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Low breast milk TGF-β2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy

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The immunological composition of breast milk differs between mothers. The reasons for these differences and the consequences for the breast-fed infants are poorly understood. The aim of this study was to evaluate the effect of probiotic *Lactobacillus reuteri* supplementation on the immunological composition of breast milk in relation to sensitization and eczema in the babies. Total IgA, secretory IgA (SIgA), TGF-β1, TGF-β2, IL-10, TNF, soluble CD14 (sCD14), and Na/K ratios were analyzed in colostrum and mature milk obtained from women treated with *L. reuteri* (n = 54) or placebo (n = 55) from gestational week 36 until delivery. Bacteriological analyses of *L. reuteri* were performed in faecal samples of the mothers. The infants were followed prospectively for 2 yr regarding development of eczema and sensitization as defined by a positive skin prick test and/or circulating allergen-specific IgE antibodies at 6, 12, and 24 months of age. Supplementation of *L. reuteri* during pregnancy was associated with low levels of TGF-β2 and slightly increased levels of IL-10 in colostrum. For TGF-β2, this association was most pronounced in mothers with detectable *L. reuteri* in faeces. Infants receiving breast milk with low levels of TGF-β2 were less likely to become sensitized during their first 2 yr of life. A similar trend was observed for development of IgE-associated eczema. The levels of total IgA, SIgA, TGF-β1, TNF, sCD14, and Na/K ratios in breast milk were not affected by the intake of *L. reuteri*. None of these parameters correlated with sensitization or development of eczema in the infant, except for high Na/K ratios that associated with increased risk of sensitization. Supplementation with *L. reuteri* during late pregnancy reduces breast milk levels of TGF-β2, and low levels of this cytokine are associated with less sensitization and possibly less IgE-associated eczema in breast-fed infants.

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Breast milk not only provides the necessary nutrients for growth and development, it also contains numerous immunological components that compensate the immature and inexperienced mucosal immune system (1). Such components include immune cells, antibodies (especially IgA antibodies), pro- and anti-inflammatory cytokines such as TNF, IL-10, and TGF-β (2) and factors that may modify immune responses to bacteria, e.g., soluble CD14 (sCD14) (3). The immunological composition of breast milk differs considerably between mothers; however, and the

factors contributing to the precise composition is not fully understood. Maternal allergy (2, 4), infections (5), inflammation (6), stress (5), and supplementation of fish oil (7) and probiotics (8) given during pregnancy have all been suggested to affect the composition of breast milk. The controversial results regarding the role of breast feeding for prevention of allergy have been suggested to be, at least partly, due to differences in breast milk composition (9). For instance, high levels of breast milk IgA (10–12) and TGF-β (13, 14) have been suggested to influence on allergy development in the child, although this is still controversial (15).

Nutritional, metabolic and immunological processes in the gut are reflected in the mammary gland and the milk through the entero-mammary link (16). Therefore, manipulation of the gut flora by probiotic supplementation may affect the immune composition of breast milk. In fact, supplementation of *Lactobacillus* GG to lactating women was associated with increased levels of TGF-β2 in breast milk collected 3 months after delivery (8), although the consequences of this for the baby are unknown.

The aim of this study was to evaluate the effect of oral supplementation of the probiotic *Lactobacillus reuteri* (*L. reuteri*) during pregnancy on the levels of several immune components in the breast milk, including total IgA, secretory IgA (SIgA), TGF-β1, TGF-β2, IL-10, TNF, and sCD14, and to relate these findings to the development of sensitization and eczema in the infants.

