EFFECT OF PROBIOTICS AND BREASTFEEDING ON THE *BIFIDOBACTERIUM* AND *LACTOBACILLUS/ENTEROCOCCUS* MICROBIOTA AND HUMORAL IMMUNE RESPONSES

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Objective To assess impact of probiotics and breastfeeding on gut microecology.

Study design Mothers were randomized to receive placebo or *Lactobacillus rhamnosus* GG before delivery, with treatment of the infants after delivery. We assessed gut microbiota, humoral immune responses, and measured soluble cluster of differentiation 14 (sCD14) in colostrum in 96 infants.

Results Fecal *Bifidobacterium* and *Lactobacillus/Enterococcus* counts were higher in breastfed than formula-fed infants at 6 months; $P < .0001$ and $P = .01$, respectively. At 3 months, total number of immunoglobulin (Ig)G-secreting cells in breastfed infants supplemented with probiotics exceeded those in breastfed infants receiving placebo; $P = .05$, and their number correlated with concentration of sCD14 in colostrum. Total numbers of IgM-, IgA-, and IgG-secreting cells at 12 months were higher in infants breastfed exclusively for at least for 3 months and supplemented with probiotics as compared with breastfed infants receiving placebo; $P = .005$, $P = .03$ and $P = .04$, respectively. Again, sCD14 in colostrum correlated with numbers of IgM and IgA cells; $P = .05$ in both.

Conclusions We found an interaction between probiotics and breastfeeding on number of Ig-secreting cells, suggesting that probiotics during breastfeeding may positively influence gut immunity. *(J Pediatr 2005;147:186-91)*

Epidemiological studies point to the early environment as a window of opportunity in shaping the infant's immune responder type.[1] During this early critical period, the gut immature immune system is confronted by increasing amounts of dietary and microbial antigens. It is not currently known how these antigens affect the maturational process of gut humoral immunity. The mother's gut mucosa modifies the structure of the dietary antigens as these are transferred to the infant via breast milk.[2] Gradually the child is given unmodified antigens in formulas and solid foods. In like manner, the establishment of gut microbiota is a gradual process, the first colonizers being enterobacteria, streptococci, and staphylococci, followed by bifidobacteria.[3] The early introduction of foreign dietary antigens and unbalanced gut microbiota, instead of a stepwise exposure, may be associated with a risk of chronic diseases such as allergic diseases, diabetes, or celiac disease later in life.[1] Probiotics have been documented to reduce the risk of gastrointestinal infection.[4] Breastfeeding promotes *Bifidobacterium* microbiota[5] and reduces the risk of infection and allergic diseases.[6-8]

The aim of this study was to assess the impact of probiotics and breastfeeding on gut microbiota composition as characterized by bifidobacteria and lactobacilli/enterococci and humoral immune responses as indirectly assessed by circulating immunoglobulin(Ig)-secreting cells (IgM, IgA, and IgG).

See editorial, p 143, and related articles, p 192, and p 197.

ELISPOT	Enzyme-linked immunospot assay	ISCs	Circulating immunoglobulin-secreting cells
FISH	Fluorescent in situ hybridization	sCD14	Soluble CD14
Ig	Immunoglobulin		

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Table. The development of bifidobacteria and lactobacilli/enterococci and total bacterial counts in fecal samples (bacterial cells/g feces) in infants receiving probiotic or placebo analyzed by FISH at 3, 6, and 12 months of age

Age (months)	(Bacterial cells/g feces) Bifidobacteria* ($\times 10^8$)	Lactobacilli/ enterococci* ($\times 10^8$)	Total cell count* ($\times 10^9$)
3 months			
Probiotic	14.0 (6.0-33.0)	2.2 (1.1-4.6)	7.0 (4.6-11.0)
Placebo	15.0 (7.3-30.0)	2.7 (1.5-4.6)	5.8 (3.8-8.7)
6 months			
Probiotic	12.0 (7.6-20.0)	2.3 (1.6-3.4)	4.1 (3.2-5.3)
Placebo	8.6 (4.4-17.0)	2.5 (1.6-3.8)	3.4 (2.8-4.5)
12 months			
Probiotic	1.2 (0.6-2.6)	0.9 (0.5-1.4)	2.4 (1.9-3.0)
Placebo	1.9 (0.9-4.0)	1.0 (0.6-1.7)	2.5 (2.0-3.1)

*Geometric mean (95% CI).

