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ORIGINAL PAPER

Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the *Bifidobacterium* microbiota in infants at risk of allergic disease

M.-M. Grönlund*†, M. Gueimonde‡, K. Laitinen‡§, G. Kociubinski‡, T. Grönroos‡, S. Salminen‡ and E. Isolauri*†

**Department of Pediatrics, Turku University Central Hospital, Turku, Finland, †Department of Pediatrics, ‡Functional Foods Forum and §Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland*

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Summary

Background The sources and the impact of maternal bacteria on the initial inoculum of the intestinal microflora of newborn infants remain elusive.

Objective To assess the association between maternal breast-milk and fecal bifidobacteria and infants' fecal bifidobacteria.

Methods Sixty-one mother–infant pairs were included, special emphasis being placed on the maternal allergic status. Bifidobacteria were analysed by a direct PCR method in fecal samples from mothers at 30–35 weeks of gestation and from infants at 1 month of age and from breast-milk samples 1 month post-partum.

Results Fecal *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* colonization frequencies and counts among mother–infant pairs correlated significantly ($P = 0.005$ and 0.02 for frequencies, respectively, and $P = 0.002$ and 0.01 for counts, respectively). Only infants of allergic, atopic mothers were colonized with *B. adolescentis*. Each of the breast-milk samples contained bifidobacteria [median 1.4×10^3 bacterial cells/mL; interquartile range (IQR) $48.7–3.8 \times 10^3$]. *Bifidobacterium longum* was the most frequently detected species in breast-milk. Allergic mothers had significantly lower amounts of bifidobacteria in breast-milk compared with non-allergic mothers [median 1.3×10^3 bacterial cells/mL (IQR $22.4–3.0 \times 10^3$) vs. 5.6×10^3 bacterial cells/mL ($1.8 \times 10^3–1.8 \times 10^4$), respectively, ($P = 0.004$)], and their infants had concurrently lower counts of bifidobacteria in feces [3.9×10^8 bacterial cells/g (IQR $6.5 \times 10^6–1.5 \times 10^9$) in infants of allergic mothers, vs. 2.5×10^9 bacterial cells/g ($6.5 \times 10^8–3.2 \times 10^{10}$) in infants of non-allergic mothers, $P = 0.013$].

Conclusions Breast-milk contains significant numbers of bifidobacteria and the maternal allergic status further deranges the counts of bifidobacteria in breast-milk. Maternal fecal and breast-milk bifidobacterial counts impacted on the infants' fecal *Bifidobacterium* levels. Breast-milk bacteria should thus be considered an important source of bacteria in the establishment of infantile intestinal microbiota.

Correspondence:
Minna-Maija Grönlund, Department of Pediatrics, Turku University Central Hospital, Kiinamyllynkatu 4-8, 20520 Turku, Finland.
E-mail: mingro@utu.fi

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Introduction

Bifidobacteria are regarded as a particularly attractive bacterial genus for intentional manipulation of the intestinal microbiota. The interest in them arises from the fact that bifidobacteria comprise the predominant intestinal bacteria in full-term, breastfed infants as early as 3–6 days of age [1–5]; they may account for up to 90% of the total bacterial count of intestinal microbiota [4, 6]. Their predominance during infancy is taken to emerge from the initial vertical transfer of bacteria from the mother's birth canal to the newborn during delivery [7–9]. This conception is, however, incomplete in that the mother's fecal bifidobacterial microbiota, reflected in the birth canal

microbiota, differs collectively by species composition from that of the newborn infant [10].

During infancy, breast-milk components such as galactooligosaccharides support the growth of bifidobacteria [11, 12]. More recently, the bifidobacterial predominance in the intestinal microbiota of breastfed infants has been linked to the direct transfer of maternal bifidobacteria to newborns in breast-milk [13, 14]. In promoting a healthy intestinal microbiota, breastfeeding has also been claimed to promote the health of the newborn by reducing the risk of atopic diseases [15]. However, some recent studies among infants of allergic mothers report opposite findings [15–17]. The inconsistencies have been explained by sharp distinctions in the immunological properties of breast-milk [18, 19]. We hypothesized here that the breast-milk of allergic as against non-allergic mothers may also differ in the concentration of bifidobacteria, and allergic mothers may have lower bifidobacteria content in their breast-milk, this leading to lowered intestinal colonization by bifidobacteria in their breastfed infants.

