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Effects of probiotic supplementation for the first 6 months of life on allergen- and vaccine-specific immune responses

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Summary

Background A reduction in microbial burden during infancy when allergen-specific memory is evolving has become a prominent explanation for the allergy epidemic.

Objective We sought to determine whether probiotic dietary supplementation in the first 6 months of life could modify allergen- and vaccine-specific immune responses.

Methods Two hundred and thirty-one pregnant women with a history of allergic disease and positive allergen skin prick test (SPT) were recruited into a randomized-controlled trial. The infants received either a probiotic (3×10^9 *Lactobacillus acidophilus* LAVRI-A1; Probiomix) or placebo (maltodextrin alone) daily for the first 6 months of life, given independent of feeding methods. One hundred and seventy-eight children completed the study; blood samples were available from 60 children in the placebo group and 58 children in the probiotic group. Infant cytokine (IL-5, IL-6, IL-10, IL-13, TNF- α or TGF- β) responses to tetanus toxoid (TT), house dust mite (HDM), ovalbumin (OVA), β -lactoglobulin (BLG), *Staphylococcus enterotoxin B* (SEB) and phytohaemagglutinin (PHA) were measured at 6 months of age.

Results Children who received the probiotics showed reduced production of IL-5 and TGF- β in response to polyclonal (SEB) stimulation ($P = 0.044$ and 0.015 , respectively). They also demonstrated significantly lower IL-10 responses to TT vaccine antigen compared with the placebo group ($P = 0.03$), and this was not due to any differences in vaccination. However, there were no significant effects of probiotics on either Type 1 (Th1) or Type 2 (Th2) T helper cell responses to allergens or other stimuli. The only other effects observed were for reduced TNF- α and IL-10 responsiveness to HDM allergens in children receiving probiotics ($P = 0.046$ and 0.014 , respectively).

Conclusions In summary, although we did not see any consistent effects on allergen-specific responses, our study suggests that probiotics may have immunomodulatory effects on vaccine responses. The significance and clinical relevance of this need to be determined in further studies.

Keywords allergen, allergy, cytokine, IL-10, infant, probiotics, tetanus, vaccine

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Introduction

The development of allergic disease is frequently associated with very early pre-symptomatic differences in allergen-specific cytokine production [1–3]. Children who develop atopy by 12 months of age have been shown to already have significantly stronger Type 2 (Th2) helper T cell responses to allergens and mitogens at 6 months of age [3]. Similarly, impaired Type 1 (Th1) IFN- γ responses in the first months of life have also been observed in children who develop inhalant sensitization by 6 years of

age [4]. This suggests that early events are critical in immune programming and highlights the potential importance of environmental influences in this period. We hypothesize that early interventions during a 'critical early window' of immune development could alter the risk of allergen sensitization and associated disease.

Bacterial exposure is one of the most significant environmental influences during this period. This is essential for normal immune development (reviewed in [5, 6]) and appears particularly important for normal post-natal maturation of antigen-presenting cells (APC), T regulatory

cell function [7] and effector Th1 responses. It has been logically proposed that microbial activation of these pathways plays a central role in reducing the risk of Th2-mediated allergic responses ([8, 9] and others). This is of most relevance in early life when immune programming is initiated.

Intestinal flora are the most abundant source of early microbial exposure and immune stimulation. A number of studies [10–14] have shown differences in colonization patterns of infants who go on to develop allergic disease (reviewed by [15]). These differences were already apparent at 1 week of age, strongly suggesting that early colonization can influence subsequent patterns of immune development. To date, there has only been one published study to assess the effects of probiotic administration during this early critical period on the prevention of allergic disease [16, 17]. Although this study did not examine the effects on cellular immune responses as we do here, they noted that administration of probiotics (*Lactobacillus rhamnosus* GG, given for the last month of pregnancy and the first 6 months of life) was associated with a 50% reduction in atopic dermatitis (AD) at 2 years of age [16] and subsequently at 4 years of age [17]. Despite this, there were no effects on allergen sensitization as detected by specific IgE. This suggests that the protective effects were not mediated through effects on allergen-specific responses, although this was not directly assessed. To address this, we performed a randomized-controlled trial to assess the effects of probiotic supplementation in the first 6 months of life on infant allergen-specific immune responses. Although food allergens are most relevant at this age, we also examined effects on early inhalant responses because we have previously noted pre-symptomatic inhalant (house dust mite (HDM)) responses at this age. We also examined the effects on vaccine antigen responses (namely tetanus toxoid (TT)), because exposure to these antigens in the first 6 months is routine, standardized and readily documented. Furthermore, our collaborators have demonstrated that functional variations in vaccine responses are a useful guide to maturation status at that age [18]. To our knowledge, this is the first study to address the effects of probiotics on these functional aspects of cellular immune responses.

