Complementary therapies in paediatric gastroenterology: prevalence, safety and efficacy studies
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Change in microbiota composition after consumption of *Bifidobacterium lactis* Bb12 in combination with *Lactobacillus paracasei* CRL431 by healthy term infants

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Abstract

Objective: To evaluate the composition of the faecal flora in the first 6 months of life after consumption of a prebiotic-containing infant formula supplemented with Bifidobacterium lactis Bb12 and Lactobacillus paracasei CRL 431.

Methods: Samples were obtained from a clinical study encompassing 54 healthy children receiving an infant formula from birth onwards supplemented with Bifidobacterium lactis Bb12 and Lactobacillus paracasei CRL 431 and 50 children receiving placebo formula. Faecal samples (taken at 1, 3, and 6 months of age) were analyzed by quantitative PCR (Q-PCR) to determine the total number of bacteria, Bifidobacteria, Lactobacilli, E. coli, Bacteroides and Clostridia.

Results: Q-PCR showed a significant increase (p<0.005) in total bacteria and bifidobacteria per gram faeces from 1 to 3 and from 3 to 6 months in both groups, reflecting the normal development of gut microbiota in early life. Bacteroides increased significantly (p<0.005) from 3-6 months in both groups. Other bacterial species remained constant. At none of the three time points, differences were seen in the numbers of Bifidobacteria, Lactobacilli, E. coli, and Bacteroides between the two study groups. Bifidobacterium lactis Bb12 and Lactobacillus paracasei CRL 431 could be detected in more than 93% and 53% resp. of the faecal samples from the probiotic intervention group, versus 20% and 9% of the placebo group (p<0.05).

Conclusions: Consumption from birth onwards of a prebiotic-containing formula supplemented with Lactobacillus paracasei in combination with Bifidobacterium lactis results in infant intestinal colonisation of these probiotic strains. It does not lead to detectable changes of the overall faecal microbiota composition at the genus level.
Introduction

Breast-feeding is the gold standard in infant nutrition. Although the composition of current infant formula closely matches the composition of human breast milk, major differences are observed between breast-fed and formula-fed infants, especially with regard to the composition of the intestinal microbiota. Breast-feeding stimulates the development of a microbiota dominated by bifidobacteria and lactobacilli, even up to 90% of the total bacteria.\textsuperscript{1,2} It is thought that this is largely the result of the abundant presence of non-digestible oligosaccharides that serve as specific substrates for these groups of bacteria. In contrast, regular formula-fed infants develop a more complex microbiota, containing also substantial numbers of \textit{Bacteroides, Enterobacteriaceae, E. coli} and \textit{Clostridium} species.\textsuperscript{2,3}

At present it is not possible to exactly mimic the composition of the oligosaccharides in infant formula. Encouraging effects have been found with the addition of so-called prebiotics to infant formula. Although the composition of currently used prebiotics, mainly galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), is by far not as complex as those in human milk, promising results have been described with an increase in the faecal proportion of bifidobacteria.\textsuperscript{4-6} An alternative approach to manipulate the intestinal microbiota is the addition of probiotics or synbiotics (a mixture of pro- and prebiotics) to infant formula. It has been shown that the addition of $10^8$-$10^{10}$ CFU per day to infants below 3 months of age can result in a clear transient colonization of the gastro-intestinal tract.\textsuperscript{7,8}

The safety of probiotics has been described in a large number of reviews, which in general conclude that the consumption of probiotics results in a neglectable risk to the consumer. A commentary by the ESPGHAN Committee on Nutrition provided an overview of the effects of probiotics in (premature) infants.\textsuperscript{9} Since then several studies have further substantiated safety and clinical efficacy of probiotics in infants.\textsuperscript{10-13} Recently, we have shown that the consumption of a prebiotic-containing infant formula, supplemented with \textit{Bifidobacterium animalis subsp. Lactis} Bb12 and \textit{Lactobacillus paracasei} subsp \textit{paracasei} (CRL-431) by healthy infants, aged 0-6 months, was safe and well tolerated.\textsuperscript{14} Growth and development was normal in all infants and no statistically significant differences were detected between the probiotics group and the control group for gain in weight, length and head circumference. Infants in the probiotics group produced softer and more frequent stools during the first three months of life. We were interested to know if the ingestion of these probiotics resulted in a change in the microbiota, and would become more comparable to that of breast-fed infants. Quantative PCR was used to examine the composition of the infant faecal flora after one, three and six months of intervention. Colonization by the two probiotic strains was also studied.
Materials and Methods