To test these hypotheses, we investigated the bifidobacteria concentration in breast-milk 1 month after delivery and compared the amounts of bifidobacteria between allergic and non-allergic mothers. We also studied the fecal bifidobacteria concentration and species composition in mothers at 30–35 weeks of gestation and their infants at 1 month of age to characterize the correlations between the bifidobacteria content of breast-milk and mothers' and infants' feces.

Methods

Subjects and study design

The study population consisted of 61 healthy full-term infants and their mothers participating in an ongoing clinical study. This is a follow-up study of high-risk allergic families where at least one family member is allergic (mother, father or sibling) [20]. Those consecutive mother–infant pairs fulfilling the sole inclusion criterion of having all three study samples (maternal fecal sample, infant's fecal sample and breast-milk sample) were included.

In this double-blind, placebo-controlled study, half of the mothers had received probiotic supplementation of a combination of *Bifidobacterium lactis* (Bb 12) and *Lactobacillus rhamnosus* (GG) from 15 weeks of gestation until the end of exclusive breastfeeding (for a maximum of 6 months post-partum) and the other half received placebo, yielding 31 mothers in the probiotic supplementation group and 30 in the placebo group. The primary end-point of the study is the prevalence of allergy in the infants. The outcome of the intervention for clinical parameters will be reported later in a separate paper. The study was approved by the Ethical Committee of the Hospital District of South-West Finland. Informed consent was obtained from the mothers.

The counts of bifidobacteria and the species composition of the *Bifidobacterium longum* group, *Bifidobacterium bifidum*, *Bifidobacterium animalis* group, *Bifidobacterium breve*, *Bifidobacterium catenulatum* group and *Bifidobacterium adolescentis* in the fecal and breast-milk samples did not differ between mothers given probiotic supplementation or placebo. Neither did the infants' fecal bifidobacteria counts differ between the probiotic and placebo groups. Because there were no differences in the bacterial counts between these groups, the data were analysed together.

Sample collection

Fecal and breast-milk sample collections were standardized for all subjects. Spot fecal samples were collected from the mothers at 30–35 weeks of pregnancy and from infants at 1 month of age. Breast-milk samples were collected 1 month post-partum. Infants were allowed to suckle for a few minutes before a breast-milk sample was collected by manual expression. Fecal and breast-milk specimens were taken into plastic containers and stored at 4 °C in home refrigerators until brought to the study clinic no longer than 24 h from collection. In the study clinic, they were kept frozen at −70 °C until analysis.

Characterization of bifidobacteria

The total quantitative count of bifidobacteria and the presence (i.e. positive or negative) of *B. longum* group (*B. longum* biotype *longum*, *B. longum* biotype *infantis* and *B. longum* biotype *suis*), *B. bifidum*, *B. animalis* group (*B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis*), *B. breve*, *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*) and *B. adolescentis* were detected in both the breast-milk and the fecal samples. However, because of the low amount of bifidobacteria in breast-milk samples, the quantitative counts of the above-mentioned *Bifidobacterium* species could be analysed only in the fecal samples.

Analysis of fecal samples

DNA extraction. DNA was extracted from feces as reported previously [21] using the Qiagen Stool minikit (Qiagen, Hilden, Germany).

Polymerase chain reaction analyses. To determine the presence of different bifidobacterial species in the fecal samples, DNA extracts were qualitatively analysed using the bifidobacterial primers and PCR conditions described in detail by Rinne et al. [22]. The levels of total bifidobacteria and those of the different bifidobacterial species

tested were determined by quantitative real-time PCR techniques, based on the use of lanthanide-labelled probes as reported previously [23].

