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ORIGINAL PAPER **Clinical Mechanisms in Allergic Disease**

Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-γ and breast milk transforming growth factor-β and immunoglobin A detection

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Clinical and Experimental Allergy

Summary

Background This study explored the effects of maternal probiotic supplementation on immune markers in cord blood (CB) and breast milk.

Methods CB plasma and breast milk samples were collected from a cohort of women who had received daily supplements of either 6×10^9 CFU/day *Lactobacillus rhamnosus* HN001 ($n = 34$), 9×10^9 CFU/day *Bifidobacterium lactis* HN019 ($n = 35$) or a placebo ($n = 36$) beginning 2–5 weeks before delivery and continuing for 6 months in lactating women. CB plasma and breast milk (collected at 3–7 days, 3 months and 6 months postpartum) were assayed for cytokines (IL-13, IFN-γ, IL-6, TNF-α, IL-10, TGF-β1) and sCD14. Breast milk samples were also assayed for total IgA.

Results Neonates of mothers who received a probiotic had higher CB IFN-γ levels ($P = 0.026$), and a higher proportion had detectable blood IFN-γ levels, compared with the placebo group ($P = 0.034$), although levels were undetectable in many infants. While this pattern was evident for both probiotics, when examined separately only the *L. rhamnosus* HN001 group showed statistically significant higher IFN-γ levels ($P = 0.030$) compared with the placebo group. TGF-β1 levels were higher in early breast milk (week 1) from the probiotic groups ($P = 0.028$). This was evident for the *B. lactis* HN019 group ($P = 0.041$) with a parallel trend in the *L. rhamnosus* HN001 group ($P = 0.075$). Similar patterns were seen for breast milk IgA, which was more readily detected in breast milk from both the *B. lactis* HN019 ($P = 0.008$) and the *L. rhamnosus* HN001 group ($P = 0.011$). Neonatal plasma sCD14 levels were lower in the *B. lactis* HN019 group compared with the placebo group ($P = 0.041$).

Conclusion The findings suggest that supplementation with probiotics in pregnancy has the potential to influence fetal immune parameters as well as immunomodulatory factors in breast milk.

Keywords allergic disease, allergy prevention, breast milk, cord blood, cytokines, 'hygiene hypothesis', IgA, infants, probiotics
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Introduction

Conflicting effects of probiotics in allergy prevention have been attributed to differences in strains, dosages, timing and duration of supplementation in addition to many other host and environmental factors. So far, most of the studies reporting potential benefits began supplementation during pregnancy [1–3], whereas studies that failed to show a preventive effect did not [4]. While differences in probiotic strains are likely, there has also been speculation that supplementation in pregnancy may have direct effects on fetal immune development. Supporting this concept, Blumer et al. recently reported that supplementation with *Lactobacillus rhamnosus* GG (LGG)

in pregnant mice was associated with significant difference in placental cytokine expression (with increased TNF-α expression and a trend for lower IL-4 expression) [5]. Offspring of the probiotic-treated group also showed significantly reduced allergic airways inflammation compared with untreated mice [5]. The findings suggest that the beneficial effects of LGG, may at least in part, be mediated via the placenta through induction of pro-inflammatory signals. To our knowledge, the effects of antenatal probiotics supplementation on neonatal immune markers have not been reported in humans.

Maternal supplementation could also have potential effects on the infant through breast milk. In the original study reporting a 50% reduction in atopic dermatitis with LGG [1], breast milk (colostrum) TGF-β levels were higher in supplemented mothers [6], suggesting additional immunomodulatory effects through breast milk. However, the effects on other cytokines and IgA levels were not reported.

The first objective of this study was to examine the effect of maternal probiotic supplementation in pregnancy on neonatal immune markers, namely cytokine production by the feto-placental unit. Other studies have reported associations with cord blood (CB) cytokine levels and the subsequent risk of allergic disease [7]. We have also shown that other dietary interventions can affect neonatal immune function [8, 9], and that this could be detected as difference in CB cytokine levels [10]. These observations were the basis of using the same strategy here.

Secondly, we further explored the effect of maternal supplementation on immune markers in breast milk, including soluble CD14 (sCD14), total IgA and cytokine levels. Again, variations in these parameters have been associated with differences in infant allergic outcomes [11–16] and may be potentially influenced by changes in early microbial exposure [15].

Methods

Study design

This analysis was performed on mother–baby pairs participating in a randomized control trial using probiotics to assess effects on allergy prevention. The study recruited 474 eligible mother–baby pairs and was designed to examine the specific effects of maternal (and subsequently infant) supplementation with *L. rhamnosus* HN001 or *Bifidobacterium lactis* HN019 compared with a placebo. Breast milk and CB samples were available on 105 mother–baby pairs, taking *L. rhamnosus* HN001 ($n = 34$), *B. lactis* HN019 ($n = 35$) or a placebo ($n = 36$), allowing comparative analysis of immune markers in these groups.