Material and methods

Subjects

In a double-blind placebo-controlled allergy prevention study, pregnant women in families with a history of allergic disease were randomized for oral supplementation of *L. reuteri* (strain American Type Culture Collection 55730, BioGaia AB, Stockholm, Sweden) or placebo from gestational week 36 until delivery (17). After birth, the baby continued with the same study product until 12 months of age. The atopic status of the mothers was revealed during an interview regarding former and present symptoms of allergic disease and by the occurrence of serum IgE antibodies to a panel of inhalant allergens as analyzed with UniCap® Pharmacia CAP System™ Phadiatop® (Pharmacia Diagnostics, Uppsala, Sweden). An experienced allergy research nurse performed the telephone interviews. Descriptive data regarding maternal age, history of allergic disease, etc., are given in Table 1.

Table 1. Descriptive data of mothers and children receiving *L reuteri* or placebo

	L. reuteri	Placebo	p-Values
Mothers	n = 54	n = 55	
Age (years), mean (range)	29 (22–38)	31 (21–44)	n.s.
Gestational (weeks), mean (range)	40 (37–43)	39 (36–43)	n.s.
No. weeks with study product, mean (range)	4 (2–7)	4 (1–7)	n.s.
History of allergic disease	39/54 (72%)	40/55 (73%)	n.s.
Positive Phadiatop®	23/50 (46%)	26/54 (48%)	n.s.
No. children, mean (range)	1.7 (1–4)	1.7 (1–5)	n.s.
Furry pets	14/54 (26%)	9/55 (16%)	n.s.
Smoking	0/54 (0%)	0/55 (0%)	n.s.
Children	n = 42	n = 44	
Breast fed (months)–exclusive/total (mean)	2.9/7.9	2.7/8.6	n.s
No. infections, mean (range)	11 (2–19)	11 (3–24)	n.s.
Antibiotic treatment	18/42 (43%)	28/44 (64%)	n.s.

One hundred and nine of the originally included 232 mother/infant pairs were included in this study. Inclusion was based on the availability of colostrum samples, obtained within the first 3 days after delivery, and mature milk samples, obtained 1 month after delivery. Fifty-four of the mothers belonged to the *L. reuteri* group and 55 to the placebo group.

Follow-ups of the infants were performed by research nurses at 1, 3, 6, 12, and 24 months of age and by structured telephone interviews with parents at 2, 4, 5, 8, 10, and 18 months. A final follow-up was done by a paediatrician at 2 yr of age. The interviews related to, e.g., symptoms of allergic disease, adverse events, infections, and use of antibiotics. Eczema was defined as a pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution, as suggested by Hanifin and Rajka and modified from Seymour (18). Eczema was classified as IgE associated if the infant also was sensitized. Skin prick tests (SPTs) were performed on the volar aspects of the forearms with egg white, skimmed cow's milk (lipid concentration 0.5%) and standardized cat, birch, and timothy extracts (Soluprick®; ALK, Hørsholm, Denmark) at 6, 12, and 24 months of age. The test was regarded as positive if the mean wheal diameter was ≥3 mm (Table 2). Histamine hydrochloride (10 mg/ml) served as positive and albumin diluent as negative control.

Venous blood samples were drawn at 6, 12, and 24 months of age. Circulating IgE antibodies specific to egg white, cow's milk, and a mixture of

Table 2. The cumulative incidence of Skin-prick test (SPT) ≥3 mm, specific IgE levels > 0.35 kU/L and eczema in infants completing the study until 2 years of age

Positive SPT ≥ 3 mm	*L. reuteri*	Placebo	p-Values
Egg	7/42 (17)	8/44 (18)	n.s.
Milk	1/42 (2)	2/44 (5)	n.s
Cat	1/42 (2)	3/44 (7)	n.s.
Birch	1/42 (2)	4/44 (9)	n.s.
Specific IgE			
OVA	5/39 (13)	8/32 (25)	n.s.
BLG	4/37 (11)	6/30 (20)	n.s.
Food allergen (fx5)	10/37 (27)	13/31 (42)	n.s.
Sensitisation	14/44 (32)	17/43 (40)	n.s.
Eczema	21/51 (41)	19/53 (36)	n.s.
IgE ass eczema	8/44 (18)	10/43 (23)	n.s.