Analysis of breast-milk

DNA extraction. DNA was extracted from breast milk samples using the Qiagen Stool Kit (Qiagen). Briefly, 1.0 mL breast-milk samples were washed twice in 1.0 mL of phosphate-buffered saline and centrifuged at 14 000 g, in order to remove PCR inhibitors. Pellets were resuspended in 200 μL of ASL lysis buffer, and bacterial cell lysis, protein digests and DNA purification were performed in accordance with the manufacturer's instructions.

Polymerase chain reaction analysis. To detect the total count of bifidobacteria in the samples, real-time PCR amplification was performed in an Applied Biosystems 7300 Fast Real-Time PCR System (Foster City, CA, USA) working in a 96-well format. Detection was by 7300 System SDS Software. Amplification reactions were conducted in a 50 μL reaction mixture composed of selected primers for *Bifidobacterium* genus [21] at a concentration of 10 μM, and a Power SYBR® Green PCR Master Mix (2 ×, Applied Biosystems, Warrington, UK) containing SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase LD, dNTPs mixture and a passive internal reference ROX™ Dye, Warrington, UK. The volume was completed with template DNA or water (2.0 μL). The amplification program consisted of one cycle of 95 °C for 10 min (step 1), then 48 cycles at 95 °C for 15 s, followed by 60 °C for 1 min (step 2), the fluorescent product being detected in the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2 °C/s increments from 60 °C to 99 °C, with continuous fluorescence collection. For quantification of the genus *Bifidobacterium*, the *B. infantis* ATCC 15 697 reference strain was used as the standard. The presence of different *Bifidobacterium* species was detected using the PCR primers and conditions indicated previously [22].

Evaluation of atopic sensitization

Sensitization to common antigens in the mothers was tested by skin prick testing (SPT) as described previously [24] during the last trimester of pregnancy. The antigens tested included cow's milk, hen's egg white, wheat and rice flour both diluted 1/10 (w/v) with 0.9% sodium chloride, gliadin diluted 1 mg/mL with an ethanol/glycroleum/ALK-diluent (Allergologisk Laboratorium A/S, Horsholm, Denmark) mixture, cod, soya bean, peanut, hazelnut, alder, mugwort, birch, six grasses, cat, dog, Dermatophagoides pteronyssimus allergen (Allergologisk Laboratorium A/S), latex (Stallergenes S.A., Antony Cedex, France) and banana, potato and carrot by the prick–prick technique. Reactions were read at 15 min, and half of the histamine reaction size or more was recorded as positive on the condition that the mean diameter of the weal was at least 3 mm and the negative control (ALK) at the same time 0 mm.

Statistical methods

The bacterial counts in feces and in breast-milk were non-normally distributed and thus non-parametric statistical methods were applied. The Mann–Whitney test was used to test differences between two groups; correlations were tested by Spearman's rank correlation test. Associations between the bacterial counts in the infants and the maternal allergic status (allergic atopic, allergic non-atopic and non-allergic mothers) were detected by the Kruskal–Wallis test and *post hoc* comparisons were performed by the Mann–Whitney test. The association between frequencies was tested using Fisher's exact test. All analyses were performed using computer software SPSS for Windows release 12.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of the study population

The infants were born between 36 and 42 weeks of gestation, at a mean gestational age of 39 weeks, and the mean birth weight was 3495 g (range 2250–4170 g). Fifty-one (83.6%) of the infants were born by vaginal and 10 (16.4%) by Caesarean delivery. At 1 month of age, 50 (82%) of the infants were exclusively breastfed and 11 (18%) partially breastfed. Four of the infants had received antibiotics before 1 month of age. Fifty-three mothers (87%) self-reported allergic disease (atopic eczema, allergic rhinitis, asthma or food allergy) and were thus regarded as allergic. SPTs were carried out in all mothers: 37 of the allergic mothers proved positive and 16 negative to SPT allergens. Those mothers yielding at least one skin prick-positive result were regarded as atopic. Eight (13%) mothers did not report any allergic disease and proved negative in SPT.

Detection of bifidobacteria in feces: evaluation of interaction between mother and infant

All mothers had bifidobacteria among the fecal microbiota comprising of one to five different species (Table 1). The *B. longum* group was that most frequently detected (98%), followed by *B. catenulatum* group (33%), *B. adolescentis* (30%), *B. bifidum* (26%), *B. animalis* group (21%) and *B. breve* (8%) (Fig. 1).