Participants and inclusion criteria in the main study

Pregnant women were recruited from Wellington and Auckland. Ethical approval was granted by the regional ethics committees in Wellington and Auckland. Informed consent was obtained from mothers at recruitment. To be eligible for the study, the woman or the infant's biological father had to have been treated for asthma, allergic rhinitis or eczema. Women were ineligible for the study if they planned to move from the study centre in the next 2 years, delivered before 37 weeks gestation or had not taken the study probiotics for $\geqslant 2$ weeks pre-birth. They were also ineligible if they were already taking probiotic supplements long term, or intended to use these in the child. Finally, subjects were then excluded if infants were less than the third percentile for weight or if they had congenital anomalies.

The supplementation

The intervention commenced 2–5 weeks before delivery. Women were randomized to receive daily supplements of either 6×10^9 CFU/day *L. rhamnosus* HN001 (Fonterra NZ, Palmerston North, New Zealand), 9×10^9 CFU/day *B. lactis* HN019 (Fonterra NZ) or a placebo (containing dextran, salt and a yeast extract; Fonterra NZ). This dosage was based on the average counts measured by the manufacturer immediately after encapsulation (although the intended dose of each probiotic had been 10^{10} CFU/capsule).

The capsule powder was either given undiluted to the infant or mixed with water, breast milk or formula and given via a teaspoon or syringe until solid food was started, when it was sprinkled on food. Parents were instructed to give the full dose on each occasion.

Probiotics were produced in a low allergenicity medium. Supplementation was continued in the postnatal period for the mother for up to 6 months if breastfeeding and for the baby, independent of feeding methods, for 2 years. Probiotic and placebo supplements were image-matched, and participants, and research scientists remained blinded to the groups for the duration of the study. Randomization and allocation of supplements occurred at a separate area from participant recruitment by persons independent from the processes. As a measure of compliance, unused capsules were counted by an independent investigator in each centre.

Clinical follow-up

Eczema prevalence was assessed using a modified version of the UK Working Party's Diagnostic Criteria for atopic dermatitis [17], at 3, 6, 12, 18 and 24 months via questionnaire and assessment of visible atopic dermatitis. Atopy was assessed at 24 months using skin prick tests (SPT), and defined as any mean weal diameter $\geqslant 3$ mm to egg white, cat pelt, *Dermatophagoides pteronyssinus*, mixed grasses, peanut or cow's milk.

Sample collection and initial processing

CB samples were collected from the placental vessels by venepuncture immediately after delivery. Plasma samples were generally isolated within 8 h of collection and stored at −80 °C or below for transport and subsequent batch analysis.

Post-colostrum breast milk was collected in the first week and at 3 months and 6 months postpartum. Mothers expressed 5–6 mL of breast milk into sterile containers by manual expression. This was frozen within 30 min and stored at −80 °C or below until analysis. Before analysis (described later), milk was thawed (warmed to 37 °C). Following centrifugation of the samples for 10 min at 7000 *g*, the aqueous milk phase was removed from below the lipid phase (with a needle and syringe) and used for cytokine, sCD14 and IgA analysis.

Cytokine and soluble CD14 detection. CB plasma and breast milk levels of IL-5, IL-6, IL-10, IL-13, TGF-β1, TNF-α and IFN-γ were quantified using time-resolved fluorometry (TRF) (DELPHIA, PerkinElmer, Life Sciences, MA, USA) [8]. Levels of sCD14 and TGF-β1 were measured using a commercial DuoSet ELISA kit (R&D Systems, Minneapolis, USA). Briefly, the TRF method was followed using paired antibodies (Pharmingen, BD, Australia), and the biotinylated antibody was detected using europium-labelled streptavidin. Fluorescence dissociation by the addition of low pH enhancement buffer was quantified using a fluorometer (WALLAC VICTOR2, PerkinElmer, Life Sciences, MA, USA). The detection limit was 2.5 pg/mL for IL-5, IL-6, IL-10, IL-13 and IFN-γ, 30 pg/mL for TGF-β1 and 6 pg/mL for TNF-α. For the Quantikine Human sCD14 Immunoassay (R&D Systems), the lower detection limit of the ELISA was 60 pg/mL.

Measurement of total immunoglobulin A. Total IgA levels in breast milk were measured using a conventional ELISA (Vasse Research Institute, Newcastle, Australia). An anti-human IgA antibody directed against the α chain (Silenus Anti-Human IgA, Melbourne, Australia) was used as capture antibody, enabling detection of all IgA, including monomeric, polymeric and secretory IgA. Samples were diluted 1/2000 in PBS/Tween before the ELISA was performed. Detection was performed using the peroxidase substrate tetramethylbenzidine (TMB; Roche Diagnostics, Castle Hill, Australia). The reaction was stopped (1 M H_3PO_4) and absorbances detected at 450 nm wavelength. The detection limit was 5 mg/mL.