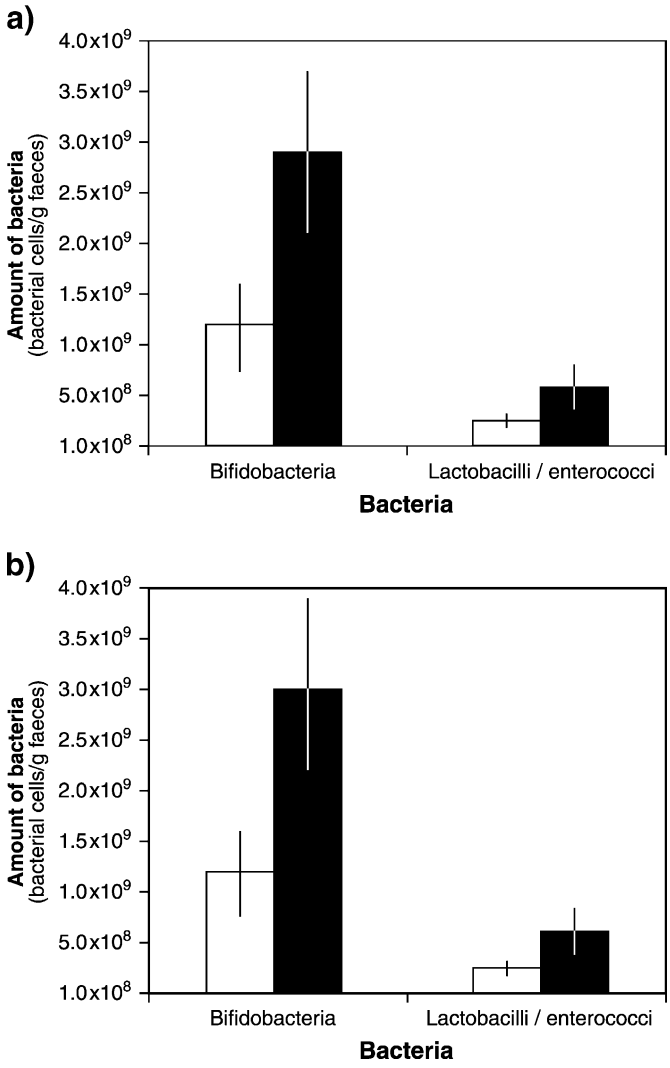

Figure 1 a, b. The effect of exclusive breastfeeding on the bacterial counts (*Bifidobacterium* and *Lactobacillus/Enterococcus*) in fecal samples (bacterial cells/g feces) analyzed by FISH at 6 months of age. The white column indicates infants exclusively breastfed for <3 months, the black column infants exclusively breastfed for at least 3 months. The number of samples analyzed in *Bifidobacterium* group was 28 and 43, respectively. The number of samples analyzed in *Lactobacillus/Enterococcus* group was 28 and 43, respectively. **b.** The effect of partial breastfeeding on the bacterial counts (*Bifidobacterium* and *Lactobacillus/Enterococcus*) in fecal samples (bacterial cells/g feces) analyzed by FISH at 6 months of age. The white column indicates infants who have been partially breastfed for <6 months, the black column infants partially breastfed for at least 6 months. The number of samples analyzed in *Bifidobacterium* group was 31 and 40, respectively. The number of samples analyzed in *Lactobacillus/Enterococcus* group was 31 and 40, respectively.