Table 1. The distribution of fecal and breast-milk samples with different numbers of bifidobacteria species

	Number of detected bifidobacteria species							
	0	1	2	3	4	5	Unidentified bifidobacteria	Total
Infant feces at 1 month of age	2 (3)	22 (36)	18 (30)	8 (13)	5 (8)	0	6 (10)	61 (100)
Mother feces at 30–35 weeks of gestation	0	20 (33)	19 (31)	11 (18)	9 (15)	1 (2)	1 (2)	61 (100)
Breast-milk 1-month post-partum	0	19 (31)	22 (36)	4 (7)	9 (15)	1 (2)	6 (10)	61 (100)

The values are numbers of samples (%).

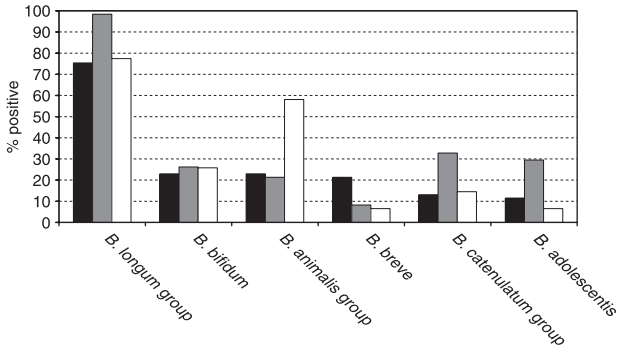

Fig. 1. The proportion of positive fecal and breast-milk samples for different bifidobacteria species. Black column: infants' feces at 1 month of age; grey column: mothers' feces at 30–35 weeks of gestation and white column: breast-milk at 1-month post-partum.

All but two infants had bifidobacteria in their fecal microbiota at 1 month of age: one of the negative infants having received antibiotics, the other born by Caesarean delivery (Table 1). The most frequently detected bifidobacteria species in infants were *B. longum* group (75%), *B. bifidum*, *B. animalis* group and *B. breve*, these being detected at frequencies of 21–23%. *B. catenulatum* group and *B. adolescentis* were found in only 12–13% of the infants (Fig. 1).

Mothers were more often colonized with the *B. longum* group (98% vs. 75%, $P < 0.0001$), the *B. catenulatum* group (33% vs. 13%, $P = 0.017$) and *B. adolescentis* (30% vs. 12%, $P = 0.024$) as compared with infants. The counts of different bifidobacteria in the culture-positive infants and mothers are given in Table 2.

Maternal fecal *B. bifidum* and *B. adolescentis* colonization frequencies were associated with corresponding frequencies in infants ($P = 0.005$ and 0.02, respectively). This was manifested in the child being more likely to be negative for these two bacteria if the mother was negative. Among infants with mothers having no fecal *B. bifidum*, 87% had no fecal *B. bifidum*, the corresponding value for *B. adolescentis* being 95%. Among infants with mothers colonized with *B. bifidum* 50% were positive, and for *B. adolescentis* 28% of the infants were positive. Further, the fecal counts of *B. bifidum* and *B. adolescentis* were positively correlated between mother–infant pairs ($\rho = 0.38$, $P = 0.002$ and $\rho = 0.32$, $P = 0.01$, respectively, data not shown). No correlation could be found in the fecal counts or colonization frequencies of other bifidobacteria species studied, nor between the total count of fecal bifidobacteria between the mothers and infants ($\rho = 0.11$, $P = 0.40$).

Detection of bifidobacteria in breast-milk: evaluation of interaction between breast-milk and infant's fecal bifidobacteria

We detected bifidobacteria in all the breast-milk samples. The bifidobacteria species compositions are shown in Fig. 1, the *B. longum* group being the most frequently detected (77% of samples), followed by the *B. animalis* group (58%), *B. bifidum* (26%), *B. catenulatum* group (15%) and *B. breve* and *adolescentis* (both 7%). The median count of the genus *Bifidobacterium* in breast-milk was 1.4×10^3 bacteria/mL [interquartile range (IQR) $48.7–3.8 \times 10^3$]. The numbers of bifidobacteria species in breast-milk varied from one to five (Table 1). There was no correlation between the total count of bifidobacteria in breast-milk and that in the infants' feces ($\rho = 0.17$, $P = 0.34$), nor was there any association between the breast-milk and infants' fecal bifidobacteria colonization frequencies at the species level (data not shown).