Data analysis

Cytokine data were analysed as continuous data, described by the median and interquartile range, and as dichotomous data (detected or not detected). For categorical determinations, cytokine levels were defined as clearly 'detectable' if they were twice the level of the 'detection limit' of the assay. Differences between the groups were determined by Mann–Whitney test for non-parametric data. Differences between the groups for dichotomous data were determined by Chi-square (or Fisher's exact test). Correlations were assessed using Spearman or Kendall's tau (τ) b (where a proportion of the variables of interest shared 'zero' values) in order to avoid the problems associated with 'ties' within the data. Factors with potential confounding effects were tested by these correlation analyses. All statistical analyses were performed using SPSS software (version 11 for Macintosh). A P-value < 0.05 was considered statistically significant for all analyses.

Results

Population characteristics

There were no significant differences in the maternal characteristics of the groups (including ethnicity, age, allergic status or income). There were also no differences in birth weight or gestational age of the infants.

While the caesarean delivery rate tended to be higher in the *L. rhamnosus* HN001 (35%) and the *B. lactis* HN019 (43%) groups compared with placebo group (25%), this was not statistically significant ($P = 0.28$). However, although caesarean delivery tended to be more common in the probiotic groups, this was not correlated with cytokine levels in this population. There were also no significant differences in the immune markers of neonates requiring special care neonatal admission (8% of placebo group, 12% of the *L. rhamnosus* HN001 group and 0% of the *B. lactis* HN019 group).

There was no significant difference in the duration of breastfeeding between study groups (75.8% in the *B. lactis* group, 80% in the *L. rhamnosus* group and 71.9% in the placebo group) breastfed for 6 months or more.

The effect of maternal probiotics supplementation on neonatal cytokine levels

The cytokine levels detected in CB are shown in Fig. 1. Although IFN-γ levels were only at low levels, neonates whose mothers had received a probiotic had higher CB IFN-γ levels ($P = 0.026$; Fig. 1a). Most of this effect was due to higher levels in the *L. rhamnosus* HN001 group ($P = 0.030$) compared with the placebo group, and although neonates in the *B. lactis* HN019 group also had detectable levels, this was not statistically significant from the placebo group ($P = 0.206$).

A greater proportion of neonates whose mothers had received a probiotic (combined groups) had detectable levels of IFN-γ ($P = 0.034$) than the placebo group, whose

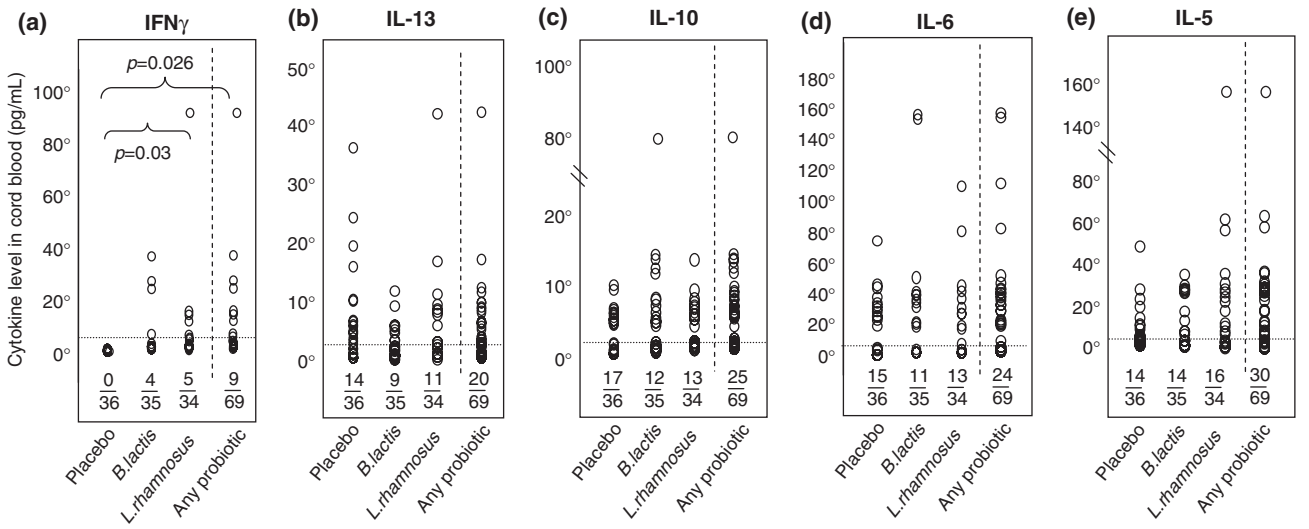

Fig. 1. Comparison of cord blood (CB) cytokine levels in the study groups. The neonatal cytokine levels (detected in CB) are shown for (a) IFN-γ, (b) IL-13, (c) IL-10, (d) IL-6 and (e) IL-5 comparing those whose mothers had received placebo, *B. lactis* HN019, *L. rhamnosus* HN001 or any probiotics (combined groups) as shown. Groups were compared by Mann–Whitney *U*-test. The fractions with detectable levels are shown at the bottom of each plot.

infants all had undetectable IFN-γ levels (<5 pg/mL). When the two probiotic groups were assessed separately, IFN-γ was detected more readily in both the *L. rhamnosus* HN001 group (11%, $P = 0.051$) and the *B. lactis* HN019 group (12%, $P = 0.054$) compared with 0% in the placebo group. Notably, most neonates had levels below detection.

