Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota

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Abstract

The aim of the study was to assess the quantitative and qualitative differences of the gut microbiota in infants. We evaluated gut microbiota at the age of 6 months in 32 infants who were either exclusively breast-fed, formula-fed, nursed by a formula supplemented with prebiotics (a mixture of fructo- and galacto-oligosaccharides) or breast-fed by mothers who had been given probiotics. The Bifidobacterium, Bacteroides, Clostridium and Lactobacillus/Enterococcus microbiota were assessed by the fluorescence in situ hybridization, and Bifidobacterium species were further characterized by PCR. Total number of bifidobacteria was lower among the formula-fed group than in other groups (P = 0.044). Total amounts of the other bacteria were comparable between the groups. The specific Bifidobacterium microbiota composition of the breast-fed infants was achieved in infants receiving prebiotic supplemented formula. This would suggest that early gut Bifidobacterium microbiota can be modified by special diets up to the age of 6 months.

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Keywords: Gut microbiota; Bifidobacteria; Infancy; Breastfeeding; Prebiotics

1. Introduction

Intestinal colonization, representing the host’s earliest contact with microbes [1], has a marked impact on the maturation of the infant’s intestinal immune system [2]. The establishment of the gut microbiota is a gradual process, the first aerobic colonizers being enterobacteria, streptococci and staphylococci, and the first anaerobic ones bifidobacteria, bacteroides and clostridia. The balance of these bacteria is influenced by breastfeeding [3]. There are data to suggest differences in the maturational composition of the intestinal microbiota between breastfed and formula-fed infants, breastfeeding strongly promoting Bifidobacterium microbiota [4]. That kind of predominance of bifidobacteria may have profound effects on later health [5]. Important questions still arise as how specific should a bifidogenic effect be.

Bifidobacteria appear after birth and within a week reaching the dominant bacterial group in healthy infants [6]. Then Bifidobacterium infantis, B. longum and B. breve are the species most often found [7].
Quantitative and qualitative differences in bifidobacterial composition are observed between breast-fed and formula-fed infants, the *Bifidobacterium* being the dominant micro-organism in the former group [4,8]. *B. infantis, B. longum* and *B. bifidum* have been reported the predominant species in breast-fed infants [9]. It has also been reported that the supplementation of infant formula with specific oligosaccharides stimulates the growth of bifidobacteria in the intestine resembling the effect of breast-feeding [10].

Probiotics are specific live active microbial cultures affecting the host by improving its intestinal microbial balance, focusing on target- and site-specific effects of clearly defined strains [11]. Prebiotics are defined as non-digestible oligosaccharides [12] which benefit the gut microbiota, e.g. by stimulating the growth of bifidobacteria or other beneficial intestinal microbiota components [13]. The current study was designed to assess the quantitative and qualitative differences of the gut microbiota in infants. For this purpose we determined the bifidogenic potential in four treatment groups at the age of 6 months: formula-fed infants, infants given prebiotic-supplemented formula, and breast-fed infants with or without maternal perinatal probiotic (B. lactis BB12) supplementation. Specifically, the *Bifidobacterium* species characterization was done at the age of 6 months.

### 2. Materials and methods

#### 2.1. Subjects and study design

The study involved 32 infants who had at least one close relative (mother, father, sibling) with atopic eczema, allergic rhinitis or asthma. In 31/32 cases the mother had an allergic disease. The children were recruited in antenatal clinics in the city of Turku (population 170,000). They had been born between 36 and 42 weeks of gestation (mean 39 weeks).

The study comprised four groups. The formula group (*n* = 8) received adapted cow milk-based formula, while group pre + formula (*n* = 8) consisted of infants given partially hydrolyzed infant formula supplemented with prebiotics, a mixture of fructo- (10%) and galacto-oligosaccharides (90%) (Omneco, Nutricia, Cuijk, The Netherlands). The criteria for inclusion in the study at 6 months of age were that the infants had used the respective formula since at least 2 months of age. The group breastmilk (*n* = 8) comprised age-matched exclusively breast-fed infants, and the group pro + breastmilk (*n* = 8) breast-fed infants whose mothers were given *B. lactis* BB 12 (Chr. Hansen Ltd., Horsholm, Denmark) in an oat-based preparation (Oy Karl Fazer Ab, Vantaa, Finland) for two weeks before and 2 months after delivery.

The study protocol was approved by the Committee on Ethical Practice of Turku University Central Hospital and infants were enrolled in the study after written informed consent was obtained from their parents.