Values in parentheses are expressed as percentages.

food allergens, including egg white, cow's milk, cod, wheat, peanut, and soy bean (fx5) (UniCap® Pharmacia CAP System™; Pharmacia Diagnostics) were analyzed in all samples (Table 2). The infants were regarded as sensitized if they had at least one positive SPT and/or detectable circulating allergen-specific IgE antibodies (Table 2). Fifty-one and 53 of the *L. reuteri* and placebo-treated mother–infant pairs, respectively, completed the study.

Milk samples were collected with a manual breast pump and aliquoted into sterile plastic tubes. Faecal samples from the mothers were collected during the first week after delivery into sterile plastic containers. Milk and faecal samples were stored at −70°C until analysis.

Immunological and chemical analyses of breast milk

After thawing, the milk samples were centrifuged to remove fat and cellular compartments as described by Böttcher et al. (2). The whey was analyzed immediately for the content of IL-10. The remaining whey was stored in aliquots at −70°C and later analyzed for TNF, TGF-β1, TGF-β2, sCD14, total IgA, SIgA, and Na/K ratio. The levels of TGF-β1 and TGF-β2 were analyzed after acid treatment to pre-activate latent TGF-β as described earlier (2), with commercial ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's protocol. The levels of sCD14 (R&D Systems) and IL-10 and TNF (CLB PeliPair reagent set, Amsterdam, the Netherlands) were also determined with commercial ELISA reagent kits. The lower limit of detection was 62.5 pg/ml for TGF-β1 and TGF-β2, 250 pg/ml for sCD14, 2.3 pg/ml for IL-10 and 7.8 pg/ml for TNF.

Total IgA and SIgA were analyzed with in-house ELISA as described earlier (19). The lower limit of detection was 31.2 ng/ml for both assays.

Sodium and potassium levels were measured at the Clinical Chemistry Department at Linköping University Hospital by ion-selective electrodes according to in-house routines.

Analyses of *L. reuteri* in faecal samples

All bacteriological analyses were performed by BioGaia, Lund, Sweden. The faeces samples were weighted, thawed, and diluted in saline buffer and plated on modified de Man-Rogosa-Sharpe (MRS) agar with 50 mg/l vancomycin and 2% sodium acetate (MRS-3; Acumedia, Ljusne, Sweden). In order to detect *L. reuteri* also in samples with low concentration of the microbe, undiluted samples was plated on the MRS agar. The plates were incubated anaerobically in anaerobic jars with GasPack Plus (Becton Dickinson) at 37°C for 48 h. *L. reuteri* colonies were confirmed by detecting reuterin production after addition of glycerol.

Statistical analysis

The Mann–Whitney *U*-test was employed for unpaired analyses, and Spearman's rank order correlation coefficient test for correlations analyses. Multiple logistic regression and ANOVA were used to analyze multivariate relationships. Differences combined with p-values of <0.05 were considered statistically significant. The calculations were performed with the statistical packages STATA version 8.2 for PC (Stata Corp LP, College Station, Texas, USA) and STATVIEW version 5.9 for PC (SAS Institute Inc., Cary, NC, USA).

Ethics

Informed consent was obtained from the parents before inclusion. Pain connected with blood sampling in the infants was minimized with topical anaesthesia. The study was approved by the regional Ethics Committee for Human Research at the Linköping University Hospital.

Results

The levels of the various immunological parameters and Na/K ratios in colostrum and mature milk samples in mothers who received *L. reuteri* or placebo are presented in Table 3. The levels of TGF-β2 were lower, whereas IL-10 was slightly higher in colostrum of mothers receiving *L. reuteri*, as compared with mothers who received placebo (Table 3). None of the other

Table 3. Levels [median (25th–75th percentiles)] of various immunological parameters and Na/K ratio in colostrum and mature milk (obtained 1 month after delivery) from mothers treated with *L. reuteri* (n = 54) or placebo (n = 55) from gestational week 36 until delivery