METHODS

Subjects and Study Design

The study population comprised 96 infants who had at least one close relative (mother, father, sibling) with atopic dermatitis, allergic rhinitis, or asthma. Children were recruited in antenatal clinics in the city of Turku, Finland. In a double-blind, placebo-controlled trial mothers were randomized to receive placebo (microcrystalline cellulose) or 1×10^{10} colony-forming units of *Lactobacillus rhamnosus* GG (ATCC 53103) once a day for 4 weeks before expected delivery, as described in detail elsewhere.[9] After the neonatal period, the assigned preparation was then given to the infants only. The contents of the capsule given to infants were administered by spoon after mixing in water. The duration of probiotic intervention after birth was 6 months. Mothers were encouraged to breastfeed their children at least up to 4 to 6 months of age. Parental smoking was discouraged, and feeding patterns of the infants were monitored.

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 the mother before the prenatal intervention.

Follow-up Visits and Sampling

The children were clinically examined at 3, 6, and 12 months of age by the same physician. The birth characteristics were evaluated; they included birth weight (g, mean and range) and the gestational age (weeks, mean and range). The diets of infants, recorded at all study visits, were classified as human milk, cow's milk-based adapted infant formula, or, in a case of documented cow's milk allergy, a hypo-allergenic formula (extensively hydrolyzed or amino acid formula). The ages at introduction of formula and solid food were recorded. In addition, the duration of exclusive and partial breastfeeding was assessed. Exclusive and partial breastfeeding are reported separately as practiced elsewhere.[10] Exclusive breastfeeding refers to breast milk as a sole source of nutrition to the infant and partial breastfeeding means giving an infant some

