According to the

Table 4. Relative amount of short-chain fatty acids (percentage of total short-chain fatty acids) in the faeces of infants, between birth and 16 weeks of age, of the different study groups

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

	5 days			10 days			4 weeks			8 weeks			12 weeks			16 weeks		
	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>
Standard formula			12			15			12			14			14			12
Acetate	84.3	3.4		70.9	2.0		71.8	2.8		74.6	2.9		73.9	2.9		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	
iC4-5 SCFA	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			11			16			15			16			13			14
Acetate	85.8	5.1		84.0*	2.4		77.7	2.2		83.5†	2.7		86.5†	2.1		82.2	5.3	
Propionate	12.0	4.7		13.5	2.3		15.4	2.0		11.4†	2.1		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*†	0.4	
iC4-5 SCFA	1.7	0.7		1.1	0.4		1.1	0.3		1.4†	0.4		1.0	0.4		1.5*	0.4	
Bb-12 formula			16			12			14			16			13			15
Acetate	80.0	3.8		73.7	4.0		75.6	3.4		71.9	2.5		66.1	3.4		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	
iC4-5 SCFA	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			32			33			28			22			24			17
Acetate	89.5	1.8		89.3	1.9		91.0	1.8		91.2	1.6		86.1	3.3		89.7	2.7	
Propionate	7.0	1.5		5.8	1.3		4.3	1.2		5.4	5.4		7.5	2.2		6.4	2.1	
Butyrate	1.6	0.4		2.3	0.5		2.6	0.6		1.9	1.9		3.0	0.7		1.6	0.4	
iC4-5 SCFA	2.0	0.4		2.6	0.4		2.1	0.4		1.6	1.6		3.5	0.8		2.2	0.5	

Total SCFA, sum of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate; iC4-5 SCFA, sum of isobutyrate, isovalerate and valerate; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; Bb-12, *Bifidobacterium animalis* strain Bb-12.

Mean values were significantly different from those of the standard formula group: * $P < 0.05$.

Mean values were significantly different from those of the Bb-12 formula group: † $P < 0.05$.

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.

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References

Adams MR & Hall CJ (1988) Growth inhibition of food-borne pathogens by lactic acid and acetic acid and their mixtures. *Int J Food Sci Technol* **23**, 287–292.

- Alander M, Matto J, Kneifel W, Johansson M, Kogler B, Crittenden R, Mattila ST & Saarela M (2001) Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. *Int Dairy J* **11**, 817–825.
- Balmer SE, Scott PH & Wharton BA (1989) Diet and faecal flora in the newborn: casein and whey proteins. *Arch Dis Child* **64**, 1678–1684.
- Benno Y, Sawada K & Mitsuoka T (1984) The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. *Microbiol Immunol* **28**, 975–986.
- Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B & Marini A (2002) Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child* **86**, F178–F181.
- Brand MJ, McVeagh P, McNeil Y & Messer M (1998) Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. *J Pediatr* **133**, 95–98.
- Bullen CL & Tearle PV (1976) Bifidobacteria in the intestinal tract of infants: an *in-vitro* study. *J Med Microbiol* **9**, 335–344.
- Chierici R, Sawatzki G, Thurl S, Tovar K & Vigi V (1997) Experimental milk formulae with reduced protein content and desialylated milk proteins: influence on the faecal flora and the growth of term newborn infants. *Acta Paediatr* **86**, 557–563.
- Edwards CA, Parrett AM, Balmer SE & Wharton BA (1994) Faecal short chain fatty acids in breast-fed and formula-fed babies. *Acta Paediatr* **83**, 459–462.
- Eklund T (1983) The anti microbial effect of dissociated and undissociated sorbic-acid at different pH levels. *J Appl Bacteriol* **54**, 383–390.
- Engfer MB, Stahl B, Finke B, Sawatzki G & Daniel H (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* **71**, 1589–1596.
- Ford K & Labbok M (1990) Who is breast-feeding? Implications of associated social and biomedical variables for research on the consequences of method of infant feeding. *Am J Clin Nutr* **52**, 451–456.