There were no significant effects of probiotic administration on the level or the frequency of detection of any other cytokines in CB (Fig. 1) including TGF-β1 ($P = 0.21$; data not shown). There were also no significant differences in the levels between the *L. rhamnosus* and the *B. lactis* groups.

The effect of maternal probiotics supplementation on cord blood soluble CD14 levels

sCD14 levels in CB plasma were significantly lower in neonates whose mothers had received the *B. lactis* HN019 compared with the placebo group ($P = 0.045$; Fig. 2). There was a similar trend for all neonates whose mothers received probiotics ($P = 0.065$, for combined groups).

The effect of maternal probiotics supplementation on breast milk immunoglobulin A levels

Mothers in the probiotic groups were significantly more likely (88%) to have any detectable IgA in early breast milk samples compared with the placebo group (61%) ($P = 0.005$). This was evident for both the *B. lactis* HN019 group (89% detection, $P = 0.008$) and *L. rhamnosus* HN001 group (88% detection, $P = 0.011$) as shown in Fig. 3a. A similar effect was also evident at 3 months, with increased detection of breast milk IgA in the women collectively on probiotics (79%, $P = 0.035$) compared with the placebo group (66%), although this was largely due to effects in the *B. lactis* HN019 group (89%, $P = 0.027$) and not the *L. rhamnosus* HN001 group (70%, $P = 0.7$). Although the women on probiotics (combined groups) were more likely to have detectable IgA (65%, $P = 0.035$) compared with the placebo group (44%) at 6 months, this was not statistically significant for either the *B. lactis* or the *L. rhamnosus* groups examined alone (Fig. 3a).

Mothers who had been on a probiotic also tended to have higher absolute IgA levels in early breast milk ($P = 0.06$) compared with the placebo group. This was seen for both the *B. lactis* HN019 group ($P = 0.07$) and *L. rhamnosus* HN001 group ($P = 0.08$) as shown in Fig. 3b. IgA levels declined over the course of lactation ($P < 0.001$), and this was seen in all the groups. At 3 months, breast milk IgA was higher in the *B. lactis* HN019 group compared with the placebo group ($P = 0.06$) and the *L. rhamnosus* HN001 group ($P = 0.005$).

Maternal history of allergic disease ($n = 81$) was not associated with any significant differences in the levels of cytokines or sCD14 in CB, compared with non-allergic women ($n = 14$). The only difference in breast milk parameters was for lower sCD14 levels in the breast milk of allergic women in the first week of life ($P = 0.022$).

The effect of maternal probiotics supplementation on breast milk cytokine and soluble CD14 levels

Over the course of lactation, there was a significant decline in all cytokines measured, including TGF-β1 ($P < 0.001$), IL-6 ($P < 0.001$), IL-10 ($P < 0.001$), TNF-α ($P < 0.001$) and IL-5 ($P = 0.004$) (data not shown). Breast milk levels of all cytokines at 3 months remained