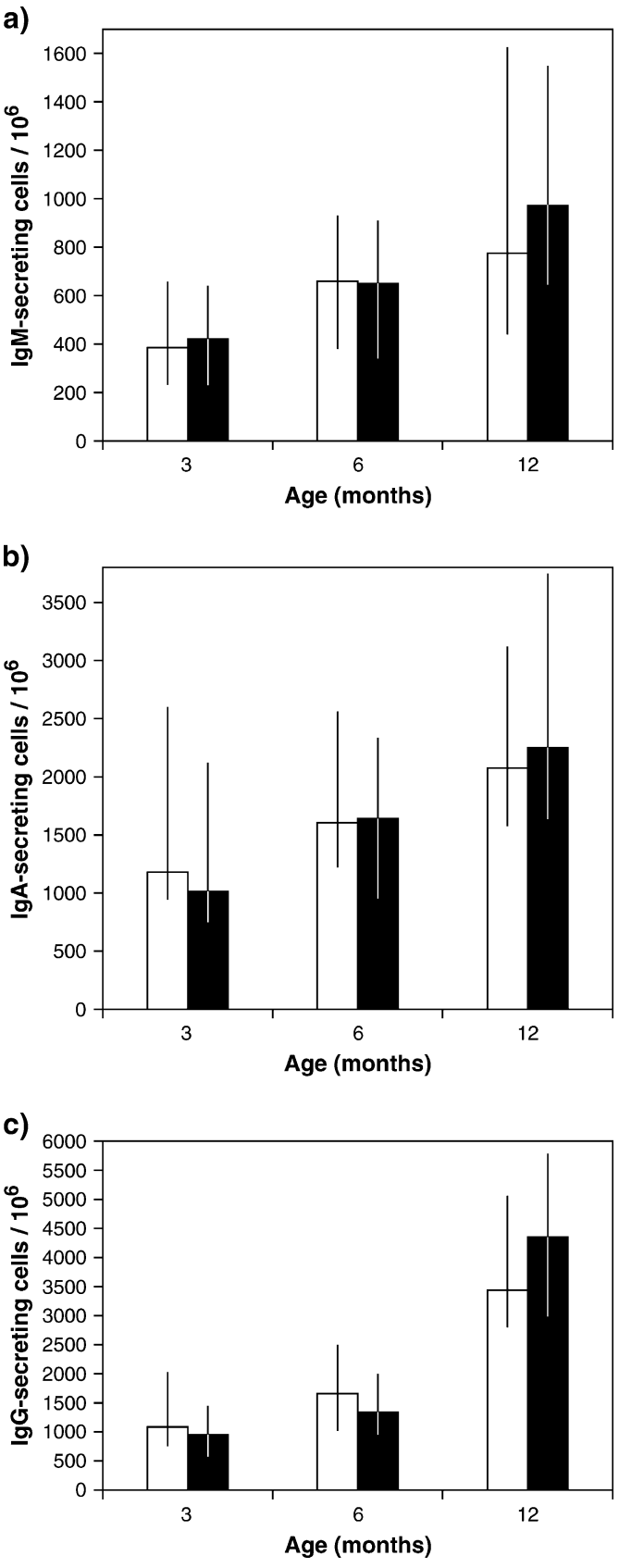

Figure 2 a. The total number of IgM-secreting cells per 10^6 mononuclear cells (medians with interquartile ranges) at 3, 6, and 12 months of age. The white column indicates infants receiving placebo. The number of samples analyzed at 3, 6, and 12 months of age were 44, 42, and 44, respectively. The black column indicates infants receiving the study probiotic; the number of samples analyzed at 3, 6, and 12 months of age were 42, 43, and 43, respectively. **b.** The total number of IgA-secreting cells per 10^6 mononuclear cells (medians with interquartile ranges) at 3, 6, and 12 months of age. The white column indicates infants receiving placebo; the number of samples analyzed at 3, 6, and 12 months of age were 44, 42, and 44, respectively. The black column indicates infants receiving the study probiotic; the number of samples analyzed at 3, 6, and 12 months of age were 42, 43, and 43, respectively. **c.** The total number of IgG-secreting cells per 10^6 mononuclear cells (medians with interquartile ranges) at the ages of 3, 6, and 12 months. The white column indicates infants receiving placebo; the number of samples analyzed at 3, 6, and 12 months of age were 44, 42, and 44, respectively. The black column indicates infants receiving the study probiotic; the number of samples analyzed at 3, 6, and 12 months of age were 42, 43, and 43, respectively.

breastfeeds and some artificial feeds, either milk or cereal, or other food.[11]

Soluble CD14 in Colostrum

The concentration of soluble CD14 (sCD14) in colostrum was measured because sCD14 in human milk has been shown to influence the innate immune responses in the intestine.[12] CD14 belongs to the so-called pattern recognition receptor family, which enables communication between bacterial products and cells.[13] CD14 is found both in membrane-bound and soluble forms that recognize not only gram-negative bacteria-derived lipopolysaccharide but also peptidoglycan, a universal cell wall component of bacteria.[14] Human intestinal epithelial cells do not express membrane CD14, but human milk contains high amounts of sCD14.[12,14] sCD14 in milk acts as an immune modulator and may prevent inflammatory conditions of the gut.[15] Altogether 44 colostrum samples were available for the sCD14 measurements, assayed at a dilution of 1:4000 (Human sCD14 Quantikine ELISA kit; R & D Systems, Inc., Minneapolis, Minn).

Fluorescent in Situ Hybridization of Gut Microbiota

Fecal specimens were collected from diapers after defecation, and the specimens were immediately cooled to 6 to 8°C and transported to the hospital within 24 hours to be frozen at −75°C. Altogether, 46 fecal samples were available for fluorescent in situ hybridization (FISH) analysis at 3 months of age, 72 samples at 6 months, and 64 samples at 12 months. Bacterial cells were harvested for FISH.[16-18] The probes for the FISH method were Bif164 (′5-CATCCGGCATTACCACCC) and Lab158 (′5- GGTATTAGCA(T/C) GTGTTTCCA, Eurogentec Ltd., Southampton, UK), specific for bifidobacteria and lactobacilli/enterococci, respectively. We used commercial synthetic probes that were 5′ labeled with fluorescent dye Cy3. Total counts were enumerated using the nucleic acid stain DAPI (4′, 6-diamidino-2-phenylindole) as described by Porter and Feig 1980.[19] Sample processing was completed as previously described,[17] and counting was done using the Leica Laaborlux D epi-fluorescence microscope (Leica Microsystems AG, Wetzlar,

Germany). At least 15 random fields were counted on each slide, and the average count was used for analysis.