	Colostrum			Mature milk		
	L. reuteri	Placebo	p-Value	*L. reuteri*	Placebo	p-Value
IL-10 (pg/ml)	6.6 (4.5–19.0)	4.8 (1.15–12.7)	0.046	1.15 (1.15–5.5)	1.15 (1.15–4.0)	n.s.
TGF-β1 (pg/ml)	570 (382–748)	653 (443–948)	n.s.	321 (231–497)	310 (219–593)	n.s.
TGF-β2 (pg/ml)	674 (455–1153)	965 (557–1691)	0.02	399 (265–672)	378 (233–810)	n.s.
TNF (pg/ml)	11 (3.9–21)	10 (3.9–23)	n.s.	4 (4–12)	4 (4–10)	n.s.
sCD14 (pg/ml)	17 (13–19)	15 (12–20)	n.s.	9 (7–10)	8 (6–10)	n.s.
Total IgA (mg/ml)	2.8 (1.7–4.0)	2.9 (1.6–4.3)	n.s.	1.2 (0.9–1.5)	1.1 (0.9–1.4)	n.s.
SigA (mg/ml)	2.2 (1.4–2.7)	2.2 (1.5–3.5)	n.s.	0.7 (0.5–1.0)	0.7 (0.6–1.0)	n.s.
Na/K	0.8 (0.7–1.1)	0.9 (0.7–1.2)	n.s.	0.7 (0.6–0.9)	0.6 (0.5–0.8)	n.s.

Groups were compared with the Mann-Whitney *U* test.

parameters differed between the *L. reuteri*- and placebo-treated groups (Table 3). The levels of all parameters in mature milk samples were similar in the two groups (Table 3).

The presence of *L. reuteri* in stool samples was also associated with low levels of TGF-β2 in colostrum [median (range) 697 (102–5600) vs. 1013 (240–2800) pg/ml, p = 0.04]. The lowest levels of TGF-β2 were found in *L. reuteri*-treated mothers with detectable *L. reuteri* in faeces; although the difference between this group and placebo-treated mothers with detectable *L. reuteri* was not statistically significant (Fig. 1). No

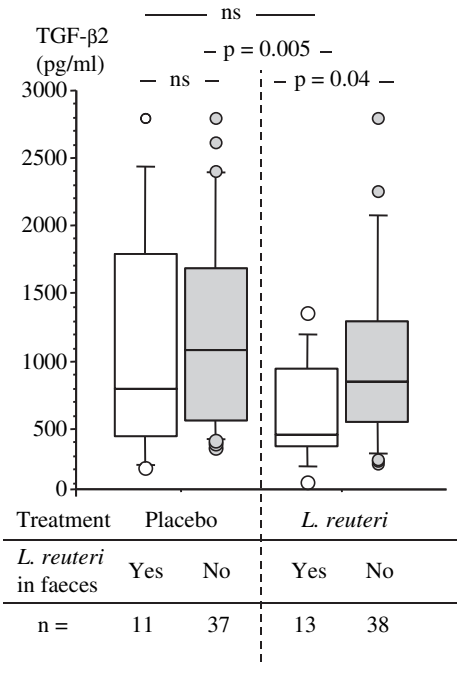

Fig. 1. The levels of TGF-β2 in colostrum were lower in mothers with detectable, as compared to mothers with no detectable *Lactobacillus reuteri* in faeces in the *L. reuteri*-treated group. No such difference was observed in the placebo-treated group. The 10th, 25th, 50th, 75th, and 90th percentiles are indicated, as well as outliers.

other immunological breast milk parameter was associated with the detection of faecal *L. reuteri*.

Maternal atopy, pets, mothers age, number of siblings, number of weeks with intake of study product and gestational weeks were not associated with any of the analyzed breast milk parameters (data not shown) and did not influence the relationship between treatment and colostrum TGF-β2 and IL-10 levels in an ANOVA model (data not shown).