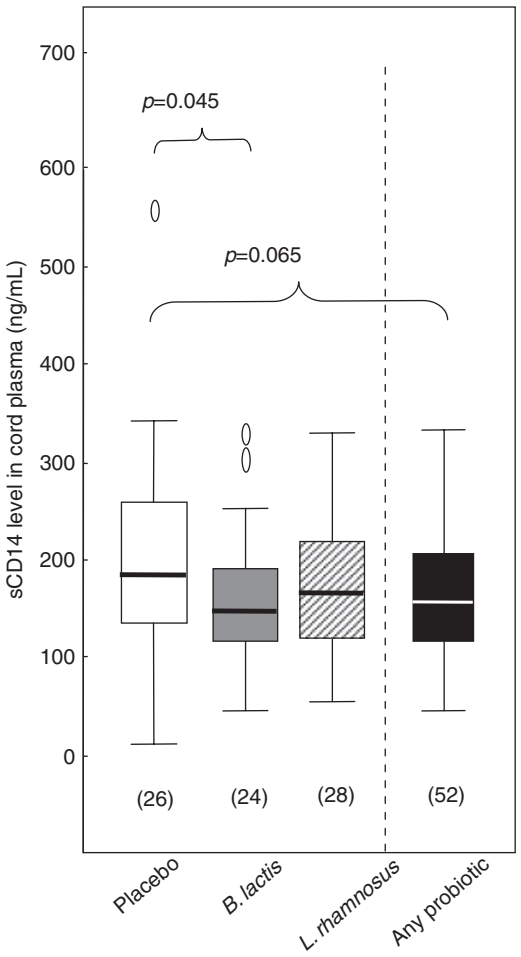

Fig. 2. Comparison of soluble CD14 levels in neonatal plasma. Detection of sCD14 in cord blood plasma is shown for the placebo group ($n = 26$, clear bar), the *B. lactis* HN019 group ($n = 24$, grey bar) and the *L. rhamnosus* HN001 group ($n = 28$, crosshatched bar) and both probiotic groups combined ($n = 52$, black bar). The data are shown as median, interquartile ranges, 95% confidence intervals and outlying values, and groups were compared by Mann–Whitney *U*-test.

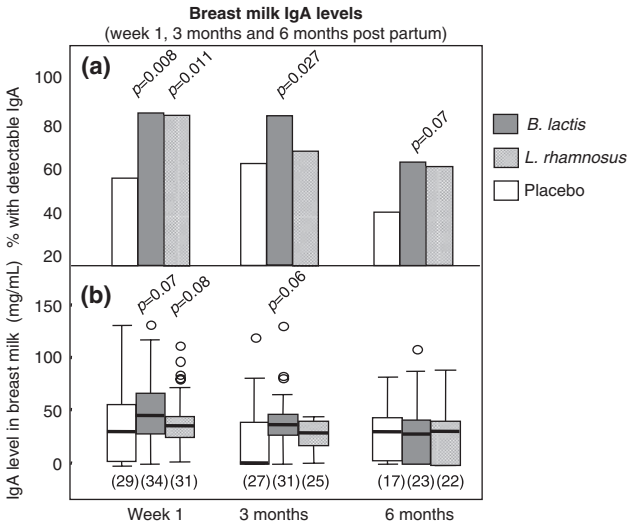

Fig. 3. Comparison of breast milk IgA levels in study groups. Detection of IgA in breast milk at 1 week, 3 months and 6 months is displayed for women in the placebo group (clear bars), the *B. lactis* HN019 group (grey bars) and the *L. rhamnosus* HN001 group (crosshatched bars). The proportion of subjects (%) with detectable IgA is shown in part (a) and groups are compared with chi-square tests. The IgA levels are shown in part (b), and groups were compared by Mann–Whitney *U*-test. The numbers or samples available at each time-point are shown in parentheses. All significance levels are shown in relation to the placebo group.

correlated with levels in the week postpartum, and this was strongest for TGF-β1 ($r = 0.5$, $P < 0.001$).

As noted in Fig. 4, women who had received a probiotic had significantly higher TGF-β1 levels than those in the placebo group ($P = 0.028$). This was most evident in the *B. lactis* HN019 group ($P = 0.041$) with similar trend in the *L. rhamnosus* HN001 group ($P = 0.075$). Significantly more women who received probiotics had detectable IL-6 levels in early breast milk ($P = 0.04$), and there was a trend for higher levels of this cytokine in breast milk of women who received the *B. lactis* HN019 ($P = 0.06$) (Fig. 4). There were no significant differences in breast milk cytokine levels detected at 3 and 6 months of lactation between the groups, even though breastfeeding women continued taking probiotic supplements for 6 months in the postnatal period (data not shown). There were also no significant differences in breast milk sCD14 levels at any of the time-points assessed (data not shown).

TGF-β1 levels were significantly correlated with IgA (at 3 months: $\tau = 0.23$, $P = 0.003$ and at 6 months: $\tau = 0.25$, $P = 0.004$), but not in the early milk sample (week 1). In early milk, IgA levels were significantly correlated with IL-6 ($\tau = 0.30$, $P < 0.001$) but not other cytokines. There were also consistent relationships between cytokine levels in breast milk. Specifically, TGF-β1 levels were positively correlated with IL-10 ($\tau = 0.20$, $P = 0.008$) and IL-6 ($\tau = 0.30$, $P < 0.001$) levels but negatively correlated with IL-5 ($\tau = -0.20$, $P = 0.03$).