#### 2.2. Clinical evaluation

The children were clinically examined at the ages of 3, 6, and 12 months. Parents recorded all episodes of infectious diseases in the infants. Any symptoms and signs of atopic disease were assessed. Atopic sensitization was evaluated by skin prick testing at the ages of 6 and 12 months. The diagnosis of atopic eczema was based on Hanifin criteria as described elsewhere [14], and that of cow’s milk allergy on a relationship between clinical symptoms and ingestion of cow’s milk. The diagnosis was confirmed in a double-blind, placebo-controlled, cow’s milk challenge as previously described [15].

#### 2.3. Fluorescent in situ hybridization (FISH)

Fecal specimens were collected from diapers after defecation and the specimens were immediately cooled to 6–8 °C and transported to the hospital within 24 h to be frozen at −75 °C. Bacterial cells were harvested for FISH to be performed as described elsewhere [16,17]. The probes for the FISH method were Bif164 (5’-CAT-CCGGCATTACCACCC), Bac303 (5’-CCAATGTGGGAGCCCTT), His150 (5’-TTATCGGTTATTAATCT(C/T)CCTTT) and Lab158 (5’-GGTATTAGCA(T/C)GTGTTTCCA) specific for bifidobacteria, bacteroides, clostridia (perfingenis/histolyticum subgroup) and lactobacilli/enterococci respectively. All probes were of commercial synthesis origin and 5’ labelled with fluorescent dye Cy3. Total counts were assessed using the nucleic acid stain 4’, 6-diamidino-2-phenylindole (DAPI) as described by Porter and Feig 1980 [18]. Sample processing was completed as previously described [17] and counting was conducted using the Leica Laaborlux D epifluorescence microscope. At least 15 random fields were counted on each slide and the average count was used for analysis.

#### 2.4. Bifidobacterium species characterization

For the characterization of the fecal bifidobacterial microbiota PCR primers were designed targeting different *Bifidobacterium* species or groups, including *B. adolescentis, B. angulatum, B. animalis* group (*B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*) according to Ventura and Zink [19]. *B. bifidum, B. breve, B. catenulatum* group (*B. catenulatum* and *B. pseudo- catenulatum*), *B. dentium* and *B. longum* group (*B. longum* biotype *longum, B. longum biotype infantis* and *B. longum* biotype *suis*) according to Sakata et al. [20] (Table 1). These oligonucleotides were purchased from Thermo Electron Corporation (Thermo
Biosciences, Ulm, Germany). To check for specificity, the sequences of the selected PCR primers were compared to the sequences available at both, the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [21] and by using the Probe Match application at the Ribosomal Database Project II (www.rdp.cme.msu.edu/html) [22].

In addition, the primers were tested against an array of 14 Bifidobacterium species (Table 2) and 21 intestinal and food micro-organisms, including Bacteroides vulgatus DSM 1447T, Clostridium butyricum DSM 10702T, C. cocoides DSM 935T, Enterobacter aerogenes DSM 30053T, Enterococcus faecalis DSM 20478T, E. faecium Gaio, Escherichia coli K12, Eubacterium cylindroides DSM 3983T, E. halii DSM 3353T, Lactobacillus acidophilus La-5, L. gasseri DSM 20077, L. jensenii DSM 20557T, L. casei DSM 20011T, L. paracasei DSM 20244, L. plantarum 299v, L. rhamnosus GG, Peptostreptococcus anaerobius DSM2949T, Anaerococcus prevotii DSM 20548T, Ruminococcus hansenii DSM 20383T, Streptococcus thermophilus DSM 20617T and Veillonella dispar DSM 20735T. DNA extractions from pure cultures of the different microorganisms and PCRs were conducted as previously indicated [23]. Fecal DNA was extracted by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

Amplification of the DNA was performed using a PCR iCycler apparatus (Bio-Rad, Espoo, Finland). The total volume of each PCR was 50 μl, employing 4 μl of DNA extract as a template. The reaction mixture was composed of 1· PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM MgCl2, 0.2 μM of each primer, 200 μM of each dNTP (Amersham Biosciences, Helsinki, Finland) and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermal cycle program consisted of the following time and temperature profile: an initial cycle of 95 °C for 10 min for denaturation and polymerase activation, 30 cycles of 15 s at 95 °C, 1 min at the annealing temperature of the corresponding primers pair (indicated in Table 2) and 45 s at 72 °C and a final extension step of 10 min at 72 °C. Amplified products were subjected to gel electrophoresis in 1% agarose gels and were visualized by ethidium bromide staining.

2.5. Statistics

The amount of bifidobacteria in gut microbiota was assessed using analysis of variance to compare different groups. The data are expressed as medians with interquartile ranges. The effect in different groups on the amount of bifidobacteria was analyzed by Fisher’s PLSD post hoc test.

All statistical analyses were performed on Statview computer software, version 4.5 (Abacus concepts, Inc., Berkeley, CA).