Low levels of TGF-β2 in colostrum were associated with less sensitization during infancy (Fig. 2). This association reached statistical significance at 24 months of age. A similar trend was observed for TGF-β2 levels in mature milk (data not shown). Furthermore, low colostrum Na/K ratios were also associated with less sensitization at 24 months of age [median (range) for sensitized vs. non-sensitized infants; 0.78 (0.42–2.8) vs. 1.1 (0.65–5.8), p = 0.004]. In a logistic regression model, controlling for possible confounders, i.e., study treatment, maternal atopy, and Na/K ratios, low levels of TGF-β2 were associated with a reduced risk for sensitization at 24 months of age, and high levels of colostrum TGF-β2 tended to be associated with an increased risk for sensitization at 6 months of age (Table 4). The relation between Na/K ratios in breast milk and sensitization at 24 months also remained in the multivariate model (Table 4). None of the other analyzed breast milk parameters were associated with infant sensitization (data not shown).

Development of eczema during the first 24 months of life was not associated with any of the analyzed breast milk parameters. The colostrum levels of TGF-β2 tended to be higher in mothers of babies with IgE-associated eczema; however Table 5, and similar trends were observed for TGF-β2 in mature milk (data not shown). The number of infants with IgE-associated eczema at 6, 12, and 24 months was low

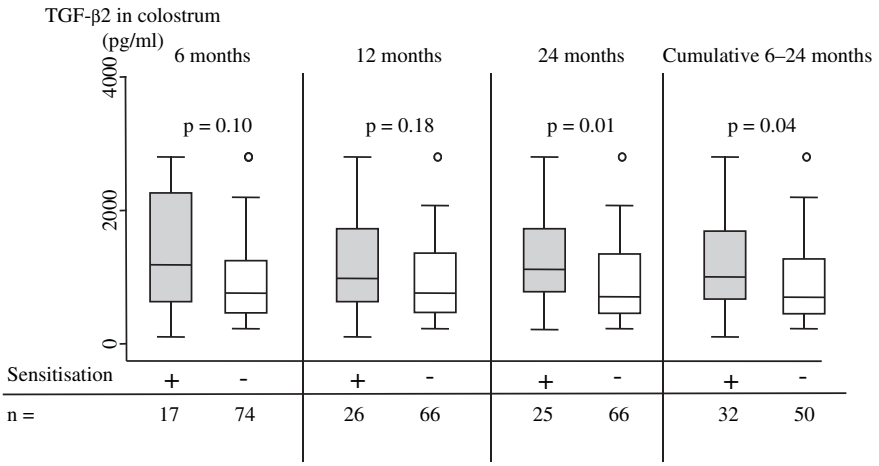

Fig. 2. Infants who were sensitized (positive SPT and/or circulating allergen-specific IgE) at 6, 12, and/or 24 months of age had received colostrum with higher levels of TGF-β2 than infants that were not sensitized. The 10th, 25th, 50th, 75th, and 90th percentiles are indicated, as well as outliers.

Table 4. Development of sensitization at 6, 12, and 24 months of age in relation to colostrum TGF-β2 levels and Na/K ratios in a logistic regression model controlling for possible confounders, i.e., study treatment (placebo or *L. reuteri*) and maternal atopy

	Crude			Adjusted		
	OR	95% CI	p-Value	OR	95% CI	p-Value
Sensitization at 6 months						
TGF-β2 (700–1400 pg/ml)	1			1		
TGF-β2 (<701 pg/ml)	1.1	0.3–4.4	n.s.	1.3	0.3–6.2	n.s.
TGF-β2 (>1400 pg/ml)	3.3	0.8–13.0	0.09	5.0	0.9–27	0.06
Na/K ratios	1.4	0.8–2.7	n.s.	1.0	0.4–2.3	n.s.
Sensitization at 12 months						
TGF-β2 (700–1400 pg/ml)	1			1		
TGF-β2 (<701 pg/ml)	0.6	0.2–1.8	n.s.	0.6	0.2–1.9	n.s.
TGF-β2 (>1400 pg/ml)	0.99	0.3–3.2	n.s.	1.1	0.3–3.9	n.s.
Na/K ratios	1.1	0.6–2.0	n.s.	0.9	0.5–1.9	n.s.
Sensitization at 24 months						
TGF-β2 (700–1400 pg/ml)	1			1		
TGF-β2 (<701 pg/ml)	0.2	0.1–0.8	0.02	0.3	0.1–0.9	0.04
TGF-β2 (>1400 pg/ml)	1.1	0.3–3.3	n.s.	0.3	0.1–2.2	n.s.
Na/K ratios	3.4	1.4–8.0	0.005	3.7	1.2–12	0.02