The relationship between cord blood and breast milk immune markers and allergy

In this study population, 96 children were assessed for allergic outcomes at 2 years of age and 93 of these had allergy SPT to assess for atopy. Of these children, 21/96 (21.8%) developed eczema and 20/93 (21.5%) had allergen sensitization. The numbers in this analysis are not sufficient to determine the effect of probiotics on clinical outcomes, which are published separately for the full cohort of 474 children [18]. However, in keeping with the significant reduction of atopic dermatitis with *L. rhamnosus* HN001 in the full study population, there was a trend for lower prevalence of atopic dermatitis in the children who received the *L. rhamnosus* HN001 (4/30; 13.3%)

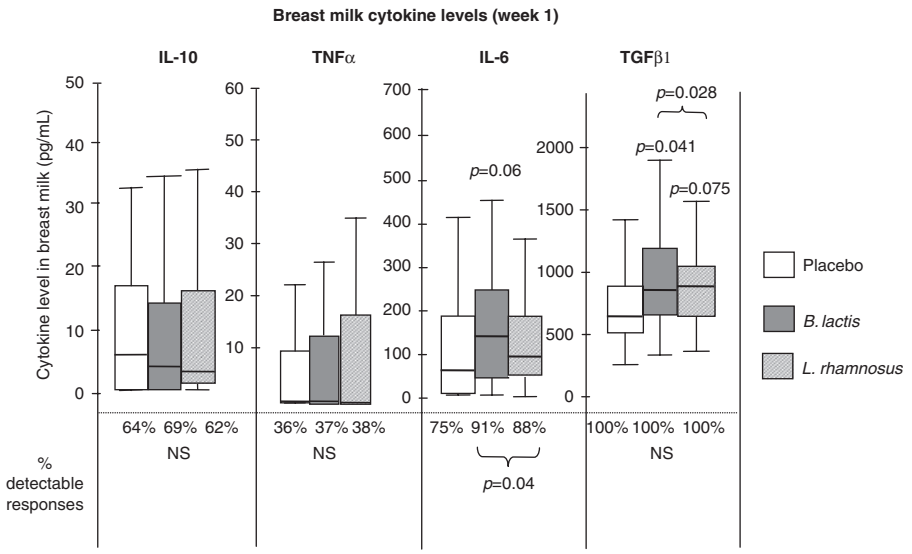

Fig. 4. Comparison of breast milk cytokine levels in study groups. The levels of IL-10, TNF-α and IL-6 are shown in breast milk samples from women in the placebo group ($n = 36$, clear bars), the *B. lactis* HN019 group ($n = 35$, grey bars) and the *L. rhamnosus* HN001 group ($n = 34$, crosshatched bars). The data are shown as median, interquartile ranges and 95% confidence intervals, and groups were compared by Mann–Whitney *U*-test. The proportion (%) with detectable levels is also shown (and compared with chi-square test). All significance levels are all shown in relation to the placebo group, including where both probiotic groups were combined for comparison with the placebo.

compared with the control group (9/33; 27.3%) and the *B. lactis* HN019 group (8/33; 24.2%), although this was not statistically significant ($P = 0.146$ and 0.219, respectively).

The development of atopic dermatitis in the first 2 years of life was associated with significantly higher levels of IL-13 ($P = 0.003$) and IL-10 ($P = 0.031$) in CB compared with children who did not develop atopic dermatitis (Fig. 5a). In logistic regression models, these relationships were independent of probiotic supplementation (for IL-13: Exp(B) = 1.13; 95% confidence intervals [CI] 1.02–1.24; $P = 0.015$ and for IL-10: Exp(B) = 1.17; 95% CI 1.01–1.35; $P = 0.035$). There were no significant relationships between CB cytokine levels and subsequent SPT sensitization, although there was a similar trend for higher IL-13 levels in the children who developed subsequent sensitization ($P = 0.057$) (Fig. 5b). Although IFN-γ was detected in fewer children who subsequently developed eczema or sensitization, this was not statistically significant.

There were no correlations between neonatal (or breast milk) sCD14 levels and subsequent allergic outcomes in this population (data not shown). There were also no relationships between breast milk cytokine or IgA levels and subsequent allergic outcomes (data not shown).

Discussion

The novel observations in this study are that maternal probiotic supplementation in pregnancy resulted in higher IFN-γ levels in CB and increased detection of IgA and cytokines (TGF-β1) in breast milk. Although IFN-γ was below detection in most neonates, the only ones that had detectable levels were in the probiotics groups. In other studies, detectable levels of IFN-γ in CB have been shown to have a protective relationship with allergic outcomes, with lower rates of subsequent asthma, wheezing and sensitization at 6 years of age [7]. The detection of other CB cytokines produced by the feto-placental unit (including TNF-α and Th2 IL-4) has also been associated with lower risk of subsequent atopy [7]. In the same study, adverse maternal exposures in pregnancy such as smoking unfavourably affected these relationships (by decreasing cytokine levels and increasing disease risk). These observations suggest that environmental factors can modulate immune function in the feto-placental unit and alter subsequent disease risk. This is highly relevant for defining how environmental modification may be of value in the prevention of disease.