Methods

Study design

We used a randomized double-blind, placebo-controlled design to assess the effect of probiotic supplementation for the first 6 months of life on cellular immune development at 6 months of age.

Peripheral blood mononuclear cell (PBMC) responses to food (ovalbumin (OVA) and β -lactoglobulin (BLG)) inhalant allergens (HDM as whole mite extract, and Der p 2

and Der p 7 peptides) and vaccine antigen (TT) were compared in infants who had received either a probiotic (*Lactobacillus acidophilus* LAVRI-A1 in maltodextrin) ($n = 89$) or placebo (maltodextrin alone) ($n = 89$) supplementation in the first 6 months of life. The number of subjects in this study was based on power calculations determined as being sufficient for the detection of a 20% decline in the clinical expression of atopic disease (as part of ongoing studies). Based on previous studies [19, 20], these numbers allow the detection of differences in immunological responses between the groups (power over 95% for all cytokines).

Study population

We recruited 231 pregnant, allergic women delivering in the Perth, Western Australia metropolitan area between July 2002 and March 2005. Allergic women were recruited as their unborn children were considered to be at high risk of developing allergic disease. Maternal allergy was defined as a doctor-diagnosed clinical history of asthma, allergic rhinitis or eczema and a positive skin prick test (SPT) to one or more common allergens (HDM; grass pollens, cat pelt, dog hair, feathers, moulds, and cockroach extracts). A weal size of ≥ 3 mm above the negative control was considered positive. Women were ineligible for the study if they smoked; had other medical problems or pregnancy complications; delivered before 37 weeks gestation; or were taking probiotic supplements themselves.

Supplementation

Group allocation occurred by block randomization, with stratification according to maternal allergy (asthma vs. other allergy), parity (first child vs. two or more children) and paternal allergy (allergic vs. non-allergic). Infants allocated to the probiotic group received 3×10^9 *L. acidophilus* LAVRI-A1 in maltodextrin (Probiomix, Sydney, Australia) daily, while those in the control group received maltodextrin alone. The products were matched in taste and appearance. Supplements were supplied as stable FD powder (in sachets) and reconstituted in 1–2 mL sterile water and administered orally via a syringe. The supplement was administered daily for the first 6 months of life, independent of feeding methods.

L. acidophilus LAVRI-A1 conformed to the FAO/WHO expert panel guidelines for probiotics (resistance to acid and bile; adherence to cells of the intestinal epithelium and colonization in the intestinal tract; antagonistic activity towards enteric pathogens and maintenance of strain identity and viability throughout shelf-life) [21, 22], as tested and verified by the commercial entity Probiomix. Preliminary studies in animals have also shown that co-administration of LAVRI-A1 with allergen is associated with down-regulation of immediate hypersensitivity reactions [23].

Cell preparation and culture

Where possible, peripheral blood was collected when the infants were 6 months of age and samples were placed into heparinized tubes. Samples were available from 118 children (60 in the placebo group and 58 in the probiotic group). Plasma was isolated and the remaining sample was reconstituted with RPMI (Life Technology, Paisley, UK) tissue culture medium. PBMC were isolated within 2 h using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis of allergen-specific cytokine responses. It has been previously shown that cryopreservation and thawing do not distort the pattern of MC cellular immune responses [24, 25].

For cytokine analysis, 2×10^6 PBMC/mL were cultured in duplicate in 96-well round-bottom plates in AIM V (Gibco, Life Technology Ltd., Paisley, Scotland) serum-free medium for 48 h without stimuli (control) or with (a) allergens, including HDM allergens [as whole mite extract 20 µg/mL (CSL, Melbourne, Australia), or Der p 2 10 µg/mL and Der p 7 10 µg/mL peptides (Der p 2 and Der p 7 allergens were kindly supplied by Professor Wayne Thomas, Molecular Biotechnology Department of ICHR, Perth, Australia)], OVA 100 µg/mL (Sigma, Castle Hill, Australia), BLG 100 µg/mL (Sigma), (b) vaccine antigen TT 0.5Lf (CSL, Australia), (c) *Staphylococcus enterotoxin B* (SEB) (Sigma) 200 ng/mL or (d) phytohaemagglutinin (PHA) mitogen 1 µg/mL (HA16, Murex, Biotech Ltd., Dartford, UK). For SEB and PHA stimulation, a concentration of 1×10^6 PBMC/mL was used. These plates were then placed in a 37 °C 5% CO₂ incubator for 48 h. After culture, the supernatants were collected and stored at –20 °C for cytokine analysis by ELISA or time-resolved fluorometry (TRF).