Effect of maternal allergic status on the bifidobacteria of breast-milk and infant's fecal microbiota

Allergic mothers had significantly lower concentrations of bifidobacteria in breast-milk compared with non-allergic mothers ($P = 0.004$) (Table 3), whereas the maternal atopic constitution had no further effect on breast-milk bifidobacteria numbers ($P = 0.55$) (Fig. 2).

In addition, maternal allergic status had a significant effect on the infant's fecal bifidobacteria. Firstly, fecal total counts of bifidobacteria were significantly lower in infants of allergic as compared with those of non-allergic mothers ($P = 0.013$) (Table 3). Secondly, when the colonization frequencies of different bifidobacteria species were compared between the infants of allergic and non-allergic mothers, it was found that infants of mothers with atopic allergy were the only ones to be colonized with *B. adolescentis* (7/37, 19%), as none of the infants of mothers with non-atopic allergy or non-allergic mothers had *B. adolescentis* (0/24, 0%, $P = 0.077$).

Table 2. Microbial counts of bifidobacteria in fecal samples of 1-month-old infants and their mothers at 30–35 weeks of gestation

	Infants			Mothers		
	Median	Minimum	Maximum	Median	Minimum	Maximum
Bifidobacterium spp.	8.70	<4.6	10.85	8.85	<4.6	10.28
B. longum group	8.36	5.18	10.08	8.25	5.25	9.43
B. bifidum	7.31	5.02	9.55	7.08	4.72	8.29
B. animalis group	7.12	6.29	8.25	6.05	<4.7	7.65
B. breve	<4	<4	8.89	<4	<4	<4
B. catenulatum group	8.28	<5.3	9.65	7.76	5.43	8.83
B. adolescentis	7.82	7.01	8.06	7.22	<5.5	8.45

Microbial counts are given in $\log_{10}$ bacterial cells/g of feces among the positive subjects.

Table 3. The bifidobacteria counts in breast-milk samples at 1 month postpartum and in fecal sampes of 1-month-old infants according to maternal allergic status

	Non-allergic mother*		Allergic mother†		
	Median	IQR	Median	IQR	*P*-value
Bifidobacteria count in breast-milk‡	3.75	3.25–4.25	3.10	1.35–3.48	0.004
Bifidobacteria count in infants' feces§	9.39	8.81–10.50	8.59	6.81–9.19	0.013

*$n = 8$ for breast-milk and fecal samples.
†$n = 52$ for breast-milk samples and $n = 53$ for fecal samples.
‡Values given in $\log_{10}$ bacterial cells/mL.
§Values given in $\log_{10}$ bacterial cells/g.
IQR, interquartile range

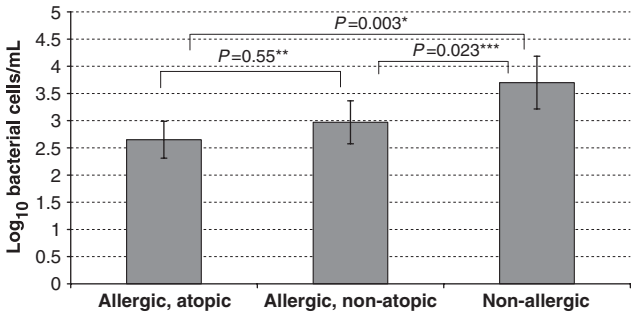

Fig. 2. The median count with interquartile range of breast-milk bifidobacteria in allergic, atopic mothers ($n = 37$), allergic, non-atopic mothers ($n = 15$) and non-allergic mothers ($n = 8$). $P = 0.014$ for overall difference among the groups, Kruskal–Wallis test. Two-group comparisons by Mann–Whitney test. *$P = 0.003$ between allergic, atopic and non-allergic mothers, **$P = 0.55$ between allergic, atopic and allergic, non-atopic mothers and ***$P = 0.023$ between allergic, non-atopic and non-allergic mothers.