With the advent of the 'hygiene hypothesis', interest in the protective effects of early microbial exposure has focused mainly on the postnatal period when infant colonization normally occurs. However, more recent studies suggest that the maternal microbial environment in pregnancy may also have significant influences on immune programming. In population studies, maternal exposure to an environment rich in microbial compounds during pregnancy has been shown to protect offspring against the development of atopic sensitization [19], suggesting that microbial exposure *in utero* can have immunomodulatory effects on the fetus. Animal models also demonstrate that administration of microbial products (endotoxin [20] and probiotics [5]) in pregnancy can prevent allergic airways disease in the offspring, with

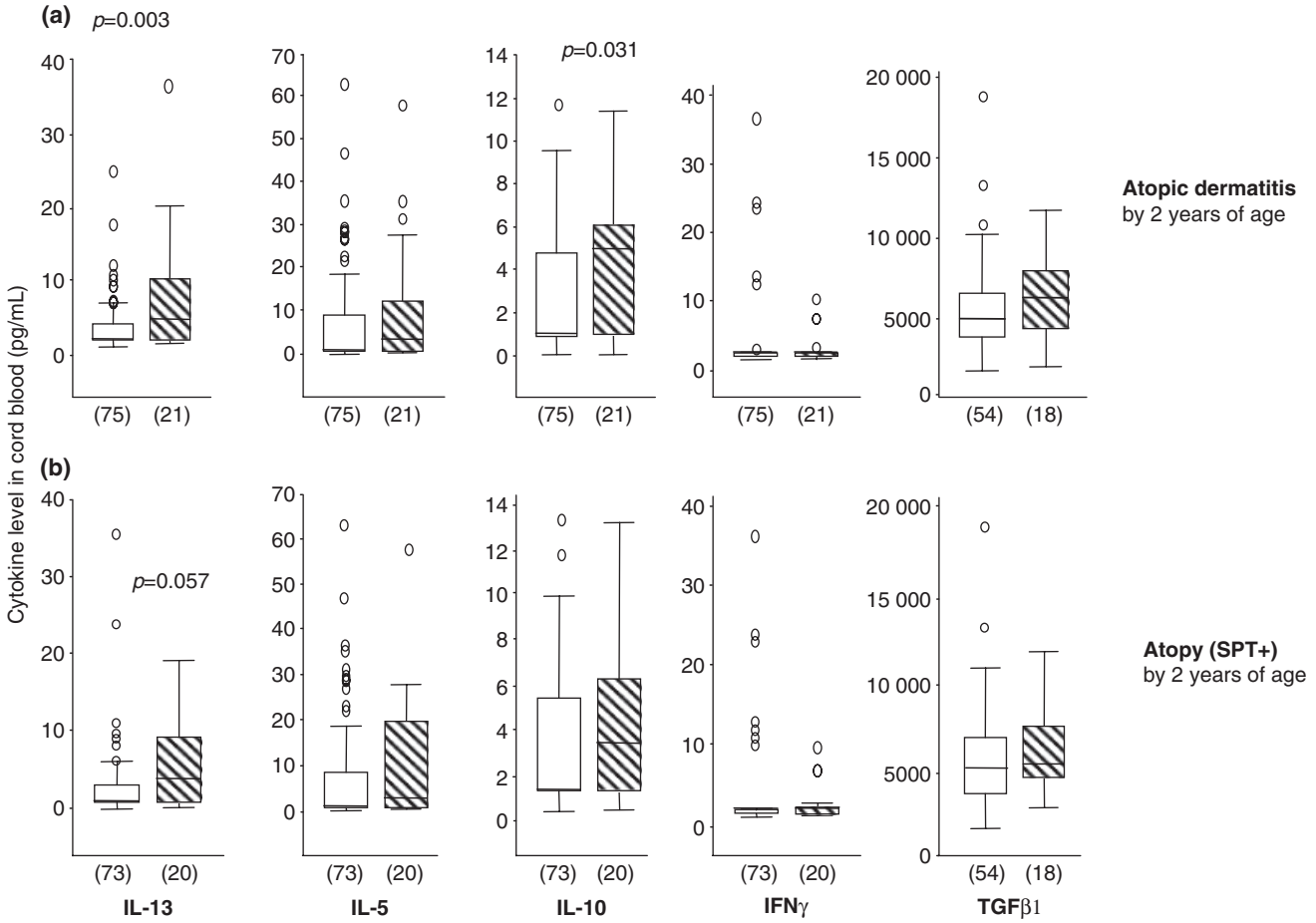

Fig. 5. Comparison of cord blood (CB) cytokine levels in relation to subsequent allergy outcomes. The levels of IL-13, IL-5, IL-10, IFN-γ and TGF-β1 are shown in CB samples from newborns who subsequently developed (a) atopic dermatitis or (b) allergen sensitization (shaded bars) compared with infants who did not develop these clinical outcomes (clear bars). The data are shown as median, interquartile ranges, 95% confidence intervals and outlying values, and groups were compared by Mann–Whitney *U*-test.

associated effects on immune function. In one of these studies, probiotics were shown to increase the expression of placental TNF-α [5] consistent with the protective relationship between neonatal TNF-α and allergic risk in other studies [7]. In humans, exposure to pathogenic flora (intrauterine infection) has been shown to increase neonatal IFN-γ production [21], but the effects of non-pathogenic commensal flora have not been documented in this context.