Cytokine assays

Supernatants were analysed for IL-5, IL-6, IL-10, IL-13, INF-γ and TNF-α using an in-house ELISA with a TRF detection system (DELPHIA, PerkinElmer, Life Sciences, MA, USA). Briefly, the ELISA method was followed using paired antibodies (Pharmingen, Sydney, NSW, Australia) and the biotinylated secondary antibody was detected using Europium-labelled streptavidin, and fluorescence was quantified using a fluorometer (WALLAC VICTOR2, PerkinElmer). Standard curves generated using serial dilutions of recombinant human IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α (Pharmingen) were linear between 3 and 30 000 pg/mL. Cytokine data are expressed as the difference between the stimulated culture and control (pg/mL). The limit of detection was 3 pg/mL for all cytokines. TGF-β was determined using an ELISA kit (R&D Systems Europe, Abingdon, UK) for detection of latent TGF-β1. The limit of detection was 3.9 pg/mL. TGF-β cytokine data are expressed as the difference between the stimulated culture and control (pg/mL).

Stool analysis

Stool samples were collected 1 month after commencing the supplement for enumeration of Lactobacilli, bifidobacteria and coliforms as follows: 10-fold diluted homogenates of the stool samples (w/v) in half-strength Wilkin–Chalgren broth (Oxoid, Unipath Ltd., Basingstoke, UK) were prepared by thawing the frozen samples in an anaerobic chamber and carrying out the homogenization in the chamber. Homogenates were serially diluted in 10-fold steps and aliquots (10 µL) were drop inoculated on MRS, Prionic acid agar or MacConkey agar for lactobacilli, bifidobacteria and coliforms, respectively. After incubation at 37 °C for 48 h for lactobacilli and bifidobacteria, and 24 h for coliforms, the colonies were counted and results were expressed as the number of colonies per gram wet weight of faeces (CFU/g). There were 106 infants with available stool and PBMC samples to assess the association between colonization and innate immune function: 54 in the placebo and 52 in the probiotic group.

Analysis

Cytokine data were analysed as (a) continuous data and (b) dichotomous data (detected or non-detected). Where the data could be normalized with logistic transformation, they are displayed as geometric mean and 95% confidence intervals, and differences between the groups were assessed by student's *t* test. Where this was not possible, the data are displayed as median and inter-quartile range and compared using non-parametric tests (Mann–Whitney *U*-test). Differences between the groups for dichotomous data were determined by χ^2 . Correlations were assessed using Spearman or Kendall's 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. The effects of potential confounding factors on the relationship between 'group' and continuous log-transformed cytokine data were determined by linear regression. All statistical analyses were performed using SPSS software (version 11.0 for Mac OS X). A *P*-value of < 0.05 was considered statistically significant for all analyses. Because of the exploratory nature of this study, we did not wish to exclude any important relationships by using stringent correction factors for multiple analyses. However, we recognize the potential for type 1 error, and data have been interpreted conservatively in this context.

Ethics

Ethical approval for the study was granted by Princess Margaret Hospital for Children (PMH), King Edward Memorial Hospital (KEMH), St John of God Hospital, Subiaco (SJOG) and Mercy Hospital, Mt. Lawley ethics committees, and all women gave written informed consent.

Results

Characteristics of the population

Two hundred and thirty-one women were initially recruited into the study. Of these, 178 mothers and their healthy full-term infants completed the study: 89 in the probiotic group and 89 in the control group. There was no difference in the dropout rates between groups. Reasons for withdrawal included (a) pre-mature delivery ($n = 4$), (b) supplements not started within the first 48 h ($n = 6$), (c) women found the study too demanding ($n = 31$), (d) colic or abdominal discomfort in the baby ($n = 4$), (e) post-natal depression ($n = 2$), (f) infant refusing to take supplement ($n = 1$), (g) unrelated infant health problems ($n = 4$) and (h) unstated reasons ($n = 1$). Blood samples were available from 118 children (60 in the placebo group and 58 in the probiotic group), which were used in this study. The characteristics of these children are shown in Table 1.