Enzyme-linked Immunospot Assay of Circulating Immunoglobulin-secreting Cells

Circulating immunoglobulin-secreting cells (ISCs) are progeny of B cells recently activated in the gut and other inductor sites. Their numbers were assayed by the enzyme-linked immunospot (ELISPOT) method, as described in detail elsewhere.[20] The ELISPOT assay indirectly indicates gut immunological events.[21-23] The total numbers of Ig-secreting cells directed against dietary antigens were assessed at 3, 6, and 12 months of age. Altogether, 86 blood samples were available for the ELISPOT at 3 months of age, 85 samples at 6 months, and 87 samples at 12 months. Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) centrifugation of lithium-heparinized blood was used to recover mononuclear cells containing mainly lymphocytes. The cells at 10^6 cells/mL were suspended in Rapid Prototyping and Manufacturing Institute (RPMI) 1640 medium (Gibco Brl, Life Technologies, Paisley, Scotland), which contained 10% fetal calf serum (Gibco Brl, Life Technologies, Eggenstein, Germany). Wells were then coated with rabbit anti-human IgM and IgA (Dako A/S, Glostrup, Denmark), and goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) at a dilution of 1/100 in phospate buffered saline (PBS), and cells were incubated overnight at room temperature; then they were overlaid by a substrate agarose. Finally, the colored spots representing individual Ig-secreting cells were counted under a microscope.

Statistics

The bacterial counts in fecal samples are expressed as geometric mean with 95% CI. The total numbers of IgM-, IgA- and IgG-secreting cells at the ages of 3,6, and 12 months are expressed as medians with interquartile ranges. The synergistic effects of exclusive breastfeeding and probiotic on the total number of IgM-, IgA-, and IgG-secreting cells are expressed as geometric mean with 95% CI after logarithmic transformation at 12 months of age. The two-way analysis of variance and analysis of variance for repeated measurements were used to compare different groups. Correlation between the sCD14 concentration in colostrum and the total number of ISCs was calculated using the Spearman rank correlation test.

All statistical analyses were performed with Statview computer software, version 4.5 (Abacus Concepts, Inc., Berkeley, Calif).

RESULTS

Clinical Characteristics

The infants were born between 35 and 41 weeks of gestation (mean, 39 weeks) in the placebo group and between 36 and 43 weeks of gestation (mean, 40 weeks) in the probiotic group. The mean birth weights of the infants in these groups were 3577 g (range, 2490-4800) and 3666 g (2840-4865), respectively. The mean duration of exclusive breastfeeding was 2.6 months (range, 2.3-3.0) and partial breastfeeding was 6.9 months (range, 6.0-7.7; 95% CI). By 6 months of age, the predominant milk was human milk in 46 of 96 (48%), adapted cow's milk-based infant formula in 43 of 96 (45%), extensively hydrolyzed formula in 4of 96 (4%), and amino acid formula in 3 of 96 (3%) infants.