3. Results

3.1. Clinical characteristics

The study groups were comparable in respect of clinical characteristics (Table 3). Altogether 27/32

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for the Bifidobacterium sp. characterization</th>
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<tbody>
<tr>
<td>Sequence 5’ → 3’</td>
<td>Orientation</td>
</tr>
<tr>
<td>TTCCAGTTGATCGATGGTGTCTTCT</td>
<td>Forward</td>
</tr>
<tr>
<td>GGCTACCCGTCGAAGCCACG</td>
<td>Reverse</td>
</tr>
<tr>
<td>GATAGGGCGCTGGACCGTTGCCG</td>
<td>Forward</td>
</tr>
<tr>
<td>CCCCCAGGGCTTGGCTCCG</td>
<td>Reverse</td>
</tr>
<tr>
<td>AATGCCGGATGCTCCATCACAC</td>
<td>Forward</td>
</tr>
<tr>
<td>GCCTTGCTCCCTAACAAGAGG</td>
<td>Reverse</td>
</tr>
<tr>
<td>TGACCGAAGTGCCTCCATGCCT</td>
<td>Forward</td>
</tr>
<tr>
<td>CCCATCCACGCCGATGAA</td>
<td>Reverse</td>
</tr>
<tr>
<td>GCCGGATGCTCCGACTCCT</td>
<td>Forward</td>
</tr>
<tr>
<td>ACCCGAAGGCTTGCTCCCG</td>
<td>Reverse</td>
</tr>
<tr>
<td>GGATCGGCTGAGGCTTGGCTCCG</td>
<td>Forward</td>
</tr>
<tr>
<td>TCACCCGAGGCTTGGCTCCCG</td>
<td>Reverse</td>
</tr>
<tr>
<td>ATCCCCGGGGTTGGCTCCCT</td>
<td>Forward</td>
</tr>
<tr>
<td>ATACCGATGGAACCTTTCCCG</td>
<td>Reverse</td>
</tr>
<tr>
<td>ACCAACCTGCCCTGTCGACCCG</td>
<td>Forward</td>
</tr>
<tr>
<td>CCATCACCGCAACAAAGCT</td>
<td>Reverse</td>
</tr>
</tbody>
</table>
(84%) of the infants had been delivered vaginally. By 6 months of age most were healthy; only one infant in the group formula had had an episode of acute otitis media (Table 3). By 12 months of age, the weight and length of the infants were comparable between the feeding groups. All infections experienced had been episodes of acute otitis media, excluding one case of streptococcal tonsillitis in the group breastmilk (Table 3). Allergic disease was the only chronic disease manifested. In the group breastmilk one infant had cow’s milk allergy, diagnosed by means of double-blind, placebo-controlled cow’s milk challenge. Atopic eczema was more common in the group formula than in other groups ($P = 0.05$).

### 3.2. Gut microbiota

The total number of bifidobacteria at 6 months of age differed between the groups ($P = 0.044$), while the total amounts of other bacteria studied were comparable (Fig. 1). The distinction was due to a significantly lower amount of bifidobacteria in the group formula, $7.6 \times 10^8$ (5.5 $\times 10^7$–2.2 $\times 10^9$), than in the groups pre + formula, $2.9 \times 10^9$ (2.2 $\times 10^9$–3.0 $\times 10^9$), and breastmilk, $2.8 \times 10^9$ (2.1 $\times 10^9$–3.6 $\times 10^9$), $P = 0.037$ and 0.0071, respectively. The amount of bifidobacteria in the group pro + breastmilk, $2.3 \times 10^9$ (1.0 $\times 10^9$–3.5 $\times 10^9$), also tended to be greater than in the group formula, $P = 0.081$. The total amount of bacteria in breastmilk group was $4.6 \times 10^9$.
The specificity of the designed *Bifidobacterium* group-specific primers is shown in Table 2. In addition, none of the primer pairs cross-reacted with any of the other 21 intestinal or food micro-organisms tested (data not shown).

To characterize the *Bifidobacterium* species composition these group-specific primers were used (Table 4). *B. longum* group was found to be the most widely spread, being present in 100% of the infants, followed by *B. bifidum* (45%) and *B. breve* (38%), whereas *B. angulatum* was not detected in any sample. Members of *B. catenulatum* group were found in 31% of the samples whilst *B. dentium*, *B. adolescentis* and members of *B. animalis* group were present in 17%, 10% and 7% of the samples respectively. Interestingly *B. bifidum* was found in six up to eight (75%) breast-fed infants and in five of the seven (71%) infants given prebiotic-supplemented formula whilst it was detected only in 1/7 (14%) and 1/8 (12%) of the infants receiving probiotics plus breastmilk or formula, respectively. Contrary to that *B. breve* was more frequently found in the group *pre + breastmilk* 4/6 (67%) than in the other groups, *breastmilk*, *pre + formula* or *formula* alone, with 25%, 28% and 37% of infants harbouring *B. breve*, respectively.