There were no significant differences in maternal age, maternal atopic status, parity or paternal atopic status between the probiotic and control groups at the time of randomization or in those who completed the study (data not shown). There were no significant differences in gestational age, birth weight, birth length, head circumference, Apgar scores or gender between the neonates in the two groups. No differences were seen between the groups in relation to breastfeeding, use of formula milk, age of solid introduction, antibiotic usage or vaccination rates (discussed further below) between the probiotic and control groups (Table 1).

Compliance was monitored by use of a sachet chart (completed by parents) and sachet counts (returned sachets counted by the pharmacy department). Compliance was good, with approximately 80% of doses administered and no difference between the groups ($P = 0.607$).

Effects of probiotic supplementation on vaccine-specific responses

Firstly, we examined tetanus vaccine responses because this is a well-defined and virtually universal exposure during the first 6 months of age. Australian children receive TT at 2, 4 and 6 months of age as part of their routine schedule. All children in this study were vaccinated (except one child), with no differences in timing or adherence to vaccination schedules between the groups. The responses measured here (at 6 months of age) reflect exposure at 2 and 4 months. This is because although 99% of children had received their 2- and 4-month injections, only 34.7% of the children in this analysis had received their 6-month injection at the time of blood collection (Table 1). There were no differences in the proportion of

Table 1. Characteristics of the 118 infants (with blood samples available at 6 months of age)

Characteristic	Placebo ($n = 60$)	Probiotic ($n = 58$)	Significance <i>P</i>
Method of delivery of neonate, n (%)			0.382
Spontaneous labour	17 (28.3)	17 (29.3)	–
Elective caesarean	21 (35.0)	18 (31.0)	–
Emergency caesarean	10 (16.7)	5 (8.6)	–
Induced	12 (20.0)	18 (31.0)	–
Perinatal characteristics			
Gestational age $\pm$ SEM (weeks)	39.4 $\pm$ 0.1	39.4 $\pm$ 0.2	0.804
Mean birth weight $\pm$ SEM (g)	3493.9 $\pm$ 49.3	3437.9 $\pm$ 59.10	0.467
Mean birth length $\pm$ SEM (cm)	50.6 $\pm$ 0.3	49.9 $\pm$ 0.3	0.078
Mean head circumference $\pm$ SEM (cm)	35.2 $\pm$ 0.2	34.9 $\pm$ 0.2	0.196
Median 5 min Apgar score*	9	9	0.523
Feeding, n (%)			
Ever breastfed	58 (96.7)	57 (98.3)	0.579
Still breastfeeding at 6 months	41 (68.3)	42 (72.4)	0.628
Duration of breastfeeding (months)* (if stopped before 6 months)	3	4	0.259
Ever received formula	42 (70.0)	45 (77.6)	0.349
Solids introduced by 6 months	56 (93.3)	53 (91.4)	0.689
Median age of first solid foods (months)*	5	5	0.473
Vaccination			
Vaccinated according to schedule			
Received 2 and 4 months'60 (100)		57 (98.3)	0.307
Received 6-month vaccination prior			
6-month visit	24 (40.0)	17 (29.3)	0.223
Infant antibiotic administration	10 (16.7)	16 (27.6)	0.153
Gender			
Male, n (%)	31 (51.7)	27 (46.6)	0.578

There were no significant differences between the groups for any of the variables as determined by Student's *t*-test for continuous data or Pearson χ^2 for all nominal data. $P < 0.05$ was considered a significant difference.

*Median determined by Mann-Whitney *U*-test.

children who had received their 6-month vaccination between the study groups ($P = 0.223$).

The cytokine responses to TT were relatively 'Th2' skewed. This IL-13-dominant response was seen in both groups (Fig. 1) and is consistent with previous reports in children at this age [18]. Administration of probiotics was

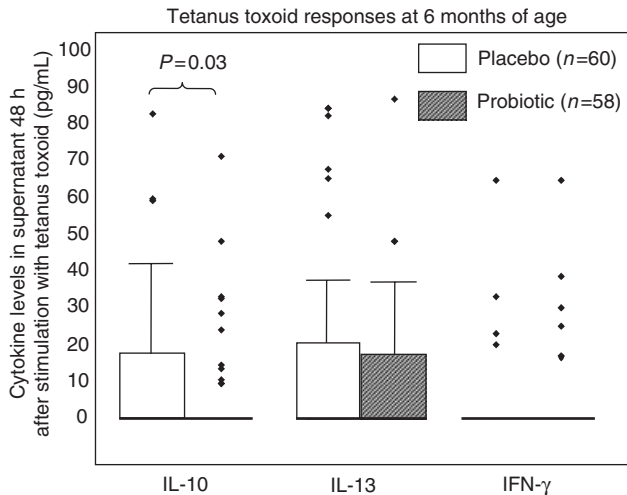


Fig. 1. Peripheral blood mononuclear cell cytokine responses to tetanus toxoid in the probiotic group (shaded) were compared with the placebo group (unshaded). Cytokine levels are expressed as median, inter-quartile ranges, 95% confidence intervals and outlying values (pg/mL). Extreme outlying values are not shown. The groups were compared by the Mann-Whitney *U*-test. Significance levels are shown, with a threshold of $P < 0.05$ for statistical significance.