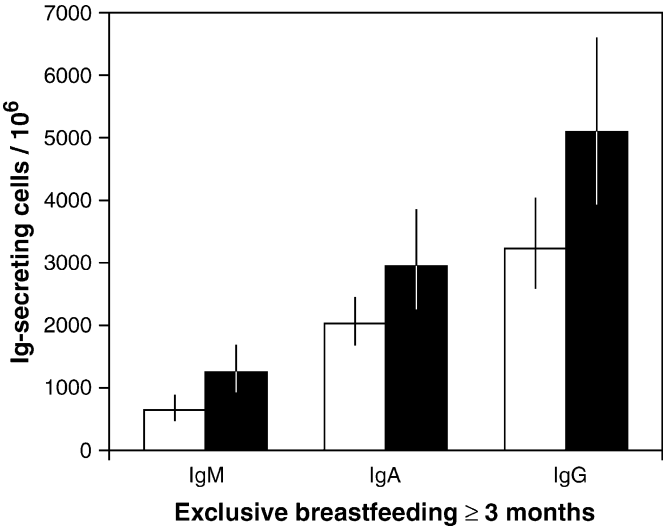

Figure 3. The synergistic effects of exclusive breastfeeding for at least 3 months and probiotic on the total number of IgM-, IgA- and IgG-secreting cells per 10^6 mononuclear cells (geometric means with 95% CI after logarithmic transformation) at 12 months of age. The white columns indicate infants receiving placebo, and the black columns infants receiving the study probiotic. The number of samples analyzed in IgM, IgA, and IgG groups in infants receiving placebo and probiotic were 18, 26; 18, 26; and 18, 26, respectively.

The Establishment of the *Bifidobacterium* and *Lactobacillus/Enterococcus* Microbiota

The total numbers of bacteria in fecal samples showed a decreasing trend from 3 to 12 months of age; P <.0001. The numbers were comparable between the probiotic and placebo groups; P = .70 (Table). Along the same lines, the *Bifidobacterium* counts followed a decreasing pattern in both placebo and probiotic groups, P <.0001 and P <.0001, respectively. The amounts of *Lactobacillus/Enterococcus* were significantly lower and remained stable throughout the study period (Table).

Breastfeeding had a strong impact on the *Bifidobacterium* microbiota during infancy (Figure 1a). The number of *Bifidobacterium* at 6 months in feces of infants exclusively breastfed for at least 3 months was 1.8×10^9 ($1.2 \times 10^9 - 2.6 \times 10^9$) as compared with those who were not, 4.4×10^8 ($2.1 \times 10^8 - 9.3 \times 10^8$); P <.0004. The number of *Lactobacillus/Enterococcus* counts in infants exclusively breastfed for at least 3 months was 2.9×10^8 ($1.9 \times 10^8 - 4.3 \times 10^8$) as compared with those who were not, 1.8×10^8 ($1.3 \times 10^8 - 2.6 \times 10^8$); P = .10. If an infant was still breastfed at 6 months of age, the promotive effect was seen on *Bifidobacterium* as well as on *Lactobacillus/Enterococcus* counts as compared with infants not being breastfed at that stage; P <.0001 and P = .01,

respectively (Figure 1b). These differences were no longer observed at 12 months of age (data not shown).

The Development of Quantity of Immunoglubulin-secreting Cells

The total numbers of IgM-, IgA-, and IgG-secreting cells in peripheral blood increased with age, as shown in Figure 2; $P < .0001$; $P < .0001$, and $P < .0001$, respectively. The increase was comparable between the placebo and probiotic groups; $P = .80$; $P = .20$; $P = .06$, respectively. The duration of exclusive breastfeeding for >3 months had no effect on the total numbers of IgM- and IgA-secreting cells, whereas a higher number of IgG-secreting cells was detected at 3 months of age in infants who had been exclusively breastfed for at least 3 months; $P = .02$.