Study Design

The samples for this study were collected during a randomized double blind controlled trial evaluating the safety and efficacy of Bifidobacterium Bb-12 and L. paracasei in a prebiotic-containing infant formula. In short, this study included two groups of infants: one group received standard infant formula, whereas the second group received the same formula supplemented with probiotics. Parents received the assigned infant formula with written instructions for its preparation and were advised to feed infants ad libitum during the study period. Solid foods were introduced at the age of 4 months.

The infant formula used was FRISO 1 (Friesland Foods, Leeuwarden, The Netherlands), a regular stage 1 formula containing 0.25 g/100 ml GOS. In addition, the experimental formula contained $1 \times 10^7$ CFU /g of Bifidobacterium animalis ssp lactis Bb-12, deposited under American Type Culture Collection (ATCC) number 27536 and $1 \times 10^7$ CFU /g of Lactobacillus paracasei ssp. paracasei (CRL-431), deposited under ATCC number 55544. Both formulas had similar taste, smell and colour and were supplied by Friesland Foods, Leeuwarden, The Netherlands. Products were manufactured according to current good manufacturing practices and coded at the manufacturing site. During storage of the product at ambient temperature, the stability of the probiotics in the product was checked monthly by selective plate counting and during the study period, the CFU/g remained stable in the product.

Infants were included in their first week of life (at latest on their 7th day of life), after a full-term pregnancy ($\geq$ 37 weeks) and written informed consent was obtained from both parents. All infants received the intervention formula with probiotics or the control formula during the first trimester. Parents of the first 80 infants who completed the first part of the study were asked to continue the use of the study feeding for another three months. Faecal samples were collected by the parents at the age of one, three and (if applicable) 6 months of age and sent to the Friesland Foods laboratory via regular mail. Time of sampling and time of delivery to the lab were noted. When received within 48 hours, the samples were frozen at -20°C until further use. The study was approved by the Medical Ethical Committee of the St. Antonius Hospital Nieuwegein, The Netherlands.

Q-PCR analysis

Total faecal DNA for Q-PCR analysis was isolated in duplicate using the QIAamp® kit, according to the manufacturers instructions (Qiagen, Venlo, The Netherlands). Determination of total number of bacteria, bifidobacteria, clostridia and Bacteroides sp. on extracted DNA was carried out by using quantitative real-time PCR. Total bacteria were analyzed with primers given in Table 1, with SYBR green as described by Fenicia
et al. and analyzed with an ABI Prism 7700 system. The quantitative PCR of the above mentioned genera was performed according to a method as derived from Kimura et al. All amplification reactions were performed with an ABI Prism 7700 sequence detector (Applied Biosystems) in a 96-well microwell plate (MicroAmp, Applied Biosystems). To this amount 12.5 μl of SYBR Green I PCR Master Mix (Applied Biosystems) or Taqman Universal PCR Master Mix (Applied Biosystems), 100 nM concentration of the forward and reverse primers (table 1) was added. To this mixture 250 nM probe (Table 1) was added in case of Lactobacilli, E. coli, Bacteroides, and Clostridia and 50 nM of probe A and probe B (Table 1) was added in case of detection of the bifidobacteria. MilliQ was used to equalize the volume to 25 μl. The reaction mixture was run at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 50°C for 2 min. QPCR of the isolated faecal DNA preparations was performed in singular. The average values of the duplicate faecal DNA QPCR analysis were converted to CFU equivalents. A subset of all faecal samples were analyzed in duplicate on a infant intestinal microarray to detect and quantitate the faecal presence of the probiotic strains (F. Schuren et al. in preparation)

### Statistical analysis

For statistical analysis, the software package SPSS (version 16.0; SPSS, Inc., Chicago, Ill.) was used. Potential differences between groups and between different time points were assessed by Mann-Whitney and Wilcoxon test. Fisher’s exact test was used to analyse differences in recovery of the probiotic strains. For all statistical analyses, statistical significance was set at the .05 level, and all tests were 2-tailed.