Discussion

Vertical transmission of vaginal and fecal bacteria from the mother to the newborn is held to be essential to the formation of the intestinal microbiota in infants. However, modern delivery practices and the high standards of hygiene currently observed in neonatal care may have reduced this vertical transmission of commensal bacteria [9]. This study demonstrated that breast-milk bacteria may in fact compensate this shortage in the case of bifidobacteria; they were found in all breast-milk samples at a median count of 1.4×10^3 bacterial cells/mL of milk. Likewise, other study groups have reported that the lactic acid bacteria in breast-milk influence the intestinal colonization of newborns much more than the vertical transfer of maternal vaginal bacteria [25, 26]. For decades, breast-milk has been known to contain a large variety of bacteria [27–29]. However, so far, these bacteria have gained little attention in the context of vertical transmission from mother to infant [30, 31].

In this study, all breast-milk samples were found by direct PCR analysis to contain bifidobacteria. Previous works using anaerobic cultivation of breast-milk samples detected no bifidobacteria [25, 30]. However, a more recent study covering a large variety of countries around the world found bifidobacteria in breast-milk samples with varying frequencies (0–100%) [13], implying that the microbial colonization of breast-milk seems to be highly dependent on the bacteriological status of the society. Our group has published one previous data covering 20 breast-milk samples from women with 1-month-old infants, and the species composition of breast-milk was comparable to the present study [14]. Similarly, the predominant species of intestinal bifidobacteria in breastfed infants varies considerably according to the country and clinic of study [32–34] (Table 4). In agreement with previous studies, here, the *B. longum* group was the most commonly found species in the fecal microbiota

Table 4. The frequency (%) of colonized infants with different bifidobacteria species in the intestinal microbiota at 1 month of age

Author	Kleessen	Matsuki	Young	Young	Present study
Country of study	Germany	Japan	UK+NZ*	Ghana	Finland
Year of study	1995	1999	2004	2004	2007
Number of infants	20	27	46	32	61
Method of detection of bifidobacteria	Classical culture	Molecular	Molecular	Molecular	Molecular
Type of feeding	Exclusively breastfed	Exclusively breastfed	Mostly breastfed	Mostly breastfed	Exclusively/partially breastfed
No. of bifidobacteria species in feces	5	11	4	22	3
Species					
B. longum group†	80	78	52	78	75
B. longum	25	37	52	6	
B. infantis	55	41	0	72	
B. bifidum	35	22	22	0	23
B. breve	30	70			21
B. catenulatum group‡		19			13
B. pseudocatenulatum	0		13	0	
B. adolescentis	5	7	9	0	12
B. animalis group§					23
B. angulatum	0	4			
B. dentium		11			

*United Kingdom and New Zeland.

†*B. longum* biotype *longum*, biotype *infantis* and biotype *suis*.

‡*B. catenulatum* and *B. pseudocatenulatum*.

§*B. animalis* ssp. *lactis* and ssp. *animalis*.

of 1-month-old infants, followed by the *B. bifidum*, *B. animalis* group and *B. breve*. Recent data on fecal bifidobacteria of infants do not cover the *B. animalis* group (Table 4), and so we cannot directly compare our results with other published data. In adults, *B. longum*, *B. bifidum*, *B. adolescentis* and *B. catenulatum* are the species commonly detected in feces [10, 33], as also seen in our results. A study of the complete genome sequence of *Bifidobacterium longum* revealed some genomic traits that can explain the capability of bifidobacteria to survive in breast-milk and their predominance in infantile fecal microbiota [35]. Namely, Schell et al. [35] found excessive number of genes associated with specialized catabolism of variety of oligosaccharides that constitute over 20% of the carbohydrate content in breast-milk.