associated with significant attenuation of IL-10 responses to tetanus antigen at 6 months of age, compared with the placebo group ($P=0.03$) and this was not due to any differences in vaccination. Children in the probiotic group were less likely to have detectable IL-10 responses (20.7%) compared with the control group (40.0%) ($P=0.023$; Table 2). There were no other significant differences in tetanus-specific cytokine responses between the groups.

IL-10 responses to tetanus were strongly correlated with both IL-13 ($\tau=0.3$, $P < 0.001$) and IFN- γ ($\tau=0.3$, $P < 0.001$) responses to this vaccine. However, this relationship was stronger for IL-13 in the probiotic group ($\tau=0.42$, $P < 0.001$) compared with the placebo group ($\tau=0.21$, $P=0.045$) and for IFN- γ in the placebo group ($\tau=0.37$, $P=0.002$) compared with the probiotic group ($\tau=0.42$, $P=0.01$). IL-10 responses to tetanus also strongly correlated with IL-10 responses to HDM in both groups ($\tau=0.5$, $P < 0.001$), as well as OVA IL-10 responses in the probiotic group ($\tau=0.24$, $P=0.02$) but not the placebo group ($\tau=0.13$, $P=0.16$).

Although these vaccine responses were 'Th2' dominant, the IFN- γ responses to TT were correlated to Th1 responses to allergens. There were consistent positive correlations between IFN- γ responses to tetanus and IFN- γ responses to BLG ($\tau=0.35$, $P < 0.001$), OVA ($\tau=0.18$, $P=0.027$) and HDM ($\tau=0.26$, $P=0.003$) allergens (when the population was examined as a whole). When the groups were analysed separately, this relationship between vaccine and allergen IFN- γ responses was only seen in the probiotic group (OVA: $\tau=0.28$, $P=0.014$ and HDM: $\tau=0.36$, $P=0.004$, respectively). However, probiotics did not have

Table 2. Frequency PBMC responses to food allergens (OVA and BLG), vaccine antigen (TT) and house dust mite (whole HDM, Der p 2, Der p 7)

Cytokine	Stimulant	Control (n/%)	Probiotic (n/%)	<i>P</i> value
IL-5	OVA	7 (11.7)	7 (12.1)	0.946
	BLG	8 (13.3)	7 (12.1)	0.837
	TT	14 (23.3)	13 (22.4)	0.905
	HDM	11 (18.3)	8 (13.8)	0.502
	Der p2	5 (9.1)	7 (14.0)	0.430
	Der p7	15 (27.8)	9 (18.8)	0.283
IL-6	OVA	60 (100)	58 (100)	–
	BLG	60 (100)	58 (100)	–
	TT	36 (60.0)	30 (51.7)	0.365
	HDM	55 (91.7)	52 (89.7)	0.707
	Der p2	55 (100)	48 (96.0)	0.134
	Der p7	24 (44.4)	27 (56.3)	0.234
IL-10	OVA	55 (91.7)	51 (87.9)	0.502
	BLG	25 (41.7)	17 (29.3)	0.161
	TT	24 (40.0)	12 (20.7)	0.023*
	HDM	28 (46.7)	21 (36.2)	0.249
	Der p2	33 (60.0)	18 (36.0)	0.014*
	Der p7	10 (18.5)	10 (20.8)	0.769
IL-13	OVA	29 (48.3)	33 (56.9)	0.352
	BLG	10 (16.7)	9 (15.5)	0.865
	TT	25 (41.7)	20 (34.5)	0.422
	HDM	17 (28.3)	16 (27.6)	0.928
	Der p2	12 (21.8)	5 (10.2)	0.110
	Der p7	9 (16.7)	4 (8.5)	0.222
IFN- γ	OVA	29 (48.3)	31 (53.4)	0.578
	BLG	6 (10.0)	4 (6.9)	0.545
	TT	4 (6.7)	5 (8.6)	0.689
	HDM	6 (10.0)	10 (17.2)	0.251
	Der p2	8 (14.5)	8 (16.0)	0.836
	Der p7	9 (16.7)	8 (16.7)	1.000
TNF- α	OVA	55 (91.7)	56 (96.6)	0.261
	BLG	18 (30.0)	12 (20.7)	0.246
	TT	3 (5.0)	2 (3.4)	0.676
	HDM	10 (16.7)	3 (5.2)	0.046*
	Der p2	20 (36.4)	16 (32.0)	0.638
	Der p7	4 (7.4)	2 (33.3)	0.487