<table>
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Results

Between November 2004 and January 2007 159 mothers agreed to participate in this study. A flowchart showing the enrolment, status of the infants and numbers of faecal samples that could be analyzed, is presented in Figure 1. One month after birth (T1) 54 faecal samples were collected in the probiotic group versus 50 in the control group. The second sample was collected at the age of three months (T3), resulting in 49 and 41 samples respectively. After the first trimester, 80 parents were asked to continue the study for the second trimester resulting in 35 and 34 samples at the age of 6 months (T6).

Analysis of infant faecal samples by Q-PCR

The total number of bacteria in both infant groups increased significantly in time: in the probiotics group the median count increased from $2.4 \times 10^{10}$ at T1 to $5.2 \times 10^{10}$ at
T3; and $9.4 \times 10^{10}$ at T6 ($P<0.01$) and from $3.5 \times 10^{10}$ (T1) to $6.4 \times 10^{10}$ (T3) and $9.0 \times 10^{10}$ at T6 ($P<0.05$, Figure 2a). Also the number of Bifidobacteria increased over time: from $2.0 \times 10^9$ at T1 to $7.3 \times 10^9$ at T6 in the probiotics group ($P<0.001$) and from $1.9 \times 10^9$ at T1 to $6.0 \times 10^9$ at T6 (Figure 2b; $P<0.01$). Lactobacilli showed a decrease between T1 and T3 in the total group from $2.0 \times 10^6$ to $3.3 \times 10^5$ (Figure 2c; $P<0.01$), and remained constant during the next months. E. coli numbers remained constant during the study (figure 2d). The number of Bacteroides bacteria
increased significantly in both groups during the 3 to 6 months period from $3.0 \times 10^8$ to $6.4 \times 10^9$ ($P<0.001$) in the probiotics group and from $6.0 \times 10^8$ to $4.7 \times 10^9$ ($P<0.05$) in the placebo group, while showing no significant difference in the 1 to 3 month period (Figure 2e).

The number of Clostridia bacteria was in most samples below the detection level of $1.0 \times 10^5$, precluding a reliable comparison between groups and between different time points (Figure 2f). Overall a trend towards a decrease in Clostridia was seen in both groups. In the probiotic group the number of samples that tested positive for Clostridia decreased from 35% at one month to 20% at 3 months and 9% at 6 months ($P<0.05$). In the placebo group these figures were 28%, 17% and 12%. No differences were seen between the study groups for all genera at the three time points.

We observed a large interindividual variation in the CFU/g faeces at all time points. On average the number of bifidobacteria (CFU/g faeces) made up about 10% of the number of total bacteria after 1 month of intervention although the range was from 0.5 to 39% of the total number of bacteria. The same large variation was observed for the other bacterial genera.

**Presence of supplemented probiotics in faecal samples**

At random, a selected group of samples was analyzed using a microarray to detect the presence of the supplemented probiotics. After one, three and six months intervention, *Bifidobacteria lactis* Bb12 was found in 14/15, 14/15 and 11/11 samples in the probiotic group. These numbers were significantly lower in the control group: 3/11, 2/11 and 2/10 respectively ($P<0.01$). *Lactobacillus paracasei* CRL-341 was found in 8/15, 8/15 and 4/11 of the probiotic samples versus 1/11, 0/11 and 0/10 in the control samples ($P<0.05$).

**Discussion**

In this study we determined the development of the composition of the gut microbiota in infants, who received a prebiotic-containing formula in combination with *Bifidobacterium lactis* Bb12 and *Lactobacillus paracasei* CRL-431 or placebo for the first 6 months of their life. The total number of faecal bacteria as well as the tested bacteria species were comparable to those found in other infant studies with prebiotics, although a detailed comparison is hampered by differences in study design and microbiological techniques, that were used. Also the large variability in the amount of the different species between infants has been described in these studies and reflects one of the characteristics of the personal gut microbiota.