In the model analyzing the relationship between TGF-β2 and sensitization, Na/K ratios was added as a potential confounder and in the model analyzing the relationship between Na/K ratios and sensitization, TGF-β2 was added as a potential confounder. The TGF-β2 levels were analyzed as a nominal variable after grouping the data in three groups: <700 pg/ml, 700–1400 pg/ml, and >1400 pg/ml. The range of the groups was based on the level distribution of the different samples. Na/K ratios were included as a continuous variable in the model.

Table 5. The levels (median and 25th–75th percentiles) of TGF-β2 (pg/ml) in colostrum of mothers of infants that at 6, 12, and 24 months had or had not developed IgE-associated eczema

	Median	25th–75th
6 months		
IgE-associated eczema	1182	514–2303
No eczema/no sensitization	780	473–1258
12 months		
IgE-associated eczema	926	515–2257
No eczema/no sensitization	760	473–1358
24 months		
IgE-associated eczema	1056	658–2165
No eczema/no sensitization	697	432–1301

(n = 11, 12, and 13, respectively), possibly explaining the lack of statistical significance. The results remained in a multiple logistic regression model including maternal atopy and study treatment (data not shown).

The Na/K ratio in colostrum correlated with TGF-β2 levels ($\rho = 0.73$, $p < 0.001$).

Discussion

Supplementation with probiotic *L. reuteri* during pregnancy is associated with lower colostral TGF-β2 and increased IL-10 levels. No such association is observed in mature milk collected 1 month after the treatment had ceased. Furthermore, low colostral TGF-β2 is associated with less sensitization and less IgE-associated eczema up to 2 yr of age in the breast-fed infants. Interestingly, we have observed similar findings in another cohort including Estonian and Swedish children followed prospectively from birth up to 5 yr of age. In that study, low breast milk TGF-β2 was associated with less SPT positivity up to 2 yr of age and tended to be correlated with reduced risk of eczema (unpublished data). These findings challenge the general idea of TGF-β as an anti-inflammatory mediator that, at least in animal and cell-line models, suppresses IgE responses (20, 21). It has previously been reported that mothers of infants with non-IgE mediated cow's milk allergy (CMA) have higher colostral levels of TGF-β1 than mothers of infants with IgE-mediated CMA (13). Furthermore, high concentration of TGF-β in colostrum has been associated with post-weaning onset of atopic diseases, whereas low TGF-β was associated with pre-weaning onset (14). In two other

clinical studies, however, there was no clear association between breast milk TGF-β and development of atopic manifestation (15, 22). The colostral TGF-β2 levels in our placebo-treated mothers were of the same magnitude as those previously reported to be present in colostrum (2, 14, 22, 23), while the levels in the *L. reuteri*-treated mothers were much lower. It is possible that any association between breast milk TGF-β and development of atopy could be bell-shaped, where intermediate levels of TGF-β would be associated with an increased risk of sensitization, whereas low and very high levels are associated with a reduced risk, possibly through different mechanisms.