Differences between the two groups were determined by Pearson's χ^2 . $P < 0.05$ is considered to be a significant difference between the groups.

any significant effects on the magnitude of IFN- γ responses to TT [although the ratio of IL-10 to IFN- γ responses to TT was significantly lower in the probiotic group ($P=0.02$)].

Effects of probiotic supplementation on allergen-specific responses

Following the 6-months supplementation period, infants in the probiotic group did not show any differences in the magnitude of cytokine production (IL-5, IL-6, IL-10, IL-13, IFN- γ , TGF- β and TNF- α) in response to food (OVA, BLG) or inhalant (HDM) allergens. The main T cell-derived cytokine responses are shown in Fig. 2. There was a

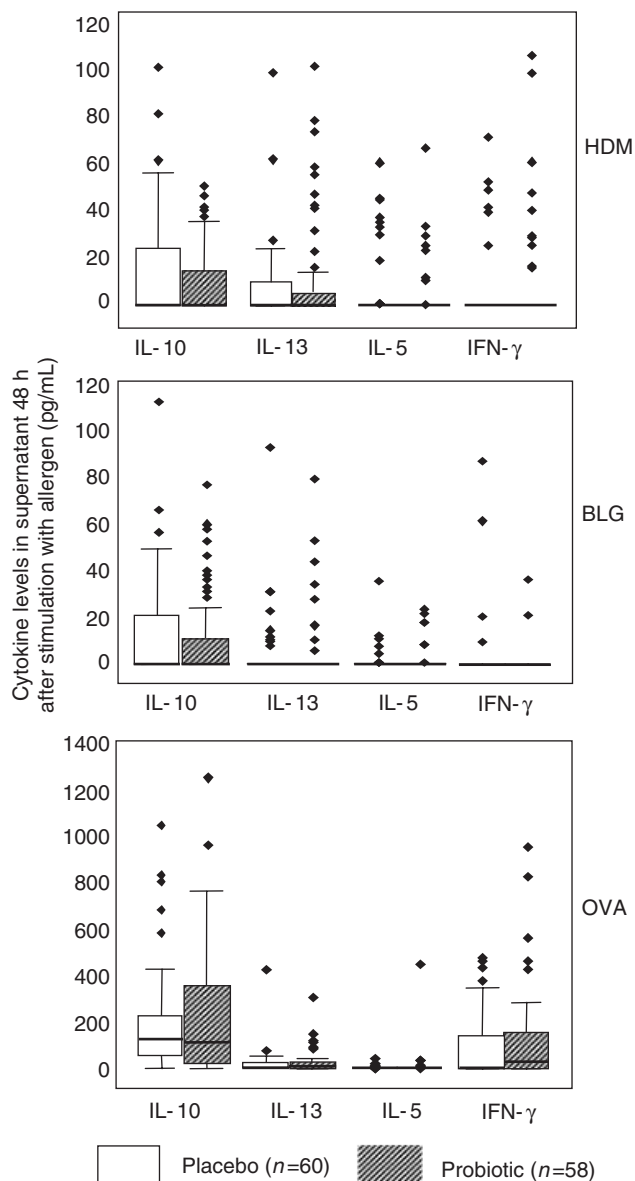


Fig. 2. Peripheral blood mononuclear cell cytokine responses to house dust mite (HDM), β -lactoglobulin (BLG) and ovalbumin (OVA) in the probiotic group (shaded) were compared with the placebo group (unshaded). Cytokine levels are expressed as median, inter-quartile ranges, 95% confidence intervals and outlying values (pg/mL). Extreme outlying values are not shown. The groups were compared by the Mann-Whitney *U*-test. A difference of $P < 0.05$ was considered to be statistically significant.

non-significant trend for lower IL-10 responses to HDM and BLG (but not OVA). IFN- γ responses were generally low level in most children, with the exception of responses to OVA (Fig. 2). There was also a non-significant trend for stronger OVA IFN- γ responses in children who had received probiotics.