- Fukushima Y, Kawata Y, Hara H, Terada A & Mitsuoka T (1998) Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. *Int J Food Microbiol* **42**, 39–44.
- Fuller R (1989) Probiotics in man and animals. *J Appl Bacteriol* **66**, 365–378.
- Gibson GR & Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* **125**, 1401–1412.
- Harmsen HJ, Wildeboer VA, Raangs GC, Wagendorp AA, Klijn N, Bindels JG & Welling GW (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30**, 61–67.
- Isolauri E (2001) Probiotics in human disease. *Am J Clin Nutr* **73**, 1142S–1146S.
- Isolauri E, Arvola T, Sutas Y, Moilanen E & Salminen S (2000) Probiotics in the management of atopic eczema. *Clin Exp Allergy* **30**, 1604–1610.
- Jansen GJ, Wildeboer-Veloo A-CM, Tonk R-HJ, Franks AH & Welling GW (1999) Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. *J Microbiol Methods* **37**, 215–221.
- Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P & Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* **357**, 1076–1079.
- Knol J, Steenbakkers BMA, van der Linde EGM, Gross S, Helm K, Larczyk M, Schopfer H & Kafka C (2002) Bifidobacterial species that are present in breast-fed infants are stimulated in formula fed infants by changing to a formula containing prebiotics. *J Pediatr Gastroenterol Nutr* **34**, 477.
- Knol J, van der Linde E, Wells JCK & Bockler HM (2003) An infant formula containing prebiotics changes the intestinal microflora of term infants. *J Pediatr Gastroenterol Nutr* **36**, 566.
- Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH & Welling GW (1995) Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microbiol* **61**, 3069–3075.
- Langhendries JP, Detry J, Van-Hees J, Lamboray JM, Darimont J, Mozin MJ, Secretin MC & Senterre J (1995) Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. *J Pediatr Gastroenterol Nutr* **21**, 177–181.
- Link-Amster H, Rochat F, Saudan KY, Mignot O & Aeschlimann JM (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol* **10**, 55–63.
- MacLean W-CJ, Fink BB, Schoeller DA, Wong W & Klein PD (1983) Lactose assimilation by full-term infants: relation of [¹³C] and H₂ breath tests with fecal [¹³C] excretion. *Pediatr Res* **17**, 629–633.
- Moon NJ (1983) Inhibition of the growth of acid tolerant yeasts by acetate, lactate and their synergistic mixtures. *J Appl Bacteriol* **77**, 412–420.
- Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B & Boehm G (2002) Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr* **34**, 291–295.
- Ogawa K, Ben RA, Pons S, de-Paolo MI & Bustos FL (1992) Volatile fatty acids, lactic acid, and pH in the stools of breast-fed and bottle-fed infants [see comments]. *J Pediatr Gastroenterol Nutr* **15**, 248–252.
- Saavedra JM, Bauman NA, Oung I, Perman JA & Yolken RH (1994) Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet* **344**, 1046–1049.
- Satokari RM, Vaughan EE, Akkermans-Antoon DL, Saarela M & de-Vos-Willem M (2001) Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial. *Syst Appl Microbiol* **24**, 227–231.
- Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM & Donnet-Hughes A (1995) Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy Sci* **78**, 491–497.
- Schmelzle H, Wirth S, Skopnik H, Radke M, Knol J, Bockler HM, Bronstrup A, Wells J & Fusch C (2003) Randomized double-blind study of the nutritional efficacy and bifidogenicity of a new infant formula containing partially hydrolyzed protein, a high β -palmitic acid level, and nondigestible oligosaccharides. *J Pediatr Gastroenterol Nutr* **36**, 343–351.
- Siigur U, Ormiston A & Tamm A (1993) Faecal short-chain fatty acids in breast-fed and bottle-fed infants. *Acta Paediatr* **82**, 536–538.
- Stahl B, Thurl S, Zeng J, Karas M, Hillenkamp F, Steup M & Sawatzki G (1994) Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* **223**, 218–226.
- Wang X & Gibson GR (1993) Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J Appl Bacteriol* **75**, 373–380.