This study was designed to investigate the effect of *Bifidobacterium lactis* Bb12 and *Lactobacillus paracasei* CRL-431 supplementation on (part of the) composition of the
intestinal microbiota. Two previous studies have investigated the effect of these two probiotics on infant faecal composition. Mohan et al. examined the stools of 69 preterm infants, supplemented with placebo or Bb12. Bifidobacterial numbers were significantly higher in the probiotic than in the placebo group. Furthermore, a reduction was seen in the number of Enterobacteria and Clostridia. Marzotto et al. demonstrated that supplementation of *Lactobacillus paracasei* increased the number of lactobacilli with a decrease in the clostridia count in 26 healthy infants. We did not find significant differences between the two study groups with respect to the number of Bifidobacteria, Lactobacilli, E. coli and Bacteroides. The number of Clostridia bacteria was in most samples below the detection level of 1.0E × 105, preventing a comparison between groups and between different time points. The main difference between above studies and ours, is the fact that all our infants also received prebiotics. It has been shown that the addition of prebiotics to infant formulas results in an increase of both bifidobacteria and lactobacilli with a decrease in Clostridia numbers. We hypothesize that the impact of the added probiotic strains on top of the prebiotic effect on the intestinal microbiota is subtle and therefore too small to detect by methodologies used in our study.

Supplementation with Bb12 and CRL-431 in this group of infants was associated with an increase in defecation frequency and softer stools during the first trimester. Because we did not find gross differences in faecal composition, except for the presence of the two supplemented strains, it is not evident what has attributed to this effect on defecation patterns. It is possible that the presence of these strains resulted in an increased production of specific short chain fatty acids, leading to an enhancement of colonic motility. Another explanation may be that the microbiological techniques used, or even the biological sample (faeces) is not an adequate way to investigate the influence of probiotics on colonic motility and stool consistency. Generally, stool samples are the preferred material to investigate the intestinal flora because of their overall availability and easy access. However, a comparison between colonic biopsies and stool samples has shown that the mucosa-associated bacterial microbiota shows substantial differences in dominant bacterial species compared with the faecal flora. Additional studies, for example examining short chain fatty acids, or the microbiota composition of different parts of the intestine may help to unravel the still unknown mechanisms by which probiotica can increase faecal moisture and enhance gastrointestinal motility.

A drawback of this study is the time between collection of the faecal samples by the parents and processing of the samples in the laboratory, which was between 24 and 48 hours. The total amount of bacterial DNA as well as the diversity of the microbiota significantly decreases after 24 hours with a preferential growth of oxygen-tolerant and robust bacteria, and a destruction of oxygen-sensitive bacteria. This may have influenced the number of samples, in which Clostridia species were found. However, the aim of this study was to examine differences in faecal microbiota composition between...
two groups. It is unlikely that the change in composition of the samples during transport was different for infants receiving probiotics and infants who did not. *Bifidobacterium lactis* Bb12 was recovered in almost all faecal samples, while *Lactobacillus paracasei* CRL-431 was found in only half of the tested samples. Our results are comparable with two previous studies, with similar differences in recovery of these two strains.24,25 *Bifidobacterium lactis* Bb12 has been shown to have the highest adhesion to human mucus of all bifidobacteria tested in both infants and adults.26 This property may have attributed to the difference in recovery rate in comparison to *Lactobacillus paracasei* CRL-431. The difference may also be explained by the fact that lactobacilli mainly colonize the small intestine while bifidobacteria are preferentially found in the colon. Bb12 was also recovered in low numbers in one or two samples of in total 5 infants of the placebo group. It is known that the bacteria which colonize the infants gut, mainly originate from the mothers gut, vaginal tract and skin. As *Bifidobacterium lactis* Bb12 is widespread in dairy products and commercially available probiotics supplements, the mothers of these infants may have been colonized with this strain.

In conclusion, this study shows that consumption from birth onwards of a prebiotic-containing formula supplemented with *Lactobacillus paracasei* CRL 431 in combination with *Bifidobacterium lactis* Bb12 results in infant intestinal colonisation of these probiotic strains. In our study, it does not lead to detectable changes of the measured part of the faecal microbiota composition at genus level. More sensitive microbiological techniques, such DNA micro-arrays, will be required to investigate in detail the effect of probiotic supplementation on the developing gut microbiota in children during the first 6 months of life.

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