We also compared the *frequency* of detectable responses with these stimuli. The only significant differences were seen in HDM responses. Children who had received

probiotics were three times less likely (5.2% compared with 16.7%) to show TNF- α responses to HDM (whole mite extract) ($P = 0.046$) as shown in Table 2. These children also had significantly less frequent IL-10 responses to HDM (Der p 2 peptide) compared with the control group ($P = 0.014$). There was also a trend for less frequent IL-13 responses to HDM allergens. These Th2 responses were almost 50% less common in the probiotic group in response to both Der p 2 and Der p 7, although this was not statistically significant (Table 2). A similar non-significant trend was seen for IL-5 responses to Der p 7. There were no differences in the frequency or patterns of food allergen responses.

Relationship between colonization patterns and antigen-specific immune responses

There were no relationships between allergen- or vaccine-specific immune outcomes and the detection of cultureable lactobacilli, bifidobacteria or coliforms in the first month of life.

Effects of probiotic supplementation on polyclonal responses

We used a standard mitogen (PHA) and a bacterial superantigen (SEB) to assess the capacity of these children to produce cytokines in response to polyclonal stimulation. In general, the administration of probiotics was not associated with any significant effects on the magnitude or frequency of responses to these stimuli (data not shown), with two main exceptions.

Firstly, although there were no differences in the *frequency* of IL-5 responses to SEB between groups (76.3% of placebo group and 79.3% of the probiotic group), the *level* of IL-5 production by the responders was significantly lower in the probiotic group compared with the placebo group ($P = 0.044$) (Fig. 3).

Secondly, TGF- β responses to SEB were also significantly lower in the probiotic group compared with the placebo group ($P = 0.015$). These responses were also detected significantly less frequently (5.7%) in the probiotic group compared with the placebo group (22%) ($P = 0.016$).

Discussion

To our knowledge, this is the first study to examine the effects of early probiotics supplementation on the development of early cellular immune responses to commonly encountered environmental antigens. We, and others, have previously shown that immunological abnormalities precede the development of allergic disease and are frequently evident in the first months of life [1–4]. The rationale for this study was to assess the potential for

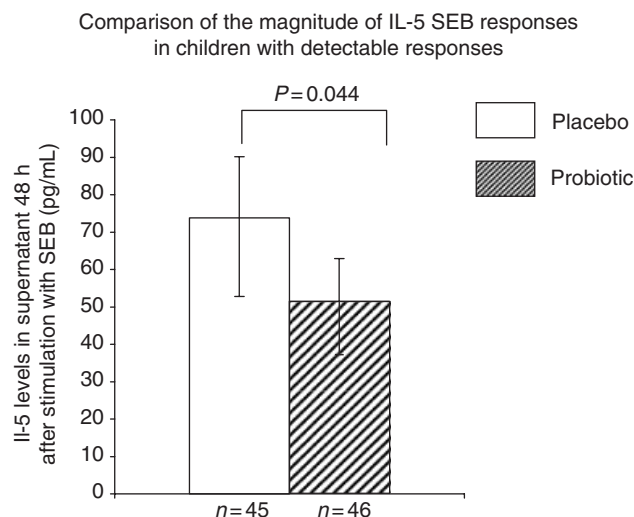


Fig. 3. The levels of IL-5 response to *Staphylococcus enterotoxin B* were assessed in children with detectable responses, making comparisons between the probiotic group (shaded) and the placebo group (unshaded). Cytokines are expressed as geometric mean and 95% confidence intervals (pg/mL). The groups were compared by the student t test. A difference of $P < 0.05$ was considered to be statistically significant.

immunomodulatory probiotic bacteria to modify immune development during this 'critical early window'. The ultimate aim of this cohort will be to determine whether this could alter the subsequent risk of allergic disease as suggested by preliminary studies [16].

The only effects of probiotics on Th2 responses were seen in children who showed IL-5 responses to SEB (Fig. 3). Although this is consistent with the hypothesis that probiotics could inhibit Th2 pathways through activation of regulatory pathways and Th1 effector mechanisms, we did not see any evidence of this. Specifically, there was no evidence that IFN- γ responses were enhanced by administration of probiotics (to any stimuli), and, if anything, the production of regulatory cytokines was either reduced (as with TT or SEB) or unaffected (all other stimuli) in the probiotic group, as discussed further below.

Despite speculation that this intervention may modify early allergen-specific responses, there were generally no significant effects on responses to either food or inhalant allergens. The only exception was for significantly less frequent TNF- α and IL-10 responses to HDM allergens in children receiving probiotics, although the significance of this is unclear. Allergen exposure can be variable at this age and is difficult to measure or standardize accurately. Thus, it is possible that there may have been differences in allergen exposure that could contribute to immunological effects.