The role of probiotics in allergy prevention is also unclear despite a recent meta-analysis documenting a reduction in the relative risk of atopic dermatitis (RR 0.66; 95% CI 0.49–0.89) following supplementation under various conditions. No benefits have been shown for other allergic outcomes. While some studies suggest a protective effect of another *L. rhamnosus* strain (LGG) either alone [1, 22] or in combination with other probiotic strains or prebiotics [2], at least two more recent studies not included in the meta-analysis found no benefits using *L. rhamnosus* (including [23] and another study yet to be published). Studies using other probiotics have found no benefits (*Lactobacillus acidophilus*) [4] or possible effects only in sub-analyses (*Lactobacillus reuteri*) [3]. There has been speculation [24, 25] that the lack of benefit seen in the study by Taylor et al. [4] could have been due to the lack of antenatal supplementation in that study. However, despite animal studies showing antenatal effects of LGG [5], at least one other human study saw no effect of LGG despite antenatal supplementation [23]. As recently reviewed [26], there may be many genetic and environmental factors contributing to these conflicting findings.

On this background of uncertainty, the present intervention trial is the first non-Nordic study to show a clinical benefit of *L. rhamnosus* in allergy prevention, with a statistically significant reduction in atopic dermatitis (Wickens et al., submitted for publication). Notably, the same trends were seen in the present sub-population where the rate of atopic dermatitis was twofold lower in the *L. rhamnosus* HN001 group compared with the

placebo group. Here, we provide evidence that maternal probiotics in human pregnancies can increase IFN-γ production by the feto-placental unit (albeit in only a small proportion of babies). Although CB cytokine levels (higher IL-13 and IL-10) were associated with subsequent atopic dermatitis at 2 years, these were not the same parameters that were modifed by probiotics in this population. However, there is mounting evidence from other studies that increased neonatal production of IFN-γ in this period is associated with lower allergic risk [27–32]. It is also notable that when the probiotic groups were examined separately, only the *L. rhamnosus* HN001 group showed significantly higher IFN-γ levels compared with the placebo group. Although sample size limited the ability to examine the effects of supplementation on clinical outcomes in this sub-population, it is of note that the clinical benefits in the larger population were also only seen with this strain [18].

Another key finding in this paper was that probiotic supplementation was associated with increased TGF-β1 and IgA in breast milk. This is consistent with the observations of Isolauri and co-workers, who observed higher TGF-β levels in the breast milk of Finnish women receiving a different *L. rhamnosus* strain for 4 weeks before birth [6]. More recent animal studies suggest that TGF-β in maternal milk is a critical factor in the development of allergen-specific tolerance [33]. While both probiotics had a similar pattern of effect on breast milk IgA and TGF-β1, it is notable that the *B. lactis* had more significant effects in the absence of any clinical effect in the larger study population (or this subgroup). This indicates that apparent immunological effects may not lead to clinical benefits, and that other pathways may be involved in the preventive effects of *L. rhamnosus* HN001. The first study to show effects of probiotics on breast milk showed increased TGF-β in mature milk (at 3 months of age), but this was only for the TGF-β2 isoform with no difference in TGF-β1 [6]. The differences between probiotic strains are further highlighted in the recent study by Böttcher et al., which showed that *L. reuteri* supplementation reduced rather than increased TGF-β2 in breast milk, but it has no effect on IgA, TGF-β1 or other cytokines [25]. There has been speculation that higher levels of IgA antibodies in colostrum and human milk may prevent antigen entry at the intestinal surface of the breast-fed infant and reduce the risk of allergic disease [34]. In the present study, we did not see any significant relationships between breast milk IgA (or cytokine levels) and allergic outcomes.

The significance of decreased neonatal plasma sCD14 levels in the *B. lactis* HN019 probiotic group is not clear. sCD14 was not associated with allergic outcomes in this study, whereas other studies have shown protective relationships between sCD14 (in amniotic fluid and breast milk but nor neonatal plasma) and subsequent infant allergy [16].

In conclusion, this study demonstrates that beginning probiotic supplementation in pregnancy may have effects through a number of pathways. While the most obvious potential avenue of effect is through maternal–infant colonization, the findings here suggest that maternal supplementation could also have (a) antenatal effects on foetal immune function and (b) potential postnatal influences through immunomodulators in breast milk. There are currently a number of probiotic intervention studies (still in progress) that have also included an antenatal supplementation, including one unique study (by Tang and co-workers, Melbourne, Australia) that has only examined the effect of antenatal supplementation (without continued postnatal intervention) (summarized in [26]). It is likely that these studies will clarify the role of maternal probiotics supplementation in allergy prevention and the mechanisms of microbial interactions between the mother and infant.

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