There was a trend towards a less diverse *Bifidobacterium* microbiota in formula-fed infants, harbouring fewer different species per infant; 1.88 ± 0.99 than those receiving breastmilk; 2.38 ± 0.74, formula supplemented with probiotics; 2.71 ± 1.11 or breastmilk plus probiotics; 3.17 ± 1.17; mean ± SD (*P* = 0.12).

### 4. Discussion

Our results demonstrate that breast-fed infants and infants given partially hydrolyzed formula supplemented with prebiotic oligosaccharides had identical levels of bifidobacteria in their feces, significantly exceeding those observed in formula-fed infants. The results further suggest that probiotics may also have effect on the exact bifidobacteria composition. The early gut *Bifidobacterium* microbiota might thus be modified by special diets.

Encouragement of breastfeeding is the first choice to this end. However, if breastmilk is not available other options must be considered.

The rationale for modulating the gut microbiota lies in the demonstration that the intestinal microbiota, a complex ecosystem, is important to the health of the host [13]. The gut microbiota is a key determinant of the intestine’s immunological development. It has been observed that breast-fed infants have less allergies, diarrhea and respiratory and gastrointestinal infections than formula-fed infants [5,24]. The health-promoting effects of breast-milk have been linked partly to its bifidogenicity [4]. Enhancement of bifidobacteria at an early age is

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**Table 4**

<table>
<thead>
<tr>
<th><em>Bifidobacterium</em> species characterization at the age of 6 months</th>
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<tbody>
<tr>
<td><strong>Number of subjects with <em>Bifidobacterium</em> sp.</strong></td>
</tr>
<tr>
<td><strong>Breast-fed</strong></td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
</tr>
<tr>
<td><em>Bifidobacterium catenulatum</em></td>
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<tr>
<td><em>Bifidobacterium bifidum</em></td>
</tr>
<tr>
<td><em>Bifidobacterium dentium</em></td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em></td>
</tr>
<tr>
<td><em>Bifidobacterium angulatum</em></td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em></td>
</tr>
<tr>
<td><em>Bifidobacterium adolescentis</em></td>
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thus justified, and functional ingredients such as prebiotics and probiotics with similar properties could therefore have beneficial effects on infant’s health. Risk groups who would especially benefit from such modification are infants born by cesarean section [25], premature infants, or infants treated with antibiotics [26].

Documentation regarding the safety and efficacy of prebiotics in infants is still scant. Recently it has been demonstrated in both premature and term infants that a prebiotic supplementation in infant formula enhances the *Bifidobacterium* microbiota in the feces [10]. The studies in question, however, were short-term and restricted to the neonatal period. Moreover, the clinical benefits of prebiotics, have not been definitively proven, nor is their safety documented. A few experiments in animal models hint that prebiotics might even have some negative effects such as dismorphology of the gut mucosa [27]. These risks attributed to prebiotic preparations have not been explored in man. Consequently, long-term follow-up studies are needed as the main target group of prebiotic formulas are young infants.

The present study would appear to offer some preliminary responses to unanswered questions regarding prebiotics even though no final conclusions e.g. on the therapeutic potential can be drawn by reason of the small study population. Recent demonstrations show that healthy and allergic infants harbor different bifidobacteria microbiota composition which may generate distinct cytokine responses [28]. These data suggest that not all bifidobacteria are necessarily health-promoting. Consequently, instead of promoting the amount of bifidobacteria per se we should focus on the overall balance of different bifidobacteria.

*Bifidobacterium* has been reported to be one of the predominant genera in the intestinal microbiota of infants [29]. *B. infantis, B. longum* and *B. breve* are the most common species found in infants whilst *B. adolescentis* and *B. catenulatum* predominate in adults [7]. Similar to our results higher levels of bifidobacteria in breast-fed than formula-fed infants have been repeatedly observed [1,4,9] *B. infantis, B. longum* and *B. bifidum* being the most common species in the former group [9]. In agreement with these results, we found that members of *B. longum* group (including *B. longum* and *B. infantis*) were the most widely present bifidobacteria. Although *B. breve* was also frequently found, *B. bifidum* was the second most common bifidobacteria due to their high prevalence in the breast-fed and prebiotic-supplemented formula-fed infants. An interesting preliminary observation can be detected in *B. adolescentis* counts. In both pre + formula and pro + breastmilk groups more *B. adolescentis* were detected than in breastmilk or formula groups. In view of the potential role of *B. adolescentis* in allergic disease more studies are required.

It was interesting to observe that infants given prebiotic-supplemented formula formed a similar bifidobacteria composition as the breast-fed infants. More studies are needed to clarify if prebiotics have the same health implications than breastmilk.

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**References**