Also high colostral Na/K ratios were associated with increased risk of sensitization. This is in agreement with one of our previous studies, where high breast milk Na/K ratios in atopic mothers were associated with development of positive SPT and symptoms of atopic disease up to 18 months of age (24). Elevated Na/K ratios in breast milk are considered indicative of increased epithelial permeability caused by inflammation and have at the mammary epithelium been reported to correlate well with IL-8 and TGF-β2 levels, i.e., two cytokines with the potential to both promote and down-regulate inflammation (25). Although the breast milk Na/K ratios were not affected by *L. reuteri* supplementation, the TGF-β2 levels and Na/K ratios correlated. This may suggest that the relation between *L. reuteri* supplementation, breast milk TGF-β2 levels, and childhood sensitization is explained by epithelial barrier function.

We have very recently reported that the *L. reuteri* treatment of mothers and their babies is associated with less IgE-associated eczema and sensitization (17). An obvious question is whether the clinical effect really is attributed to the effect of *L. reuteri* on TGF-β2 in colostrum, or if it is because of a separate mechanism associated with the *L. reuteri* supplementation to the infants the first year of life. The relationship between low levels of TGF-β2 in colostrum and subsequent sensitization in the offspring remained in a logistic regression model adjusting for treatment regime and Na/K ratios; however, indicating that one explanatory mechanism to the clinical effect of *L. reuteri* may be via lowering breast milk TGF-β2 levels. The mechanism is unknown, and it is still possible that TGF-β2 only is a marker for a co-existing effect on the mother and, via the mother, on the foetus. Recently, Taylor et al. (26) reported the outcome of an allergy prevention study using probiotic *L. acidophilus*. The results in that study differ significantly from ours and two previous Finnish studies (27, 28), in which sensitization were more common in infants receiving probiotics, whereas the incidence of any eczema was not affected. Besides, the use of a different bacterial strain, their study design differed from ours and the Finnish studies in that the mothers were not supplemented with the study product during pregnancy. Together with our report on the effect of colostrum in the present study, the results of these four prevention studies may therefore suggest that the supplementation to the mothers during late pregnancy is important.

Notably, in contrast to our prevention study, *L. rhamnosus* GG did not have any preventive effect on sensitization in the two Finnish prevention studies, while there was an effect on the incidence of eczema symptoms in their studies but not in ours (27, 28). In a previous Finnish study, supplementation with *L. rhamnosus* GG during pregnancy and lactation was associated with increased TGF-β2 levels in mature breast milk obtained at 3 months after delivery (8), in contrast to our study, in which TGF-β2 was lower in colostrums from mothers in the probiotic group. No direct association between breast milk TGF-β2 levels and development of sensitization or atopic disease in the infant was reported in the Finnish study. The highest levels of TGF-β2 were found in mothers to babies with detectable levels of cord blood IgE (8), however. Since elevated cord blood IgE levels have been identified as a risk factor for later sensitization (29), the results from Rautava and colleagues might, in this respect, point in the same direction as ours.

The different findings in these studies are possibly consequences of different host responses to different strains of lactobacilli (30). Furthermore, bacterial colonization was not confirmed in the study by Rautava et al. The association between *L. reuteri* supplementation and low TGF-β2 in our study was most evident for mothers in whom *L. reuteri* were found in faeces.

The levels of IL-10 in colostrum were slightly higher in the *L. reuteri*, as compared to placebo-treated mothers. *L. reuteri* has been reported to induce IL-10-producing regulatory T cells *in vitro* (31) and an IL-10 inducing capacity by *L. reuteri* supplementation also affecting the levels is possible. The breast milk IL-10 levels were not associated with sensitization or development of eczema in the infants.

None of the other analyzed breast milk parameters, i.e., IgA, SIgA, TGF-β1, TNF, and sCD14 differed between the *L. reuteri* and placebo-treated mothers and were not related to

sensitization or development of eczema in the infants which is in agreement with our previous findings (15).

In conclusion, supplementation with *L. reuteri* during late pregnancy reduces breast milk levels of TGF-β2, and low levels of this cytokine is associated with less sensitization and possibly also less IgE-associated eczema in breast-fed infants.

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