The Combined Effects of Breastfeeding and Probiotics on the Amounts of ISCs

There was a significant interaction between probiotics and breastfeeding in respect to the amounts of ISCs in peripheral blood. The total numbers of IgM, IgA, and IgG at 12 months of age were higher in those infants who had been breastfed exclusively for at least 3 months and supplemented with probiotics as compared with those breastfed infants receiving placebo; $P = .005$, $P = .03$ and $P = .04$, respectively (Figure 3). Interestingly, at the same age of 12 months, a positive correlation between the total numbers of IgM- and IgA-secreting cells and the sCD14 concentration in colostrum was detected; $P = .05$, $P = .05$, respectively. At 3 months of age the total number of IgG-secreting cells was higher in breastfed infants supplemented with probiotics, 1210 (942-1553), as compared with those receiving placebo, 1196 (871-1643); $P = .05$. Again, the concentration of sCD14 in colostrum correlated with the total number of IgG-secreting cells at 3 months of age; $P = .001$.

DISCUSSION

Characterization of the maturation of the gut immune responses and the compositional development of the gut microbiota in health is essential in improving our understanding of the development of host-microbe interaction. The present findings suggest that probiotic therapy and breastfeeding exert synergistic effects on these processes and may thereby generate the necessary initial step for a healthy maturation of the functional adaptive immune system. Identification of the immunomodulatory components may have promise in the attempt to mimic these events in at-risk children.

The establishment of the gut microbiota has traditionally been considered a stepwise process. In our present study, the total bacterial counts in fecal samples were at their peak by 3 months, thereafter decreasing with age. Bifidobacteria appear after birth and within a week, reaching the dominant bacterial group in healthy infants.[24] The unbalanced gut microbiota is often characterized by a low number of bifidobacteria.[25] Indeed, recent studies applying modern molecular methods demonstrate that bifidobacteria may constitute from 60% up to 90% of the total fecal microbiota in breastfed infants, whereas lactic acid bacteria appear to account for <1% of the total microbiota.[26,27] Bifidobacteria are genetically adapted to utilizing human milk and human milk oligosaccharides as the major substrates for growth and thus are particularly adapted to the environment in the breastfed infant gut.[28] Oral administration of specific strains of *Lactobacillus* species, including *Lactobacillus rhamnosus* GG, stimulated the *Bifidobacterium* microbiota.[29] Combination of this type of probiotic microbes with breastfeeding may thus explain the synergistic effect on gut humoral immunity and microbiota modification. In addition, human milk was identified as a possible source of lactic acid bacteria for the infant gut.[30] This suggestion if proven in future subsequent studies, further strengthens the importance of the composition of the mother's gut microbiota during delivery and breastfeeding.

The results presented here suggest that some human milk-derived compounds may be mandatory for probiotics to stimulate humoral immune responses, or vice versa, supporting the general principle of collaboration of the innate and adaptive immune system in the maturation of normal immune responses in the gastrointestinal tract. Human milk-derived CD14 participates in the activation of the innate immune responses in the intestine and possibly in modulating the immunological balance in the infant's intestine.[12] There are several soluble molecules such as toll-like receptor 2 in breast milk that influence the gut maturation together with CD14.[31] A recent study demonstrated that administration of sCD14 to mice enhanced Ig secretion in vivo.[14] In parallel, we found here that the sCD14 concentration in colostrum correlated with IgA and IgM at 12 months of age, suggesting a real dependence of the infant's own Ig production on humanmilk-derived sCD14. Infant's IgG and sCD14 in human milk correlated at 3 months of age but not later. It is likely that IgG at this age originated mostly from the mother, and the correlation may not be causal. *Lactobacillus* GG has been shown to promote local antigen-specific immune responses (particularly in the IgA class), which is further supported by the well-documented stimulatory effects of transforming growth factor β on the IgA production.[8] Moreover, total numbers of Ig-secreting cells were increased among breastfed probiotic-supplemented infants as compared with those breastfed infants with placebo. This would point to sCD14 in human milk as a potential mediator of beneficial probiotic effect on humoral immune responses. Human milk sCD14 protected against the later development of atopic eczema,[32] with which low IgA is connected.

Our findings suggest that the incorporation of probiotics in the mother's diet before delivery and in the infant diet during breastfeeding may positively influence the maturation process of gut immunity.

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