For this reason, we also examined the effects of the intervention on vaccine responses, as over 99% of the participating children in this study were vaccinated against tetanus according to the schedule. The most

significant finding was that probiotic administration was associated with attenuated IL-10 responses to vaccine antigens (TT) at 6 months of age. These responses are of interest in this context because early vaccine responses reflect the overall maturational status of the developing immune system [18, 26]. Others have previously shown a high degree of variability in vaccine responses in this age group, which can vary with atopic propensity [18, 26, 27]. Most notably, children with an allergic propensity have stronger early Th2 responses to vaccine antigens [18, 26]. Consistent with this, we observed that IFN- γ responses to tetanus correlated significantly with IFN- γ responses to all allergens assessed (OVA, BLG and HDM). This was only evident in the children who received probiotics (for OVA and HDM responses). The significance of this is unclear, but suggests that vaccine IFN- γ responses in these children are more likely to reflect more general Th1 function. In our study, there was no evidence of an inhibitory (inverse) relationship between IL-10 and other cytokines. In contrast, IL-10 was generally positively correlated with both Th1 IFN- γ responses and IL-13 (and IL-5) Th2 responses. Although IL-10 has been implicated in priming Th2 responses in some studies [28, 29], probiotics did not appear to have any direct effects on either Th1 or Th2 responses to vaccines (or other stimuli).

Using polyclonal stimulants (PHA or SEB), we did not see any differences in the 'capacity' to produce IL-10-regulatory cytokine. However, we did observe that the capacity to produce TGF- β another key regulatory cytokine was attenuated in children receiving probiotics ($P = 0.015$).

These findings are unexpected given previous reports that probiotics favour the production of IL-10, TGF- β and other aspects of regulatory immune function [30–36]. However, many of these studies have involved stimulating mononuclear cells directly with probiotic species *in vitro* [32–34, 36], which may not reflect *in vivo* effects. There is only one report of increased serum IL-10 levels in young children following probiotic supplementation [35]. Animal studies also support the notion that intestinal flora induce regulatory populations (bearing TGF- β) [31] associated with clinical benefits (an amelioration of colitis) [31].

It is probable that the peripheral blood immune responses measured in the present study do not directly reflect events in the gastrointestinal tract. Furthermore, at this age we are still examining 'evolving' immune responses and it is possible that with increasing age, the significance of these early differences in vaccine antigens will become more apparent, or that effects on allergen-specific responses could become evident. This cohort will also be followed further to examine the effects on the development of allergic disease. As previously reported, Kalliomaki et al. [16] reported that early probiotic supplementation was associated with a 50% reduction in AD by

2 years of age in a Finnish birth cohort of a similar size. Despite these clinical effects, they did not see any effects on atopic sensitization as measured by both RAST and SPT at 3, 12 and 24 months as well as subsequently at 4 years in a further follow-up analysis [17]. Although they did not measure effects on cellular immune responses, the lack of effect on sensitization suggests that other mechanisms are involved. This is consistent with lack of effect on allergen-specific responses seen in our study, at least at 6 months of age. There are a number of methodological differences between our study and the previously published study by Kalliomaki et al. [16], which may also become relevant when the subsequent clinical outcomes are compared. We used a *L. acidophilus* species, whereas the Finnish group used a *L. rhamnosus* GG. While biological differences between strains have been noted [36], *in vitro* immunological effects of the LAVRI strain are evident [23] (and personal communication from Patricia Conway). In our study, all infants received the probiotic regardless of the feeding method, whereas the Finnish children only received the probiotic directly if they were bottle-fed (and it was otherwise given to their mothers). The Finnish infants were also exposed to probiotics in pregnancy (only for the last 2–4 weeks). While it is known that pre-natal exposure to microbial products (living in farming environments) may protect from subsequent asthma and allergic sensitization [37, 38], the significance of 2–4 weeks of extra probiotics exposure in the pre-natal period is not clear. It is generally accepted that more studies are needed to assess the clinical role of probiotics in allergy prevention before recommendations can be made. Our cohort will also be used to address this issue in subsequent studies.

In summary, although we did not see any consistent effects on allergen-specific responses, our findings suggest that probiotics may have immunomodulatory effects on vaccine responses. The significance and clinical relevance of this need to be determined in further studies. This and the effects of *L. acidophilus* on the prevention of allergic disease will also be assessed in future studies of this population.

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