Bifidobacteria have been demonstrated to have a species-specific influence on gut immunity [34, 36, 37], and thus the early composition of bifidobacteria may have a major impact on the naïve immune system. Allergic infants have indeed been found to be colonized by bifidobacteria less often and with lower concentrations [38–41]. It is also noteworthy that *B. adolescentis* is found more often in the intestinal microbiota in allergic than in non-allergic children [42]. Because the offspring of allergic mothers evince a higher incidence of atopic diseases as compared with non-allergic mothers [17, 43, 44], our results provide a new, potential mechanism for previous findings. Allergic mothers had significantly lower breast-milk concentrations of bifidobacteria than non-allergic mothers, and concurrently their infants had lower fecal counts of bifidobacteria. Secondly, *B. adolescentis* was detected only in the fecal samples from infants of allergic mothers. These findings would indicate that the transfer of bacteria via breast-milk may be deviant, thus partly explaining the differences between allergic and non-allergic infants, and underlining the impact of the primary inoculums. They can further be interpreted as a reflection of the common genetic background in mother and child, which has indeed been shown in murine studies to regulate the primary colonization of the intestine [45]. Here, information on the allergic status of the offspring in later infancy was not yet available and thus the colonization results between allergic and non-allergic infants will be reported later.

Because exclusively breastfed infants may receive up to 1000 mL of breast-milk every day, correlating to up to 7.5×10^5 bifidobacteria, the breast-milk bifidobacteria numbers were expected to correlate with those in the feces in infants. The reasons why this was not the case are unclear. One explanation could be that the impact of the bacteriological status of the surroundings has such a major effect on the primary colonization that it exceeds the importance of the breast-milk bacteria [46, 47]. Secondly, the differences in the other bifidogenic factors in breast-milk, including the individually different galactooligosaccharide composition, may have an impact on bifidobacteria concentrations and species composition in the infant's intestine. Lastly, intestinal colonization may also differ greatly from the colonization detected in fecal samples, as has been shown in previous studies [48, 49], thus indicating a need for mucosal sampling.

The route of bifidobacteria to breast-milk is still open. Bifidobacteria are inhabitants of the oral cavity [50, 51] and transfer of bacteria from the infant's mouth to the milk ducts and consequently to breast-milk samples before analysis is one possibility. However, it has been shown that breast-milk contains similar bacteria both before and after breastfeeding [31]. Further, Martin and colleagues also demonstrated that pregnant mice can transfer orally administered bacteria to the fetal gut and to the mammary glands of the mother. This has been proposed to take place by transfer of intestinal bacteria within the phagocytosing cells from the gut to breast-milk [31]. Dendritic cells can penetrate the gut epithelium and take up commensal bacteria directly from the gut lumen [52]. Further, such bacteria remain alive in small numbers for several days [53]. Although in animal experiments dendritic cells loaded with enteric commensal bacteria were restricted to the mucosal immune compartment by the mesenteric lymph nodes [53], an active entero-mammary circulation of immune cells during lactation is known to take place [54, 55]. It is known that pregnancy increases the mucosal vascular addressin MadCAM-1 in the mammary gland, which interacts with the gut-homing receptor $\alpha 4\beta 7$ [56] and that 20 times as many of the breast-milk lymphocytes express intestinal-homing receptors as compared with the blood-derived lymphocytes [55]. Because colostrum is rich in mononuclear cells (80% of all immune cells of breast-milk), these could contain commensal-loaded cells originating from the intestinal tract, which could explain the colonization of breast-milk by intestinal bacteria. For pathogenic *Salmonella typhimurium*, this kind of entero-mammary colonization has been proposed recently [57].

Our findings here clearly demonstrate that breast-milk contains bifidobacteria and that a constant supply of bifidobacteria to the infant's intestine is thus assured during breastfeeding. These results call for further studies on the species composition of breast-milk bacteria to clarify their potential impact on the succession of infantile microbiota. As the composition of intestinal bacteria is claimed to be one of the major contributors to the development of immune functions in newborn infants, thereby affecting the growing problem of allergies, this important factor should be characterized. The routes of transmission of indigenous bacteria and the factors that disturb the normal step-wise colonization process need to be examined by modern molecular techniques, extending information to the strain level to clarify where and how these disturbances in the intestinal microbiota of atopic infants take place.

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